Serum Bile Acid Profiles Improve Clinical Prediction of Nonalcoholic Fatty Liver in T2DM patients

Tao Wu

Shanghai University of Traditional Chinese Medicine <https://orcid.org/0000-0003-1567-1512>

Ming Yang

Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Hanchen Xu

Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Lei Wang

Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Huafeng Wei

Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Guang Ji (✉ jiliver@vip.sina.com)

Institute of Digestive Disease, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Research

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Abstract

Background: The present study aimed to assess the ability of serum bile acid profiles to predict the development of nonalcoholic fatty liver (NAFL) in type 2 diabetes mellitus (T2DM) patients.

Methods: Using targeted ultraperformance liquid chromatography (UPLC) coupled with triple quadrupole mass spectrometry (TQ/MS), we compared serum bile acid levels in T2DM patients with NAFL ($n=30$) and age-gender matched T2DM patients without NAFL ($n=36$) at the first time. Second, an independent cohort study of T2DM patients with NAFL ($n=17$) and age-gender matched T2DM patients without NAFL ($n=20$) was used to validate the results. The incremental benefits of serum biomarkers, clinical variables alone or with biomarkers were then evaluated using receiver operating characteristic (ROC) curves and decision curve analysis. Area under the curve (AUC), integrated discrimination improvement (IDI) and net reclassification improvement (NRI) were used to evaluate the biomarker predictive abilities.

Results: The serum bile acid profiles in T2DM patients with NAFL were significantly different from T2DM patients without NAFL, as characterized by the significant elevation of LCA, TLCA, TUDCA, CDCA24G and TCDCA, which may be potential biomarkers for identification of NAFL in T2DM. Based on the improvement in AUC, IDI and NRI, addition of 5 bile acids to a model with clinical variables statistically improved its predictive value. Similar results were found in the validation cohort.

Conclusions: These results highlight detected biomarkers may contribute to the progression of NAFL in T2DM patients, and these biomarkers particularly in combination may help diagnosis of NAFL and allow earlier intervention in T2DM patients.

1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has increased in the world. Nonalcoholic fatty liver disease (NAFLD) has become the most frequent chronic liver disease in T2DM patients[1, 2]. NAFLD includes simple steatosis, i.e. nonalcoholic fatty liver (NAFL), to nonalcoholic steatohepatitis (NASH), cirrhosis or finally hepatocellular carcinoma (HCC)[3]. Studies have shown that NAFLD may be present in up to 70% of patients with diabetes[4], however the prevalence of biopsy-proven NASH was 20% in asymptomatic T2DM patients with normal liver function [5].

The presence of both NAFLD and T2DM increases the possibilities of the development of complications of diabetes (including both macro- and microvascular complications) and raises the risk of more severe NAFLD, including cirrhosis and HCC [6]. The newest guidelines for the diagnosis and management of NAFLD from the American Association for the Study of Liver Diseases mention two points, that it is important to assess the possibility of NAFLD and NASH in T2DM patients and that the ratio of morbidity and mortality of cardiovascular disease (CVD) in NAFLD patients is very high[7]. Moreover, HCC happens more commonly in T2DM with NASH patients compared to those patients without T2DM [1]. Nagpal et al[8] found that serum cytokeratin-18 levels could be used to predict the development of T2DM in adult patients with NAFLD. However, there were no related studies describing biomarkers which can predict the

development of NAFLD in T2DM patients, which could cause the high risk of occurrence of CVD or severe liver diseases. Therefore, it is urgent and important to find simple, non-invasive diagnostic biomarkers that can help early detect and understand the mechanism involved in the formation of NAFLD in T2DM patients in order to prevent the occurrence of CVD and HCC.

Metabolomics is an emerging analytical technology which simultaneously analyzes many metabolites in biological samples. Bile acids (BAs) are amphipathic steroid molecules which are synthesized in the liver from cholesterol and excreted into the bile, and have been acknowledged as key signaling molecules that regulate glucose, lipid and energy metabolism due to their micelle-forming properties[9, 10]. BAs are secreted by hepatocytes into the canaliculus actively and are nearly completely (95%) absorbed by an active uptake process after reaching the terminal ileum. It is well-known that BAs not only facilitate the intestinal absorption of lipids and lipid-soluble vitamins such as vitamins A, D, K, and E, but also mediate cellular and molecular signal transduction by activating the nuclear receptors, i.e., the farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D receptor (VDR) and the G-protein-coupled bile acid receptor (GPBAR1 or TGR5) and sphingosin-1-phosphate receptor 2 (S1PR2)[11, 12]. Caussy et al[13] found that there was no significant differences of the total serum BAs between NAFLD vs non-NAFLD and NAFL vs NASH, but serum BAs were significantly increased progressively as liver fibrosis increased. Our former study based on untargeted metabolomics, has shown that serum differential metabolites, including bile acids, fatty acids, amino acids, lipids, carbohydrates, steroids, and tricarboxylic acids, were involved in the formation of T2DM complications according to a cohort study in 292 T2DM patients with different complications[14]. We recently summarized many different metabolomics studies on diabetic complications[15]. However, it is still unclear whether the concentrations of BAs are different in the T2DM patients with or without NAFL. We then hypothesized that BAs contribute to the formation of NAFL in T2DM patients and performed a serum targeted metabolomics study on T2DM patients with and without NAFL.

In the present study, we quantified the serum levels of bile acids in T2DM patients with or without NAFL using targeted metabolomics. The purpose of this study was to observe the metabolic differences between diabetes patients with different complications and to assess the performance of the new screening approach for the diagnosis of NAFL in patients with T2DM. We anticipate that this study could provide metabolomics support for the early intervention in diabetic complications, and offer new insights into the approach of targeting metabolites and pathways in the treatment of this serious global health problem.

2. Materials And Methods

2.1 Chemicals and Reagents

A total of 41 BAs, including their corresponding glycine and taurine conjugates, were obtained from Steraloids, Inc. (Newport, RI), including cholic acid (CA), glycocholic acid (GCA), taurocholic acid (TCA), chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid

(TCDCA), deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), ursodeoxycholic acid (UDCA), glyoursodeoxycholic acid (GUDCA), taoursodeoxycholic acid (TUDCA), lithocholic acid (LCA), glycolithocholic acid (GLCA), tauroolithocholic acid (TLCA), hyocholic acid (HCA), glycohyocholic acid (GHCA), taurohyocholic acid (THCA), α -muricholic acid (α MCA), tauro- α -muricholic acid (TaMCA), β -muricholic acid (β MCA), tauro- β -muricholic acid (T β MCA), ω -muricholic acid (ω MCA), tauro- ω -muricholic acid (T ω MCA), hyodeoxycholic acid (HDCA), glycohyodeoxycholic acid (GHDCA), taurohyodeoxycholic acid (THDCA), muricholic acid (MuroCA), dehydrocholic acid (DHCA), glycodehydrocholic acid (GDHCA), taurodehydrocholic acid (TDHCA), 3-dehydrocholic acid (3-DHCA), 7-dehydrocholic acid (7-DHCA), isodeoxycholic acid (isoDCA), apocholic acid (apoCA), 6-ketolithocholic acid (6-KLCA), 7-ketolithocholic acid (7-KLCA), 12-ketolithocholic acid (12-KLCA), 23-nordeoxycholic acid (23norDCA), dehydrolithocholic acid (DHLCA), lithocholic acid-3 sulfate (LCA-3S) and acyl CDCA-24 glucuronide (CDCA-24G). Cholic acid-2,2,4,4-D₄ (CA-D₄) and lithocholic acid-2,2,4,4-D₄ (LCA-D₄) were also obtained from Steraloids, Inc. (Newport, RI) as deuterated internal standards (IS). HPLC grade methanol, acetonitrile and formic acid were from Sigma-Aldrich (St. Louis, MO).

2.2 Sample population

There were two study groups. We first conducted a cross-sectional study of 30 T2DM patients with NAFL and 36 without NAFL. Second, an independent cohort composed of 17 T2DM patients with NAFL and 20 without NAFL was used to validate the former results. All patients were obtained at Longhua Hospital, Shanghai University of Traditional Chinese Medicine, China, from Sep 2015 to Dec 2016. According to the criteria by the World Health Organization in 1999[16], T2DM was defined as fasting plasma glucose (FPG) of ≥ 7.0 mmol/L, 2 h plasma glucose (2 h PG) of ≥ 11.1 mmol/L, or a history of oral hypoglycemic or insulin use, or both. NAFLD was defined according to the guidelines for management of NAFLD by the Chinese Liver Disease Association in 2010[17]. NAFLD can be diagnosed if the patient matches the following the criteria: 1) no history of alcohol consumption or alcohol equivalent to < 70 g per week (female), < 140 g per week (male); 2) fatty liver disease was not caused by other factors including viral hepatitis, drug-induced liver disease, total parenteral nutrition, Wilson's disease and autoimmune liver disease; 3) having obtained characteristic histological changes of fatty liver disease from histological examination. Considering that it is hard to achieve histological diagnosis, 3) could be substituted for 4) having obtained characteristic diffuse fatty liver imaging results; and/or 5) serum levels of transaminases, including alanine aminotransferase (ALT), and/or aspartate aminotransferase (AST) and γ -glutamyl-transpeptidase, have increased continuously for over half a year in patients with several components of metabolic syndrome, such as overweight and (or) visceral obesity, with elevated fasting blood glucose, dyslipidemia and hypertension. The imaging results of characteristic diffuse fatty liver are as below: if ultrasonography examinations have the following two items of three abdominal ultrasound manifestations 1) the near-field echo of the liver is diffuse, enhanced and stronger than that of the kidney; 2) the far-field echo of the liver gradually attenuates; 3) the structure of hepatic duct is unclear. Or computed tomography (CT) shows diffuse decreased liver density and the CT value of liver/spleen is ≤ 1 . The diagnosis of NAFLD in the present study was based on ultrasonography examinations. Patients were excluded if they were pregnant or breast-feeding, or had intellectual dysfunctions or psychiatric

disorders. Serum samples were stored at -80 °C until analysis. All patients who participated in the study provided signed informed consent. All methods were in accordance with the relevant guidelines and regulations. All the studies were approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine and have been performed in accordance with the ethical standards in the 1964 Declaration of Helsinki.

2.3 Sample preparation and bile acid detection

Serum BAs levels were measured according to the previously reported approach [18–20]. Briefly 50 µL of serum was used, and the final extracts were reconstituted with 40 µL of acetonitrile and 40 µL of water. Next, 5 µL of supernatant was injected for analysis. Serum BAs were analyzed using the Waters ACQUITY ultraperformance liquid chromatography (UPLC) (BEH C18 1.7 µm 2.1 × 100 mm column) system coupled with Waters Xevo Triple Quadrupole mass spectrometry (TQ/MS). The raw data acquired from UPLC-TQ/MS were first processed by TargetLynx software v 4.1 (Waters, USA), and then manually checked to ensure data quality. Total bile acids (TBA) concentrations were determined by calculating the molar sum of all detected BAs.

2.4 Statistical analysis

In the present study, we proposed a 3-step analytic approach, and several classification models were considered as follows. First, we used multivariate analysis to perform a global test to examine whether clinical characteristics (Model 1) or the entire set of all metabolites (Model 2) were related to a given outcome (NAFL development in T2DM). Second, if the metabolism panel was associated with the outcome, then as a second step, we further performed a feature selection procedure to identify the discriminative metabolites based on both univariate analysis (Kruskal-Wallis test) and multivariable analysis (orthogonal projections to latent structures discriminant analysis, OPLS-DA). The metabolites with $p < 0.01$ and variable influence on projection (VIP) > 1.0 were considered as potential biomarkers in accounting for class discrimination. Subsequently, Model 3 was established based on these informative predictors. Finally, we investigated whether the addition of a panel of metabolites to clinical characteristics could improve the accuracy for classification. Here, we considered Model 1 as a baseline model, and Model 4 and Model 5 were established based on the combined predictors, respectively. The added discriminative power offered by the addition of metabolites to clinical characteristics was analyzed by comparison of model performance. All models were constructed based on exploration cohort, and their power of prediction was tested on validation cohort. All outliers have been included to analysis.

Model performances were compared in a pairwise manner by calculating the improvement in area under the curve (AUC), integrated discrimination improvement (IDI) and net reclassification improvement (NRI). To obtain the robust assessments, a 10000 bootstrap resamples were used to estimate the improvement in model performance assessments. IDI was used to determine the average increase in predicted probabilities[21], and NRI to assess model reclassification[22]. IDI > 0 and NRI > 0 represent the improvement of the prediction of outcome in the model.

We also evaluated the clinical usefulness and net benefit of the new predictive models using decision curve analysis according to the method described in JAMA Guide to Statistics and Methods[23]. Decision curve analysis is a method to evaluate the benefits of a diagnostic test [23]. The X-axis shows threshold values for NAFL risk, and the Y-axis represents the net benefit for threshold probabilities. A decision curve that is far away from the “None” line (i.e., assume no NAFL case) and the “All” line (i.e. assume all NAFL cases) for prediction model can provide a high net benefit [24].

All values are shown as the mean $\pm$ SD for continuous variables and frequencies for categorical variables. Multivariate models were constructed using OPLS-DA. The number of orthogonal components was optimized using 10-fold cross-validation, and the models were assessed using cross-validation based metrics (R²_X, R²_Y, Q²_Y values) and permutation analysis. The continuous variables were analyzed using non-parametric Kruskal-Wallis test, however the categorical variables were analyzed with Fisher’s exact test. AUC from receiver operator characteristic (ROC) curves was performed to assess the incremental value of the metabolic markers for risk prediction of NAFL in T2DM patients. All calculations and multivariate analysis were performed using R software version 3.5.2 (www.r-project.org). A *p*-value of < 0.05 (two-tailed) was considered statistically significant. GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) was used to generate histograms.

3. Results

3.1 Clinical and metabolic characteristics of T2DM patients with or without NAFL

We recruited a total of 56 T2DM patients without NAFL and 47 T2DM patients with NAFL respectively. According to the time of enrolment, the first 30 T2DM patients with NAFL and 36 T2DM patients without NAFL were included in the exploration cohort, while the subsequent 17 with NAFL and 20 without NAFL were included in the validation cohort. The demographic, clinical and biochemical characteristics of these patients are shown in Table 1, including age, gender, body mass index (BMI), waist-hip ratio (WHR), fasting plasma glucose (FPG), post-load plasma glucose (2 h PG), ALT, triglyceride (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Of note, important clinical characteristics such as age, gender and BMI, as well as serum levels of FPG, 2 h PG, HDL, LDL and TG, showed no significant differences between the two groups either in exploration cohort or in validation cohort. However, T2DM patients with NAFL had higher ALT levels than T2DM patients without NAFL both in exploration cohort and in validation cohort. The above information verified the comparability between T2DM patients with and without NAFL either in exploration cohort or in validation cohort.

Table 1
Demographic and Clinical Manifestations of patients

Variables	Exploration cohort			Validation cohort		
	T2DM patients without NAFL(n = 36)	T2DM patients with NAFL(n = 30)	<i>P</i> -value	T2DM patients without NAFL(n = 20)	T2DM patients with NAFL(n = 17)	<i>P</i> -value
Age (years)	67(58-77.25)	71.5(64.25–74.75)	0.503	70.5(60-74.5)	75(70–76)	0.060
Gender (Male/Female)	24//12	25//5	0.162	18//2	12//5	0.212
BMI (kg/m ²)	23.84 (21.76–26.35)	25.39 (24.01–26.17)	0.144	24.55(23.29-26.00)	25.38 (24.38–26.22)	0.217
WHR	0.93(0.91–0.98)	0.92(0.89–0.96)	0.309	0.94(0.90–0.98)	0.90 (0.86–0.95)	0.106
FPG (mmol/L)	7.20(5.23–11.13)	7.85(6.92–9.59)	0.652	7.93(6.40–9.60)	7.41(6.90–8.16)	0.402
2 h PG (mmol/L)	12.15(8.19–16.20)	10.95(9.23–13.58)	0.615	12.55(9.28–15.53)	10.4(8.40–12.10)	0.085
ALT (U/L)	19(15-26.25)	22.5(17.85–36.58)*	0.031	16(12.75–20.50)	25.10(21.40–30.70)***	0.001
TG (mmol/L)	1.13(0.96–1.38)	1.07(0.93–1.31)	0.704	1.06(0.81–3.20)	1.3(1.10–1.88)	0.573
HDL (mmol/L)	1.11(0.94–1.46)	1.227(1.08–1.438)	0.158	1.10(0.98–1.30)	1.25(1.09–1.38)	0.100
LDL (mmol/L)	2.36(1.91–3.08)	2.55(2.30–2.98)	0.491	2.20(1.88–2.59)	2.42(2.21–2.8)	0.135
Note: Data are mean ± SEM or frequencies. * <i>p</i> < 0.05, ** <i>p</i> < 0.01, *** <i>p</i> < 0.001, compared with T2DM patients without NAFL. T2DM, type 2 diabetes mellitus; NAFL, nonalcoholic fatty liver; BMI, body mass index; WHR, waist-hip ratio; FPG, fasting plasma glucose; 2 h PG, 2 h plasma glucose; ALT, alanine aminotransferase; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.						

3.2 Serum concentrations of bile acids in T2DM patients with or without NAFL

Twenty-four bile acids were quantitatively detected in the serum of T2DM with NAFL (n = 30) and T2DM without NAFL (n = 36) (Supplementary Fig. S1, Table S1). BAs synthesized in the liver are termed primary BAs such as CA and CDCA, and those generated by bacteria are secondary BAs such as DCA and UDCA. Overall, several BAs were significantly different in T2DM with compared to those without NAFL, including GCA, LCA, TLCA, CDCA, GCDCA, UDCA, GUDCA, CDCA-24G, and 3-DHCA. Nevertheless, TBA, total CDCA,

total UDCA, and total glycine-conjugated BAs were higher in T2DM patients with NAFL than in T2DM patients without NAFL.

3.3 Multivariate analysis based on serum BAs in T2DM patients with or without NAFL

First, we used multivariate analysis to perform a global analysis to examine whether clinical characteristics or the total set of all metabolites were related to NAFL development in T2DM or not. In exploration cohort, it was shown that the AUC statistic values of the clinical model alone including age, sex, BMI, WHR, FPG, 2 h PG, ALT, TG, HDL and LDL (Model 1), and all metabolites model alone (Model 2) were 0.731 (95%CI: 0.625–0.837) and 0.822 (95%CI: 0.731–0.914) respectively (Table 2, Supplementary Fig S2). It means that both clinical factors and all metabolites could predict the development of NAFL in T2DM.

Table 2
Performance of different models for the prediction of NAFLD in T2DM patients

Model	Features	Number of Features	Exploration cohort			Validation cohort		
			AUC	95%CI	<i>P</i> -value#	AUC	95%CI	<i>P</i> -value#
Model1	Clinical	10	0.731	0.625–0.837	< 0.001	0.703	0.551–0.855	0.016
Model2	All Metabolites	33	0.822	0.731–0.914	< 0.001	0.735	0.613–0.858	0.001
Model3	Biomarker Metabolites	5	0.744	0.641–0.848	< 0.001	0.765	0.642–0.887	< 0.001
Model4	Clinical + All Metabolites	43	0.797	0.698–0.896	< 0.001	0.824	0.706–0.941	< 0.001
Model5	Clinical + Biomarker Metabolites	15	0.808	0.713–0.904	< 0.001	0.794	0.674–0.915	< 0.001
Note#: Overall <i>P</i> value against a null model was calculated using the Wilcoxon signed rank test. AUC, area under the curve. Model 1, clinical factors; Model 2, all metabolites; Model 3, biomarker metabolites; Model 4, clinical factors plus all metabolites ; Model 5, clinical factors plus biomarker metabolites.								

Subsequently, we performed OPLS-DA models in order to find the distinct bile acids which contribute to the classification more significantly. OPLS-DA score plots showed a clear separation between the two groups based on all metabolites: R2X = 0.523, R2Y = 0.480, Q2Y = 0.248 (Fig. 1A). Permutation analysis showed the reliability of the model (Fig. 1B). Combined with the results of VIP value > 1 and *p* < 0.01, it was shown that the contribution of TLCA, CDCA24G, TUDCA, LCA and TCDCA for the classification was 2.175, 1.980, 1.831, 1.793, 1.084 respectively. The serum concentrations of TLCA, CDCA24G, TUDCA, LCA

and TCDCA were marginally higher in T2DM patients with NAFL than those without NAFL (Fig. 2A; Supplementary Table S1). Spearman correlation analysis was performed to evaluate the relationships among the 5 NAFL-associated bile acids in the exploration cohort. Strong correlations were observed between LCA and TLCA ($\rho = 0.562$); TLCA and TCDCA ($\rho = 0.34$); TUDCA and CDCA24G ($\rho = 0.278$); TUDCA and TCDCA ($\rho = 0.677$); CDCA24G and TCDCA ($\rho = 0.33$) (Fig. 2B, Supplementary Table S2, Fig S3).

3.4 Predictive ability of the 5 NAFL-associated bile acids

In order to further test the predictive value of the above selected biomarkers, we then analyzed the AUC of biomarkers (Model 3). It was found that AUC was 0.744 (95%CI: 0.641–0.848). It should be noted that the number of features used to develop Model 3 was only five. Then Model 4 and Model 5 were developed based on clinical factors combined with all metabolites and these biomarkers, respectively. As a result, both models display good performance with higher AUC than 0.79 (Table 2, Supplementary Fig S2). In order to compare the discriminatory capacity of models with clinical variables alone or with biomarkers, several performance metrics (difference in AUC, IDI, and NRI) were estimated based on bootstrap techniques. All the performance measures calculated for a pairwise comparison are shown in Table 3. It was shown that the increased discrimination (difference in AUC 0.133[95%CI: 0.039–0.244], $p = 0.001$, IDI 0.365[95%CI: 0.136–0.593], $p = 0.001$ and NRI 0.265[95%CI: 0.007–0.569], $p = 0.027$) were observed when the metabolites added to the clinical prediction model (Model 4 vs Model 1). The biomarkers also improved risk classification when added to the clinical prediction model (difference in AUC 0.108[95%CI: 0.030–0.211], $p = 0.001$; IDI 0.285[95%CI: 0.110–0.487], $p < 0.001$; NRI 0.218[95%CI: 0.022–0.498], $p = 0.034$) (Model 5 vs Model 1). In summary, the combined models (Model 4 and Model 5) provide significant improvements ($p < 0.05$) in discrimination performance over the baseline model (Model 1) in terms of all metrics, while the differences between Model 4 and Model 5 are not statistically significant ($p > 0.05$). These results indicate that our approach successfully made a good optimization by eliminating the redundant metabolites, and a small set of informative biomarkers was achieved.

Table 3
Discriminatory capacity of models with clinical variables alone or with biomarkers

Comparison	Index	Data Set	Estimate#	ci95low#	ci95up#	pvalue#
Model 4 vs Model 1	Difference in AUC	Exploration	0.133	0.039	0.244	0.001
		Validation	0.181	0.015	0.338	0.017
	IDI	Exploration	0.365	0.136	0.593	0.001
		Validation	0.375	0.073	0.645	0.006
	NRI	Exploration	0.265	0.007	0.569	0.027
		Validation	0.360	0.015	0.691	0.021
Model 5 vs Model 1	Difference in AUC	Exploration	0.108	0.030	0.211	0.001
		Validation	0.141	0.027	0.271	0.008
	IDI	Exploration	0.285	0.110	0.487	< 0.001
		Validation	0.267	0.047	0.551	0.005
	NRI	Exploration	0.218	0.022	0.498	0.034
		Validation	0.284	0.009	0.585	0.028
Model 5 vs Model 4	Difference in AUC	Exploration	-0.025	-0.083	0.030	0.811
		Validation	-0.041	-0.177	0.106	0.726
	IDI	Exploration	-0.081	-0.266	0.111	0.805
		Validation	-0.108	-0.391	0.252	0.765
	NRI	Exploration	-0.047	-0.245	0.154	0.662
		Validation	-0.075	-0.409	0.241	0.645
Note #: Estimated by bootstrap resampling 10000 times. AUC, area under the ROC curve; IDI, integrated discrimination improvement; NRI, net reclassification improvement. Model 1, clinical factors; Model 2, all metabolites; Model 3, biomarker metabolites; Model 4, clinical factors plus all metabolites ; Model 5, clinical factors plus biomarker metabolites.						

We also used decision curve analysis in order to assess predictors of NAFL in T2DM and the performance of the predictive models while combining biomarkers with clinical factors (Table 3, Fig. 3 and Supplementary Table S3). The decision curve analysis plot shows distinct net benefit of the biomarkers when added to clinical factors, which does justify their clinical use in decision-making (Fig. 3A).

3.5 Validation Cohort of the 5 NAFL-associated bile acids

To validate the discrimination of the model, we used another independent group comprising 20 T2DM patients without NAFL and 17 T2DM patients with NAFL in the validation cohort to prove the performance. 24 bile acids were quantitatively detected in the serum of these patients (Table S1, and Supplementary Fig. S4, Fig S5). The summary information of predictors in models is shown in Table 3. It was shown significant differences of TLCA, CDCA24G, TUDCA and TCDCA among the 5 bile acids between the two groups. OPLS-DA score plots showed a clear separation between the two groups based on the total 24 metabolites, $R^2X = 0.552$, $R^2Y = 0.828$, $Q^2Y = 0.660$ (Supplementary Fig. S6). Strong correlations were observed between LCA and TLCA ($\rho = 0.435$); TLCA and CDCA24G ($\rho = 0.405$); TUDCA and CDCA24G ($\rho = 0.494$); TUDCA and TCDCA ($\rho = 0.73$); CDCA24G and TCDCA ($\rho = 0.53$) (Supplementary Table S2, Fig S3, Fig S7).

In the validation cohort, the AUC statistic values of the Model 1, Model 2, Model 3, Model 4 and Model 5 were 0.703, 0.735, 0.765, 0.824 and 0.794 respectively. The comparison results of Model 4 vs Model 1, Model 5 vs Model 1 and Model 4 vs Model 5 in the validation cohort were similar with those in the exploration cohort (Table 3, Fig. 3B). These results further confirm that the addition of biomarkers improves the predictive value of the baseline clinical model, and the combined models achieve better performance in classification task that allows for the identification of NAFL in T2DM patients on the basis of bile acid characteristics (Table 2, Supplementary Fig S2).

4. Discussion

In this study, we quantitatively profiled, for the first time, bile acids in the serum of T2DM patients with or without NAFL based on targeted metabolomics, and combined traditional clinical characteristics to predict the development of NAFL, then further validated the results by another independent cohort study. Finally, we defined 5 serum bile acids extracted from the whole metabolites and combined traditional clinical characteristics to predict the NAFL status in T2DM patients. Interestingly, serum bile acid profiles in T2DM patients with NAFL were significantly different from T2DM patients without NAFL, as characterized by the significant elevation of LCA, TLCA, CDCA24G, TUDCA and TCDCA, which may be potential biomarkers for diagnosis of NAFL in T2DM based on UPLC-TQ/MS and OPLS-DA. To explore the potential ability of the biomarkers to discriminate NAFL status in T2DM patients, model performances were compared and it was shown that the addition of five bile acid species (selected based on the exploration cohort, then validated in the validation cohort) to the clinical variables, improved the AUC, and categorical IRI, NRI for predicting the NAFL development in T2DM. Thus, the above evidences showed these biomarkers contributed to the improved model performance in predicting the development of NAFL in T2DM patients.

At the same time, these results suggest that the increase in bile acids may contribute to the formation of NAFL in T2DM patients, which could cause the high risk of occurrence of CVD and HCC. The increased five bile acids may cause the progression of NAFL in T2DM patients with insulin resistance. Our motivation for performing this study was that biomarkers that improve discrimination of NAFL in T2DM

patients will not only support the prediction of NAFL development but also promote to further acknowledging the roles of bile acids. However, the detailed mechanisms have been unclear until now.

It is shown that recent increase in the incidence of HCC is driven by NAFLD, especially in Western Countries [25]. Nevertheless, the morbidity and mortality of CVD in NAFLD patients are also very high[17]. It is therefore crucial to prevent the progression of metabolic diseases, including T2DM, dyslipidemia and NAFLD; moreover, elucidation of the pathogenesis of NAFLD in T2DM patients has become increasingly more important.

Arab et al [26] summarized current available data on the relationships of BAs to NAFLD. Cyrielle et al[13] found although serum total BAs were no significant differences between NAFLD and non-NAFLD participants, the proportion of serum CA, CDCA conjugates were higher in NAFLD, while serum GHCA was lower than those non-NAFLD participants. Jiao et al [27] found that total serum bile acid levels in patients with NASH were about 3 times that of healthy controls. Total plasma bile acid levels were higher in obese and T2DM subjects than in healthy controls [28, 29]. However, there is no systematical analysis about serum BAs between T2DM with or without NAFL. Puri et al[30] found that NAFLD was related to significantly changed circulating BA composition, unaffected by T2DM, and associated with histological features of NASH. These observations provided the foundation for future hypothesis-driven studies of specific effects of BAs on specific aspects of NASH.

In the present study we mainly found two classes of bile acids metabolites between the two groups including (A) CDCA and its metabolites (LCA, TLCA, CDCA24G and TCDCA) and (B) UDCA metabolite (TUDCA) as illustrated in Fig. 4.

(A) CDCA and its metabolites

CDCA plays an important role in controlling cholesterol homeostasis. Hydrophobic bile acids are known to induce hepatocyte injury experimentally, and the mechanisms are supposed as several different mechanisms, 1) mitochondrial and endoplasmic reticulum (ER) oxidative stress, 2) activate the death receptors FAS and tumor necrosis factor-related apoptosis-inducing ligand receptor 2 (TRAIL-R2) solubilization of the hepatocellular plasma membrane directly [31]. Here we found serum levels of CDCA metabolites such as LCA, TLCA, CDCA24G and TCDCA were higher in T2DM with NAFL than those without NAFL.

LCA increased in patients with liver disease as the most potent endogenous chemical causing liver toxicity [32]. LCA is converted from the primary BA-CDCA in the distal small intestine and colon after undergoing deconjugation and dehydroxylation[33]. LCA can be reabsorbed passively and constitute a portion of the total BA pool in the enterohepatic circulation[34]. Fickert et al [35] found that segmental bile duct obstruction, destructive cholangitis and periductal fibrosis in mice were caused by LCA feeding. LCA was reported to alter the levels of phospholipids, cholesterol, free fatty acids and TGs [36], and LCA disrupted phospholipid/sphingolipid homeostasis through TGF- β signaling [37]. Duboc et al[38] found that a decrease in serum LCA was implicated in the development of coronary atheromatous plaques.

However it was shown significant difference of LCA between the two groups only in exploration cohort, the possible reason may be the small sample size of the validation cohort.

TLCA is capable of impairing BA flow and inducing cholestasis [39]. It was shown that the contribution score of TLCA was high with high VIP score (Fig. 2; Supplementary Table S1). However, serum concentration of TLCA was quite low compared to primary BA (CA, CDCA and their conjugates) (Fig. 1; Supplementary Table S1). These results suggested that LCA and TLCA are key products for developing NAFLD in patients with T2DM. It was shown that TLCA upregulated AP-1 proteins cFos and JunB in HepG2-Ntcp hepatoma cells [40]. Amongyingcharoen et al [41] showed that TLCA induced cholangiocarcinoma cell growth via the muscarinic acetylcholine receptor (mAChR) and epidermal growth factor receptor (EGFR) /extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. However, the detailed mechanisms of the increased serum TLCA level in T2DM patients with NAFL than those without NAFL were unclear. Marialena et al[42] reported that patients with NASH had increased fecal BA synthesis and high fecal levels of CA and CDCA, which suggested a possible role for BAs in the progression of NAFL to NASH. Li et al[43] found that children with NAFLD had higher levels of CDCAs, CDCA and CA, and lower levels of total DCA, DCA, TDCA, GDCA, GLCA, and total conjugated LCA (GLCA + TLCA) compared to subjects without NAFLD. Similar to the progression of NAFLD to NASH, the formation of NAFLD in T2DM patients likely involves not just one pathway but rather repeated injuries over time[44].

Trottie et al [45] concluded that UGT1A3 was the main UGT enzyme for the formation of hepatic CDCA-24G and glucuronidation inhibited the ability of CDCA to act as an FXR activator.

TCDCA as cytotoxic bile acids, changes in bile flow, biliary excretions of bile acids[46]. It was shown serum levels of ALT and AST were highly elevated in all rats given TCDCA[46], while another study showed that TCDCA as a signaling molecule showed obvious anti-inflammatory and immune regulation properties via protein kinase C (PKC)/Jun N-terminal kinase (JNK)-dependent pathway[47]. Song et al [48] quantitatively analyzed 15 biliary bile acids in cholangiocarcinoma (CCA) (n = 30), benign biliary disease (n = 57) and pancreatic cancer (n = 17) patients and discovered GCA and TCDCA as specific CCA biomarkers. However, until now, the roles of CDCA-24G and TCDCA in the process of NAFLD in T2DM patients are unclear.

(B) UDCA metabolite

UDCA, a hydrophilic bile acid, is used to treat a number of cholestatic disorders. There was a significant improvement in the levels of aminotransferases and steatosis in NAFLD patients treating with UDCA [49]. However, it was shown that UDCA did not offer a histological benefit compared with placebo in NASH patients[50]. Therefore, till now UDCA has not been recommended to treat NAFLD/NASH. Daniel et al[51] found that 24-norursodeoxycholic acid (NorUDCA), a side-chain shorted derivative of UDCA, may be a promising new approach in the treatment of cholestatic and metabolic liver diseases with anti-fibrotic, anti-inflammatory and anti-lipotoxic properties. The detailed mechanism of the increased serum level of TUDCA in T2DM patients with NAFL than those without NAFL remains unclear. Beuers et al [39]

investigated that UDCA conjugates may improve the impaired secretory abilities of cholestatic hepatocytes.

It was reported that in the liver TUDCA improves insulin sensitivity and have cardiovascular protective effects by reducing ER stress [52]. It was reported that TUDCA attenuated hepatocarcinogenesis by suppressing carcinogen-induced ER stress-mediated cell death and inflammation without stimulating tumor progression [53]. However, until now there is no related research describing the effect of TUDCA on liver diseases.

There are still several limitations in our study to be addressed in the future studies. First, the sample size of groups may not be sufficient to achieve more powerful data although we validated the results in an independent cohort study. Second, although fast serum samples were used for analysis, the information about diet, sedentary lifestyle was not available. Nevertheless, there is no information on other confounding factors that may influence bile acid concentration such as treatments (e. g. statins) or the degree of insulin resistance[54, 55]. Thus we could not entirely exclude the effect of those treatments on metabolism. Third, the diagnosis of NAFL in our study was based on ultrasonography but not confirmed by histological examination. The guidelines for NAFL in Europe, America and China all stipulate that liver histological examinations are only necessary in clinical trials of new drugs or clinical studies of NASH. In the present study, the patients are all NAFL patients, and it is not obligatory to perform histological examinations considering the risk and benefit of patients. However, it is better to perform histological examination than ultrasonography if condition permits. Nevertheless, it is better to further illustrate the mechanistic insight into the changes in the BA profiles in the pathogenesis of NAFL in T2DM from experimental studies using animal models and/or cell lines. In addition, an integrative omics method with other -omics, such as proteomics, transcriptomics and genomics, should be explored. We also intend to perform both in vivo and in vitro studies in the near future, to confirm the mechanisms of selected BAs, such as LCA, TLCA, TUDCA, CDCA24G and TCDCA, in the formation and development of NAFL in T2DM patients.

5. Conclusion

To the best of our knowledge, it is the first study of this magnitude to provide a comprehensive association of bile acids and the progression of NAFL in T2DM patients. Our key finding is that the development and progression of liver disease in patients with T2DM (i.e., without NAFL→NAFL) are associated with metabolism disorders of serum bile acids. In summary, T2DM patients with NAFL have altered serum bile acid profiles compared to T2DM patients without NAFL, characterized by higher serum levels of LCA, TLCA, TUDCA, CDCA24G and TCDCA. Further understanding the role of bile acids in the pathogenesis of NAFL in T2DM patients may provide insights for the diagnosis of NAFL as well as potential therapeutic targets for treatment.

Abbreviations

T2DM	Type 2 diabetes mellitus
NAFLD	Nonalcoholic fatty liver disease
NAFL	Nonalcoholic fatty liver
NASH	Nonalcoholic steatohepatitis
HCC	Hepatocellular carcinoma
CVD	Cardiovascular disease
BAs	Bile acids
FXR	Farnesoid X receptor
PXR	Pregnane X receptor
VDR	Vitamin D receptor
GPBAR1 or TGR5	G-protein-coupled bile acid receptor
S1PR2	Sphingosin-1-phosphate receptor 2
CA	Cholic acid
GCA	Glycocholic acid
TCA	Taurocholic acid
CDCA	Chenodeoxycholic acid
GCDCA	Glycochenodeoxycholic acid
TCDCA	Taurochenodeoxycholic acid
DCA	Deoxycholic acid
GDCA	Glycodeoxycholic acid
TDCA	Taurodeoxycholic acid
UDCA	Ursodeoxycholic acid
GUDCA	Glycoursodeoxycholic acid
TUDCA	Tauroursodeoxycholic acid
LCA	Lithocholic acid
GLCA	Glycolithocholic acid
TLCA	Taurolithocholic acid
HCA	Hyochoolic acid
GHCA	Glycohyochoolic acid

T2DM	Type 2 diabetes mellitus
THCA	Taurohyocholic acid
α MCA	α -muricholic acid
T α MCA	Tauro- α -muricholic acid
β MCA	β -muricholic acid
T β MCA	Tauro- β -muricholic acid
ω MCA	ω -muricholic acid
T ω MCA	Tauro- ω -muricholic acid
HDCA	Hyodeoxycholic acid
GHDCA	Glycohyodeoxycholic acid
THDCA	Taurohyodeoxycholic acid
MuroCA	Muricholic acid
DHCA	Dehydrocholic acid
GDHCA	Glycodehydrocholic acid
TDHCA	Taurodehydrocholic acid
3-DHCA	3-Dehydrocholic acid
7-DHCA	7-Dehydrocholic acid
isoDCA	isodeoxycholic acid
apoCA	Apocholic acid
6-KLCA	6-ketolithocholic acid
7-KLCA	7-ketolithocholic acid
12-KLCA	12-ketolithocholic acid
23norDCA	23-nordeoxycholic acid
DHLCA	Dehydrolithocholic acid
LCA-3S	Lithocholic acid-3 sulfat
CDCA-24G	acyl CDCA-24 glucuronide
CA-D4	Cholic acid-2,2,4,4-D4
LCA-D4	Lithocholic acid-2,2,4,4-D4
IS	Internal standards

T2DM	Type 2 diabetes mellitus
CT	Computed tomography
UPLC	Ultraperformance liquid chromatography
TQ/MS	Triple Quadrupole mass spectrometry
TBA	Total bile acids
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
AUC	Area under the curve
IDI	Integrated discrimination improvement
NRI	Net reclassification improvement
PCA	Principal component analysis
ROC	Receiver operator characteristic
BMI	Body mass index
WHR	Waist-hip ratio
FPG	Fasting plasma glucose
2 h PG	Post-load plasma glucose
ALT	Alanine aminotransferase
TG	Triglyceride
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
TRAIL-R2	Tumor necrosis factor-related apoptosis-inducing ligand receptor 2
mAChR	Muscarinic acetylcholine receptor
EGFR	Epidermal growth factor receptor
ERK1/2	Extracellular signal-regulated kinase 1/2
PKC	Protein kinase C
JNK	Jun N-terminal kinase
NorUDCA	24-norursodeoxycholic acid
PKC	Putative protein kinase C
ER	Endoplasmic reticulum

Declarations

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Authors' contributions

G.J. and T.W. contributed to design the research and review of the final version of the manuscript. T.W. contributed to conduct/data collection, analysis of the research data and wrote the manuscript. M.Y. contributed to analysis of the paper. H.C.X., L.W. and H.F.W. contributed to data collection. G.J. revised the paper. All authors contributed to and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All patients who participated in the study provided signed informed consent prior to their participation. All the studies were approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine.

Consent for publication

All authors have read the final manuscript and have provided their consent to publish.

Competing interests

The authors declare that no competing financial interests exist.

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Figures

Figure 1

OPLS-DA analysis of 24 BAs from T2DM patients with and without NAFL in exploration cohort. (A), 3D OPLS-DA Plots, $R^2X=0.340$, $R^2Y=0.465$, $Q^2Y=0.176$; (B), Permutation analysis. T2DM, type 2 diabetes mellitus; NAFL, nonalcoholic fatty liver; OPLS-DA, orthogonal projections to latent structures discriminant analysis.

Figure 2

Serum concentrations of BAs and spearman correlations among the 5 NAFL-associated bile acids in T2DM patients with or without NAFL analyzed by UPLC-TQMS in exploration cohort. (A), Serum

concentrations of BAs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared with T2DM patients without NAFL. represents T2DM with NAFLD, represents T2DM without NAFLD. (B), Strong correlations were observed between LCA and TLCA ($\rho=0.562$); TLCA and TCDCA ($\rho=0.34$); TUDCA and CDCA24G ($\rho=0.278$); TUDCA and TCDCA ($\rho=0.677$); CDCA24G and TCDCA ($\rho=0.33$). “” means no significant correlation between the two BAs, ie $P>0.05$, otherwise no “” means significant correlation. T2DM, type 2 diabetes mellitus; NAFL, nonalcoholic fatty liver; UPLC-TQMS, ultra-performance liquid chromatography-triple quadrupole mass spectrometry; LCA, lithocholic acid; TLCA, tauroolithocholic acid; TUDCA, tauroursodeoxycholic acid; CDCA-24G, acyl CDCA-24 glucuronide; TCDCA, taurochenodeoxycholic acid.

Figure 3

Comparison of model performance from Decision Curve analysis in exploration and validation cohort. (A): exploration cohort; (B): validation cohort. Model 1, clinical factors; Model 4, clinical factors plus all metabolites; Model 5, clinical factors plus biomarker metabolites.

Figure 4

Schematic illustration of detected bile acids in T2DM patients with vs. without NAFL. (A), bile acid composition in patients; (B), possible mechanisms of increased bile acids. T2DM, type 2 diabetes mellitus; NAFL, nonalcoholic fatty liver; LCA, lithocholic acid; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; CDCA-24G, acyl CDCA-24 glucuronide; TCDCA, taurochenodeoxycholic acid; LysoPC, lysophosphatidylcholine; SM, sphingomyelins; mAChR, muscarinic acetylcholine receptor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; UGT 1A3, UDP-glucuronosyltransferase 1A3.

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