Secondary bile acids improve risk prediction for non-invasive identification of mild liver fibrosis in nonalcoholic fatty liver disease

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Abbreviation list:

NAFLD, nonalcoholic fatty liver disease; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NITs, non-invasive tests; BA, bile acid; OB, obesity; NOB, non-obesity; MetS, metabolic syndrome; No-MetS, non-metabolic syndrome; AUROC, area under receiver operating characteristic curve; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; S, steatosis; L, lobular inflammation; B, ballooning; NAS, NAFLD Activity Score; HFS, Hepatic Fibrosis Score; FIB-4, Fibrosis-4 index; NFS, NAFLD Fibrosis Score; WC, waist circumference; WBC, white blood cell count; TLCA, taurolithocholic acid; 6-ketoLCA, 6-ketolithocholic acid; dehydroLCA, dehydrolithocholic acid; 7-DHCA, 7-ketodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; NorCA, norcholic acid; UCA, ursocholic acid; TCA, taurocholic acid; βCA, 3β-cholic acid; βUCA, β-ursocholic acid; TCDCA,

taurochenodeoxycholic acid; GCA, glycocholic acid; HDCA, α-hyodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GGT, γ- glutamyltransferase; PLT, platelets; isoLCA, isolithocholic acid; CA, cholic acid; CDCA, chenodeoxycholic acid; 7-ketoLCA, 7-ketolithocholic acid; HCA, hyocholic acid; GDHCA, glycodehydrocholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; GDCA, glycodeoxycholic acid; TDCA, taurodeoxycholic acid.

Summary

Dysregulated bile acid (BA) metabolism has been linked to steatosis, inflammation, and fibrosis in individuals with nonalcoholic fatty liver disease (NAFLD). However, whether circulating BA levels may accurately stage liver fibrosis in NAFLD is currently uncertain. We recruited 550 Chinese adults with biopsy-proven NAFLD and varying stages of fibrosis. Ultra-performance liquid chromatography coupled with tandem mass spectrometry was performed to quantify a total of 38 serum BAs. We found that compared with those without fibrosis, patients with NAFLD and mild fibrosis (stage F1) had significantly higher secondary BAs, as well as higher values of diastolic blood pressure (DBP), serum alanine aminotransferase (ALT), body mass index, and waist circumstance (WC). The combination of BA biomarkers with WC, DBP, ALT, or HOMA-estimated insulin resistance performed well in identifying mild fibrosis, especially in men and women, and in subjects with or without obesity, with AUROCs of 0.80, 0.88, 0.75 and 0.78 in the training set (n=385), and 0.69, 0.80, 0.61, and 0.69 in the testing set (n=165), respectively. In comparison, the combination of BA and clinical biomarkers performed less well in identifying significant fibrosis (F2-4). In women and in non-obese subjects, the AUROCs were 0.75 and 0.71 in the training set, and 0.65 and 0.66 in the validation set, respectively. However, these AUROCs were higher than those observed for other commonly used non-invasive fibrosis scores, including the fibrosis-4 index, NAFLD fibrosis score, and Hepamet fibrosis score. In conclusion, secondary BA levels were significantly increased in individuals with NAFLD, especially in those with mild fibrosis. The combination of BA markers and clinical risk factors for identifying mild fibrosis is worthy of further assessment.

Keywords

nonalcoholic fatty liver disease; liver fibrosis; secondary bile acids; risk prediction

Introduction

It has been estimated that nonalcoholic fatty liver disease (NAFLD) affects up to a third of the world's adult population and the global prevalence of NAFLD will increase markedly in the next decade (1, 2). NAFLD includes a spectrum of potentially progressive liver conditions, ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) and cirrhosis (3). About 20% of patients with NASH may progress to cirrhosis (4, 5) and it has been reported that the severity of liver fibrosis is the strongest histological predictor of liver-related outcomes and mortality in NAFLD (6, 7). To date, liver biopsy remains the "gold standard" method for staging fibrosis in NAFLD (8), but this method is invasive, expensive, can cause morbidity, and cannot be routinely used for monitoring disease progression or treatment responses in clinical practice (9, 10).

Dysregulated bile acid (BA) metabolism has been implicated in the pathophysiology of chronic liver diseases, including NAFLD (11, 12). Primary BAs are synthesized from cholesterol in the liver. Following their synthesis, BAs are conjugated to an amino acid such as taurine and glycine and then secreted into bile, concentrated in the gall bladder, and released into the intestine after food ingestion. BAs carry out their important digestive functions aiding in the absorption of fats and fat-soluble vitamins (13). Besides, primary BAs are transported into the distal small bowel from where they are actively reabsorbed by the gut epithelium and return to the liver via enterohepatic circulation. Additionally, BAs pass into the colon and are bio-transformed into secondary BAs by intestinal microbiota through multiple different reactions, including deconjugation, 7α-dehydroxylation, 6α-hydroxylation, or epimerization (14). These secondary BAs are also absorbed and diversify the BA pool in the body. During this process, BAs can enter into the systemic circulation, and act as biologically active signaling molecules to regulate glucose and lipid homeostasis (15), mainly through the activation of specific receptors, such as farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) (16). Dysregulated BA homeostasis and impaired

BA signaling can lead to liver damage, thereby contributing to the development and progression of NAFLD (17). Hepatic BA accumulation leads to hepatocyte apoptosis, mitochondrial damage, and endoplasmic reticulum stress (18). Both conjugated and unconjugated BAs at cholestatic levels also lead to a release of multiple proinflammatory cytokines, which can activate hepatic stellate cells and induce hepatic fibrogenesis (19). On this background of evidence, it is conceivable that modulation of BA synthesis and metabolism could become a valid therapeutic option for NAFLD and its related metabolic diseases (20, 21).

It is known that the high heterogeneity of NAFLD may result from a complex and multilayered dynamic interaction between different factors, such as sex, obesity, diabetes, and other coexisting metabolic disorders (22, 23), which are also closely associated with BA synthesis and metabolism. BA synthesis is higher in men than women with a wider inter-individual variation (24, 25). Sex-related differences in BA synthesis and metabolism have been shown in steatosis, NASH, and hepatocellular carcinoma (26). The differential BAs, related gut microbiota and signaling pathways need to be further investigated to better understand their effects on disease heterogeneity (27). Moreover, individuals with lean NAFLD have an obesity-resistant phenotype that could be, at least in part, mediated by higher levels of certain BAs and different gut microbiota composition (with higher amounts of microbes involved in BA metabolism), thus contributing to explain their milder liver disease and more favorable metabolic profiles compared to NAFLD individuals with obesity (28). Distinct signatures of gut microbiome and BAs have been also identified in the stool samples of individuals with lean NAFLD and fibrosis (29). Thus, it is reasonable to assume that a better understanding of BA profiles in different subgroups of NAFLD individuals can also help to better decipher the clinical heterogeneity of NAFLD and to develop more targeted pharmacotherapies for NAFLD and NASH.

Therefore, in a large cohort of Chinese adults with biopsy-confirmed NAFLD and

fibrosis, we aimed to examine the differences in a large panel of circulating BA levels in patients with varying levels of liver fibrosis. In addition, we developed and validated prediction models using serum BAs and clinical/biochemical biomarkers, alone or in combination, for the non-invasive identification of mild and significant fibrosis, both in the whole cohort and in different subgroups of patients stratified by sex, and the presence or absence of obesity and metabolic syndrome.

Materials and Methods

Patient recruitment

This is a retrospective analysis of our well-characterized Prospective Epidemic Research Specifically of NASH (PERSONS) cohort. All NAFLD patients in this study were consecutively recruited from 2016 to 2019 at the First Affiliated Hospital of Wenzhou Medical University in Wenzhou (China). The inclusion and exclusion criteria have been described extensively elsewhere (30). Briefly, patients were initially diagnosed with suspected NAFLD based on the presence of imaging-defined hepatic steatosis and/or persistently elevated serum transaminase levels with coexisting metabolic risk factors (such as overweight/obesity, type 2 diabetes, or metabolic syndrome), in the absence of significant alcohol consumption (≥140 g/week in men or ≥70 g/week in women). All these patients underwent a diagnostic liver biopsy. Subsequently, we excluded from the analysis patients with at least one of the following criteria: (1) those with chronic liver disease from other etiologies (such as viral hepatitis or autoimmune hepatitis); (2) those chronically treated with drugs potentially inducing steatosis; (3) those with liver cancers or other extrahepatic malignancies; and (4) those with liver fat content <5% on histology. According to these exclusion criteria and the availability of serum samples, 550 Chinese adults with biopsy-proven NAFLD were included in the present study. Overweight/obesity was defined as BMI≥25. Metabolic syndrome was defined as having three or more of the following criteria: overweight/obesity, high triglyceride level (≥ 1. 7 mmol/L), reduced HDL cholesterol levels (HDL-c < 1.03 mmol/L for men and < 1.29 mmol/L for women), high blood

pressure ≥ 130/85 mmHg, and elevated fasting glucose level (≥ 5.6 mmol/L) or diagnosed with type 2 diabetes. Written informed consent was obtained from each subject before study participation. The research protocol was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University (2016-246, 1 December 2016) and registered in the Chinese Clinical Trial Registry (ChiCTR-EOC-17013562).

Liver histology assessment

An ultrasound-guided liver biopsy was performed using a 16-gauge Hepafix needle (Gallini, Modena, Italy). All biopsy specimens were analyzed by an experienced liver pathologist, who was blinded to participants' clinical and laboratory data. The histologic features of NAFLD were scored according to the NASH-Clinical Research Network (NASH-CRN) scoring system (31). The stage of fibrosis was quantified according to Brunt's criteria (32). Mild and significant fibrosis was defined as fibrosis F1 and ≥F2 on histology, respectively.

Clinical and laboratory parameters

In all participants, demographic characteristics and anthropometric measurements were collected on the day of the liver biopsy examination. Venous blood samples were obtained after overnight fasting for standard laboratory biochemical tests, including serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), bilirubin, albumin, glucose, insulin, lipids, creatinine, uric acid, and complete blood count. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated according to the following formula: fasting insulin (μU/mL) * fasting glucose (mmol/L) / 22.5 (33). Three commonly used non-invasive fibrosis scores were also calculated using established equations (34-36), including the fibrosis-4 (FIB-4) index, NAFLD fibrosis score (NFS), and Hepamet fibrosis score (HFS).

Serum BA measurement

A 20 μL serum sample together with 180 μL of acetonitrile/methanol (8:2) containing 10 internal standards was added into a 96-well plate. The metabolite extraction was centrifuged at 10°C and 1,500 rpm for 20 min. After centrifugation, the supernatant was transferred to a microcentrifuge tube for lyophilization using a FreeZone freeze dryer equipped with a stopping tray system (Labconco, Kansas City, MO, USA). The supernatant was transferred to a 96-well plate for ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) analysis (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA) (37). The BA standards were obtained from Steraloids Inc. (Newport, RI, USA) and TRC Chemicals (Toronto, ON, Canada), and 10 stable isotope-labeled standards were obtained from C/D/N Isotopes Inc. (Quebec, Canada) and Steraloids Inc. (Newport, RI, USA). Column ACQUITY UPLC Cortecs C18 1.6 μM VanGuard pre-column (2.1×5 mm) and ACQUITY UPLC Cortecs C18 1.6 μM analytical column (2.1 × 100 mm) were used. Column temperature and sample manager temperature were 30°C and 10°C, respectively. The mobile phases were water with formic acid (pH =3.25) (A) and acetonitrile/methanol (80:20) (B). The gradient conditions at a flow rate of 0.4 mL/min were as follows: 0-1 min (5% B), 1-3 min (5-30% B), 3-15 min (30-100% B), 15-16 min (100-5%B), 16-17 min (5%B). The source temperature and desolvation temperatures were 150°C and 550°C, respectively. Raw data generated by UPLC-MS/MS were processed using the TargetLynx software to perform peak integration, calibration, and quantitation for each BA metabolite. Missing values were preprocessed using the quantile regression imputation of left-censored data (QRLIC) method (38). A total of 38 BAs were identified and quantified in serum samples (**Supplementary Table 1**). Serum BAs were classified into 8 categories according to their chemical structures, including primary glycine or taurine conjugated BAs, primary unconjugated BAs, secondary glycine or taurine conjugated BAs, secondary unconjugated BAs, sulfated BAs, and glucuronidated BAs, respectively.

Statistical analysis

R software (version 3.6.3, R Foundation for Statistical Computing, Vienna, Austria) was applied for statistical analysis and visualization. The normal distribution of variables was initially tested using the Shapiro-Wilk test. Then, parametric tests (i.e., the Student's t-test and the one-way ANOVA) were performed on variables with normal distribution. Meanwhile, non-parametric tests (i.e., the Mann-Whitney U test and the Kruskal Wallis test) were used for variables that were not normally distributed. The chi-square test was used for categorical variables. Spearman's rank correlation coefficients were calculated to examine the associations between liver fibrosis, BAs, and other clinical and biochemical parameters. Random forest analysis was applied for biomarker selection. Binary logistic regression models were then performed to establish predictive models for fibrosis. Areas under receiver operating characteristic curves (AUROC) were calculated to evaluate the performance of non-invasive predictive models for identifying mild (F1) or significant fibrosis (F2-4). Two-tailed $P<0.05$ value was considered to be statistically significant. All *P* values were further adjusted for multiple testing corrections by the Benjamini and Hochberg statistical procedure.

Results

Clinical and biochemical biomarkers associated with mild and significant fibrosis

The study design is summarized in **Figure 1**. A total of 550 Chinese adults with biopsy-proven NAFLD were included in the study. They were further divided into six subgroups according to sex, and the presence or absence of obesity (OB) or metabolic syndrome (MetS). They were also divided into patients without fibrosis (stage F0), patients with mild fibrosis (stage F1), and those with significant fibrosis (stages F2-4). Clinical characteristics and BA profiles were then examined at different fibrosis stages for each patient subgroup. Meanwhile, all these 550 patients with NAFLD were randomly subdivided into the training (n=385) and validation (n=165) sets, according to a ratio of 7:3 (39), to develop and validate predictive models for the non-invasive diagnosis of mild and significant fibrosis.

The main clinical, biochemical, and histological characteristics of the whole cohort of NAFLD patients, stratified by increasing fibrosis stages are summarized in **Table 1**. Adiposity measures, diastolic blood pressure (DBP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), HOMA-IR, hemoglobin A1c (HbA1c), white blood cell count (WBC) and hemoglobin increased significantly across fibrosis stages. Similarly, the histological severity of hepatic steatosis, lobular inflammation, and NAFLD Activity Score (NAS) increased significantly with increasing fibrosis stages. The Hepatic Fibrosis Score (HFS) emerged as the best non-invasive score for staging fibrosis compared to FIB-4 and NFS scores.

Baseline clinical and biochemical parameters for discriminating mild and significant fibrosis were further evaluated and compared in the (aforementioned) six patient subgroups. **Figure 2A** shows the heatmap of their fold-change values by comparing either F1 vs. F0 or F2-4 vs. F0-1, respectively. DBP was increased significantly in most patient subgroups with mild fibrosis ($P<0.001$), except for women. Adiposity measures (BMI and WC) were increased in men and OB, MetS patient subgroups with mild and significant fibrosis. Serum liver enzymes (ALT, AST, and GGT) were increased in mild fibrosis as compared to F0. HbA1c and WBC were increased in significant fibrosis. The histological severity of steatosis and lobular inflammation increased with increasing fibrosis stages in most patient subgroups (all $P<0.01$). Also, NAS was a significant marker for discriminating mild fibrosis both in men and women, as well as in OB and MetS, subgroups. Among the three commonly used non-invasive fibrosis scores, HFS was better than FIB-4 and NFS scores to stage fibrosis. To summarize, twelve differential markers for mild or significant fibrosis identified in more than three patient subgroups are reported in **Figure 2B**, showing their differences among F0, F1, and F2-4 subgroups in the whole cohort.

Overall BA profiles in NAFLD with mild and significant fibrosis

Overall, we identified that secondary unconjugated BAs, primary glycine-conjugated

BAs, and primary unconjugated BAs covered more than 80% of the BA pool in the serum (**Figure 3A)**. The secondary unconjugated BAs were increased in patients with mild and significant fibrosis. The top eleven abundant BAs were GCDCA, βUDCA, CDCA, CDCA, βCDCA, GCA, DCA, GUDCA, GLCA-3S, CA, and UDCA, respectively (**Figure 3B**). The chemical names of individual BAs and their classifications are reported in **Supplementary Table 1.** The circus plots showed differential BAs (P<0.05 highlighted in red) and their fold changes by comparing F1 vs. F0 (mild fibrosis, **Figure 3C**), and F2-4 vs. F0-1 (significant fibrosis, **Figure 3D**), respectively. TCA and nine secondary BAs were increased in mild fibrosis, while UCA, βUCA, 7-DHCA, and NorCA were increased in significant fibrosis.

BA changes in mild and significant fibrosis in different subgroups

We compared the changes of individual BAs in the presence of mild and significant fibrosis across the six different subgroups of NAFLD patients (**Figure 4A**). In general, BA profiles changed more significantly in patients with mild fibrosis than in those with significant fibrosis. Specifically, secondary BAs (HDCA, UCA, βCA, 7-DHCA, βUCA, dehydroLCA, 6-ketoLCA, and TLCA) were significantly increased in patients with mild fibrosis (F1) compared to those without fibrosis (F0). However, in the presence of significant fibrosis (F2-4 vs. F0-1), CA and CDCA increased in women but decreased in men and the no-MetS subgroup. Three secondary BAs (βCA, 7-DHCA, and βUCA) were increased in women and the OB and MetS patient subgroups. In the whole cohort of patients, ten serum BAs increased progressively across fibrosis stages (**Figure 4B**, all $P<0.05$).

Spearman's rank correlation analyses showed that twelve secondary BAs and two primary BAs were significantly associated with fibrosis, including NorCA, UCA, βCA, 6-ketoLCA, HDCA, 7-DHCA, βUCA, dehydroLCA, TLCA, βCDCA, TCA, and TCDCA (**Figure 4C**). Meanwhile, six biochemical parameters (HbA1c, fasting glucose, HOMA-IR, ALT, AST, and WBC) and four demographic and anthropometric

parameters (weight, BMI, WC, and DBP) were significantly associated with liver fibrosis, together with HFS, NFS, S, and L indices. These aforementioned BAs and clinical/biochemical parameters were ordered by the number of connections between them in the Sankey plot, which indicated that NorCA, TCA, and TCDCA were more strongly (darker red color) associated with clinical/biochemical parameters.

Biomarker discovery and validation for identifying fibrosis severity

Through biomarker selection by random forest analysis and predictive modeling by logistic regression analysis, the combination of serum BAs and clinical/biochemical biomarkers enabled us to obtain optimal non-invasive predictive models for identifying liver fibrosis (**Figure 5** and **Table 2**). Notably, the performance of these non-invasive predictive models differed in the six subgroups of NAFLD patients. In particular, we obtained four predictive models that had good performance in identifying mild fibrosis in men and women, as well as in OB and NOB patient subgroups, with AUROCs of 0.80, 0.88, 0.75, 0.78 in the training set (threshold >0.7), respectively, and 0.69, 0.80, 0.61, 0.69 in the validation set (threshold >0.6), respectively. Among these, the predictive model for identifying mild fibrosis in women was the best one. Accordingly, the predictive model for the whole cohort also achieved a relatively good performance in identifying mild fibrosis with AUROC values of 0.77 in the training set and 0.64 in the validation set, respectively. We also compared three commonly used non-invasive scores of fibrosis and identified that the HFS had the best performance in most of our NAFLD patients, while FIB-4 had the best performance in the no-MetS patient subgroup. However, both of these non-invasive fibrosis scores failed to achieve good diagnostic performance for identifying mild fibrosis (AUROC value < 0.7 in the training set or <0.6 in the testing set).

In comparison, the predictive model for identifying significant fibrosis showed overall good performance in women and the NOB subgroup with AUROCs of 0.75 and 0.71 in the training set, and 0.65 and 0.66 in the validation set, respectively (**Figure 5** and **Table**

2). Similar to the above-mentioned predictive models for mild fibrosis, the three commonly used non-invasive scores of fibrosis failed to achieve a good performance in both the training and validation sets for identifying significant fibrosis, with AUROCs less than 0.60.

Discussion

Our novel results show that compared with those without fibrosis, patients with NAFLD and mild fibrosis (stage F1) had significantly higher secondary BAs, as well as higher values of DBP, ALT, BMI, and WC. The combination of BA biomarkers with WC, DBP, ALT or HOMA-estimated insulin resistance performed well in identifying mild fibrosis, especially in men and women, and in subjects with or without obesity. The combination of BA biomarkers and clinical risk factors performed less well in identifying significant fibrosis (F2-4) although in this fibrosis group prediction was better in women and non-obese subjects. Importantly, the AUROCs including BAs were higher than those observed for other commonly used non-invasive fibrosis scores, including the fibrosis-4 index, NAFLD fibrosis score, and Hepamet fibrosis score.

BA changes and their associations with liver fibrosis

Increased serum and hepatic BAs have been recognized as important metabolic factors in the pathophysiology of NAFLD and reported to be associated with greater severity of NAFLD and liver fibrosis (29, 40-46). However, most previously published studies had a case-control design and compared BA profiles between NAFLD patients and healthy controls or non-NAFLD individuals (**Supplementary Table 2**). In this cross-sectional study of Chinese adults with biopsy-confirmed NAFLD, we measured a large panel of circulating BA levels and compared their changes in the presence of mild and significant liver fibrosis among different subgroups of NAFLD patients. We found an altered BA profile in the presence of mild fibrosis that was specifically characterized by increased primary BAs, mainly represented by CA, TCA, and GCA in the female and MetS patient subgroups, and by secondary BAs, mainly HDCA, UCA, CA, 7-

DHCA, UCA, dehydroLCA, 6-ketoLCA, and TLCA, in the whole patient population (**Figure 4A** and **Figure 4B**). The overall secondary BA profiles were also increased in the presence of significant fibrosis, but their increases as compared to mild fibrosis (F2-4 vs. F0-1) were not as significant and consistent as the increases observed in mild fibrosis (F1 vs. F0). Specifically, CA and CDCA were significantly increased in women but decreased in men and the no-MetS subgroup. Secondary BAs were found significantly increased in women and the OB, and MetS patient subgroups. Moreover, those BAs that were closely associated with liver fibrosis were also significantly correlated with serum liver enzymes and glycemic parameters (**Figure 4C**).

It is possible to hypothesize that significant changes in secondary BA metabolism might be causally linked with intestinal dysbiosis and greater severity of liver fibrosis (47-49). In our study, we found that LCA species were significantly increased in patients with mild fibrosis, including TLCA, 6-ketoLCA, and dehydroLCA, and were closely associated with fibrosis severity (**Figure 4**). LCA species also increased significantly in the OB patient subgroup with significant fibrosis (**Figure 4**). A previous cross-sectional study of 390 Mexican-American subjects screened with liver elastography also reported that higher serum LCA levels were associated with significant fibrosis (50). Compared to primary BAs, secondary BAs (e.g., DCA and LCA) can more effectively activate TGR5 which is expressed in Kupffer cells and hepatic stellate cells(HSCs) (51, 52). LCA is considered to be hepatotoxic as the most hydrophobic BA (53), and it has been used to produce a model of cholestatic liver damage (54). Serving as a physiological sensor of LCA, the pregnane X receptor (PXR) could be activated to protect against severe liver damage induced by LCA (55). However, experimental studies revealed that LCA and its derivatives may inhibit the activation of HSCs and have anti-inflammatory effects on liver fibrosis by inhibiting glycolysis and promoting oxidative phosphorylation, thus leading to macrophage polarization toward the M2 phenotype (56). From these and other studies emerges that secondary BAs that are produced and/or modified through gut microbiota, and their enterohepatic circulation

and conjugation in the liver, could play a role in the early stage of fibrosis in NAFLD.

Predictive models combining clinical and BA markers for fibrosis severity

Several non-invasive fibrosis scores, such as FIB-4, NFS, and HFS scores, have been regarded as sufficiently reliable biomarkers for ruling out advanced fibrosis in NAFLD (34-36). In our study, HFS was better than FIB-4 and NFS to predict mild and significant fibrosis, but in any case, HFS did not achieve a good performance in identifying fibrosis (AUROC <0.6) in most subgroups of our NAFLD patients. The diagnostic performance of these three commonly used non-invasive fibrosis scores might vary with ethnicity, age, sex, disease severity, comorbidities, and treatment of patients (57, 58). Here, the integration of machine learning and logistic regression analyses allowed us to build non-invasive predictive models for identifying mild and significant fibrosis by combining clinical and BA biomarkers. DBP, ALT, AST, HOMA-IR, fasting insulin, WBC, and HbA1c were closely associated with the severity of liver fibrosis, together with WC and BMI (**Table 1, Figures 2** and **4**). The predictive model by combining WC, DBP, and ALT with 6-ketoLCA, HDCA, dehydroLCA, TLCA, isoLCA, and βCA performed well in identifying mild fibrosis in our whole patient population (**Figure 5** and **Table 2**). WBC and BMI were the two biochemical biomarkers selected for predicting significant fibrosis with 7-DHCA and TUDCA.

Clinical heterogeneity of NAFLD

A spectrum of variables, including sex, obesity, and metabolic disorders, may determine the heterogeneity of NAFLD observed in clinical practice (59). Thus, more accurate and refined characterization and stratification of this common liver disease are needed for precision medicine in NAFLD (60, 61). Applying a targeted metabolomics approach and integrating clinical biomarkers can help us to better define the specific metabolic features and biochemical snapshots among different patient subgroups that might contribute to precision medicine in NAFLD. In our study, increased serum aminotransferase levels and impaired glycemic control were associated with increased

liver fibrosis, particularly in men and in the OB and MetS patient subgroups (**Figure 2**). In contrast, these associations were weaker in women and the NOB and no-MetS patient subgroups. These results are consistent with the existence of sex-related differences in NAFLD, supporting that men are at higher risk of visceral adiposity and MetS (62, 63). Meanwhile, we identified that BAs were closely associated with mild and significant fibrosis in women and the OB and MetS patient subgroups (**Figure 4A**). The non-invasive predictive models combining clinical markers and BAs showed good performance in identifying mild fibrosis in both sexes and the OB/NOB patient subgroups, but not so good in the MetS/no-MetS patient subgroups. For identifying significant fibrosis, we found that the predictive model performed better in women and the NOB patient subgroup. Thus, these results suggest that the effect of dysregulated BA metabolism in hepatic fibrogenesis of NAFLD may play a differential role in different patient subgroups. It is, therefore, of great clinical importance to consider disease heterogeneity for exploring diagnostic and prognostic biomarkers of liver fibrosis in NAFLD.

Taken together, this is one of the largest cross-sectional cohorts of Asian adults with biopsy-proven NAFLD focusing on the biomarker discovery for mild and significant liver fibrosis. The changes of secondary BAs observed in mild fibrosis, instead of in significant fibrosis, suggest an important role of these molecules in the early development of liver fibrosis in NAFLD. Different from some previous case-control studies, our study aims to identify the specific metabolic features among different NAFLD patient subgroups. However, it should be noted that a limitation of our study design is that our exploratory sub-group analyses have limited power to detect differences between groups. In the six different subgroups, the sample size in each group is not large enough and further studies are needed to verify our findings in other ethnic groups. Moreover, other studies are required to examine whether BA profiles may significantly differ between NAFLD and chronic liver diseases from other etiologies, and to elucidate whether different etiologies of chronic liver disease may

differentially impact BA pools for any given stage of fibrosis. Finally, gut microbiota composition in relation to BA metabolism and their complex cross-talk are future priorities in the NAFLD research arena.

Conclusion

The results of this large cross-sectional study show that the circulating levels of secondary BAs (including LCA species) were biomarkers for predicting mild liver fibrosis in Chinese adults with biopsy-proven NAFLD. In addition, the combination of BAs and clinical biomarkers had good performance in identifying liver fibrosis in NAFLD. Our newly developed predictive models achieved a better diagnostic performance in identifying mild fibrosis than significant fibrosis. We suggest further studies in different ethnic populations are now required to validate our findings.

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Authorship Contribution Statement

YN and MHZ conceived and designed this project. YN, ANL, and YRL drafted the manuscript. YN and CFX performed the data processing, statistical analysis, and visualization. ANL, LJ, and MMS performed the sample processing and BA analysis

using UPLC-MS/MS platform. DQS, LJT, and PWZ collected biological samples and performed biochemical tests. SDC conducted histopathological analysis of liver tissues. MHZ, FJF, WYL, and XDW took clinical assessment and diagnosis. GT, CDB, VWW, and RL contributed to manuscript writing and revision.

Declaration of competing interests

Vincent Wai-Sun Wong served as a speaker and/or consultant for Echosens. Mingming Su is employed by Shanghai Keyi Biotech., Shanghai, China. Other authors have no conflicts of interest. RL serves as a consultant to Aardvark Therapeutics, Altimmune, Anylam/Regeneron, Amgen, Arrowhead Pharmaceuticals, AstraZeneca, Bristol-Myer Squibb, CohBar, Eli Lilly, Galmed, Gilead, Glympse bio, Hightide, Inipharma, Intercept, Inventiva, Ionis, Janssen Inc., Madrigal, Metacrine, Inc., NGM Biopharmaceuticals, Novartis, Novo Nordisk, Merck, Pfizer, Sagimet, Theratechnologies, Terns Pharmaceuticals and Viking Therapeutics. In addition, his institutions received research grants from Arrowhead Pharmaceuticals, Astrazeneca, Boehringer-Ingelheim, Bristol-Myers Squibb, Eli Lilly, Galectin Therapeutics, Galmed Pharmaceuticals, Gilead, Intercept, Hanmi, Intercept, Inventiva, Ionis, Janssen, Madrigal Pharmaceuticals, Merck, NGM Biopharmaceuticals, Novo Nordisk, Merck, Pfizer, Sonic Incytes and Terns Pharmaceuticals. Co-founder of LipoNexus Inc.

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Figure legends

Figure 1. The flowchart of biomarker discovery and validation in patients with biopsy-proven NAFLD.

Figure 2. Comparison of clinical parameters in six subgroups of NAFLD patients in the training cohort. (A) The heatmap of fold changes of demographic, biological, and histological variables and non-invasive diagnostic indices between F1 and F0, and between F2-4 and F0-1 in the six patient subgroups of the training cohort. *P* values were tested by parametric or non-parametric tests (as appropriate). (B) The changes of potential clinical risk factors among NAFLD patients with F0, F1, and F2-4 stages. $P<0.05$ is demonstrated as an asterisk (*); $P<0.01$ is demonstrated as two asterisks (**); $P<0.001$ is demonstrated as three asterisks (***); and $P<0.0001$ is demonstrated as four asterisks (****).

Figure 3. Overall bile acid profiles of patients with NAFLD. (A) The bar plot of relative abundances of different groups of bile acids from F0, F1 to F2-4 stages. (B) The bar plot of relative abundances of top-ten abundant bile acids from F0, F1 to F2-4 stages. (C-D) The circus plot of differential bile acids (fold changes) by comparing F1 vs. F0, and F2-4 vs. F0-1, respectively. The reference circle value is defined as a fold-change value of 1.

Figure 4. The bile acid changes with the development of fibrosis in six subgroups of NAFLD patients in the training cohort. (A) The heatmap of fold changes of bile acids between F1 and F0, and F2-4 and F0-1 in the six patient subgroups of training cohort. *P* values were determined by non-parametric tests (as appropriate). (B) The bar plots of differential bile acid biomarkers among patients with F0, F1, and F2-4 fibrosis. $P<0.05$ is demonstrated as an asterisk (*); $P<0.01$ is demonstrated as two asterisks (**); $P<0.001$ is demonstrated as three asterisks (***); and $P<0.0001$ is demonstrated as four asterisks (****). (C) The Sankey network of correlations among bile acids, clinical

parameters, and fibrosis in patients with NAFLD. Bile acids, clinical parameters, and liver fibrosis stage are shown as different colors of dots. The connecting lines between dots represent the correlation, with red lines referring to positive correlation, and green lines referring to negative correlation. And the width of connecting lines is depending on the correlation coefficients. *P* values and correlation coefficients were assessed by Spearman's analysis. Only Spearman's correlation analyses with a *P* value <0.05 were depicted.

Figure 5. Predictive models for mild and significant liver fibrosis. The figure shows the area under the receiver operating characteristic curves (AUROC) with recommended prediction models with an excellent diagnostic performance marked with a red star.

Table 1. Demographic, biochemical, and histological characteristics of patients with biopsy-confirmed NAFLD (n=550), stratified by increasing stages of liver fibrosis.

Characteristics	F0 (n=145)	F1 (n=273)	F2-4 (n=132)	*P*-value	*P*-value*
Demographics					
Age (y)	42.34 ± 12.26	42.82 ± 12.07	43.45 ± 13.28	0.900	0.949
Male sex, n (%)	104 (72%)	203 (74%)	89 (67%)	0.345	0.345
Height (cm)	167.14 ± 8.44	167.63 ± 8.24	166.58 ± 9.34	0.607	0.763
Weight (kg)	72.11 ± 12.28	75.86 ± 13.24	76.29 ± 14.89	1.66E-02	5.00E-02
BMI (kg/m^2)	25.68 ± 2.96	26.88 ± 3.61	27.4 ± 4.46	2.84E-04	1.80E-03
WC (cm)	89.1 ± 7.91	92.08 ± 8.42	93.75 ± 9.49	2.30E-05	3.00E-04
SBP (mmHg)	125.43 ± 16.16	128.83 ± 14.95	128.27 ± 16.74	0.127	0.291
DBP (mmHg)	77.68 ± 10.07	83.07 ± 9.98	81.43 ± 10.82	2.37E-06	4.60E-05
Obesity, n (%)	101 (70%)	219 (80%)	111 (84%)	8.25E-03	1.40E-02
Type 2 diabetes, n (%)	51 (35%)	126 (46%)	72 (55%)	4.90E-03	1.20E-02
Hypertension, n (%)	73 (50%)	178 (65%)	81 (61%)	1.22E-02	1.50E-02
MetS, n (%)	80 (55%)	194 (71%)	103 (78%)	1.04E-04	5.20E-04
Biochemical parameters					
ALT (U/L)	63.9 ± 92.88	73.35 ± 61.25	73.46 ± 67.85	4.11E-03	1.80E-02
AST (U/L)	41.36 ± 44.45	45.26 ± 32.7	49.6 ± 36.93	5.52E-03	2.20E-02
ALP (U/L)	89.06 ± 44.86	86.33 ± 27.22	85.7 ± 36.78	0.806	0.916
GGT (U/L)	71.48 ± 97.04	70.55 ± 59.48	78.49 ± 100.94	0.221	0.384
TBIL (µmol/L)	14.74 ± 12.04	14.14 ± 7.09	14.04 ± 6.7	0.951	0.951
DBIL (µmol/L)	5.83 ± 9.35	4.8 ± 2.35	4.87 ± 2.2	0.332	0.498
IBIL (µmol/L)	8.92 ± 4.44	9.34 ± 5.03	9.16 ± 4.91	0.547	0.711
Total protein (g/L)	76.83 ± 5.42	76.85 ± 5.87	76.93 ± 5.58	0.83	0.916
Albumin (g/L)	45.97 ± 3.85	45.79 ± 4.29	45.55 ± 4.22	0.943	0.951
Globulin (g/L)	31 ± 4.1	30.95 ± 3.82	31.38 ± 4.15	0.473	0.659
A/G ratio	1.51 ± 0.24	1.5 ± 0.21	1.48 ± 0.26	0.469	0.659
Glucose (mmol/L)	5.67 ± 1.86	5.82 ± 1.57	6.01 ± 1.94	0.072	0.186
Insulin (pmol/L)	121.34 ± 125.77	125.76 ± 109.18	136.71 ± 119.09	0.181	0.371
HOMA-IR score	4.94 ± 7.79	5.04 ± 6.35	5.4 ± 5.5	3.62E-02	0.101

HbA1c (%)	6.07 ± 1.75	6.25 ± 1.52	6.57 ± 1.51	6.28E-04	3.50E-03
Creatinine (μmol/L)	67.77 ± 14.53	67.73 ± 13.91	65.86 ± 15.91	0.158	0.343
Uric acid (μmol/L)	384.57 ± 101.22	388.24 ± 101.55	381.17 ± 111.2	0.771	0.916
Total cholesterol (mmol/L)	5.12 ± 1.15	5.15 ± 1.17	5.15 ± 1.27	0.846	0.916
Triglycerides (mmol/L)	2.31 ± 1.69	2.21 ± 1.33	2.51 ± 2.92	0.821	0.916
HDL-c (mmol/L)	1.04 ± 0.24	1.01 ± 0.22	1 ± 0.24	0.296	0.482
LDL-c (mmol/L)	3.1 ± 0.9	3.03 ± 0.89	2.99 ± 1.02	0.499	0.671
WBC ($\times 10^9$/L)	5.92 ± 1.35	6.27 ± 1.53	6.47 ± 1.76	1.40E-02	5.00E-02
RBC ($\times 10^9$/L)	4.96 ± 0.49	4.97 ± 0.51	4.89 ± 0.56	0.226	0.384
Hb (g/L)	146.52 ± 14.63	149.53 ± 14.21	145.06 ± 16.29	1.55E-02	5.00E-02
PLT ($\times 10^9$/L)	248.37 ± 63.88	240.21 ± 59.46	252.42 ± 66.12	0.207	0.384
Liver histology features					
Steatosis, n (%)					
0	43 (30%)	42 (15%)	17 (13%)	4.26E-05	4.10E-04
1	51 (35%)	85 (31%)	37 (28%)		
2	19 (13%)	56 (21%)	31 (23%)		
3	32 (22%)	90 (33%)	47 (36%)		
Ballooning, n (%)					
0	0:21 (14%)	64 (23%)	20 (15%)	0.203	0.384
1	80 (55%)	134 (49%)	74 (56%)		
2	44 (30%)	75 (27%)	38 (29%)		
Lobular inflammation, n (%)					
0	19 (13%)	14 (5%)	2 (2%)	2.55E-07	1.00E-05
1	112 (77%)	200 (73%)	89 (67%)		
2	13 (9%)	56 (21%)	37 (28%)		
3	1 (1%)	3 (1%)	4 (3%)		
NAS score	3.41 ± 1.65	3.93 ± 1.78	4.28 ± 1.59	7.72E-05	6.00E-04
Non-invasive fibrosis scores					
FIB-4	14.73 ± 6.63	14.99 ± 6.86	17.33 ± 10.53	0.325	0.498
NFS	0.55 ± 1.41	0.76 ± 1.38	0.95 ± 1.68	0.076	0.186
HFS	0.06 ± 0.09	0.09 ± 0.12	0.12 ± 0.18	3.00E-03	1.50E-02

Abbreviations: BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; MetS, metabolic syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ- glutamyltransferase; TBIL, total bilirubin; DBIL, direct bilirubin; IBIL, indirect bilirubin; A/G, the ratio of albumin to globulin; HOMA-IR, homeostasis model assessment of insulin resistance; HbA1c, hemoglobin A1c; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; PLT, counts of platelet; S, steatosis; B, ballooning; L, lobular inflammation; FIB-4, Fibrosis-4 index; NFS, NAFLD Fibrosis Score; HFS, Hepatic Fibrosis Score. P-values are assessed by parametric or non-parametric tests (as appropriate). *P-values assessed by the Benjamini–Hochberg procedure after multiple testing corrections.

Table 2. Predictive diagnostic performance of established non-invasive models for different stages of liver fibrosis in the training and testing sets.

Stage F1 vs. F0				
Group	**Panel**	**Content**	**Training AUC**	**Testing AUC**
All (n=550)	BA + Clinical panel	**WC + DBP + 6-ketoLCA + HDCA + dehydroLCA + TLCA + isoLCA + ALT + βCA**	**0.77**	**0.64**
	Non-invasive diagnostic index	HFS	0.58	0.55
Men (n=396)	BA + Clinical panel	**DBP + WC + 6-ketoLCA + TLCA + βCA + AST + HOMA-IR + CDCA-3Glu**	**0.80**	**0.69**
	Non-invasive diagnostic index	HFS	0.67	0.54
Women (n=154)	BA panel	**NorCA + UCA + THCA + TUDCA + TLCA + TCDCA + GHCA**	**0.88**	**0.80**
	Non-invasive diagnostic index	HFS	0.75	0.65
Obese (n=431)	BA + Clinical panel	**DBP + ALT + TLCA + βUCA + βCA + TCA + GCA**	**0.75**	**0.61**
	Non-invasive diagnostic index	HFS	0.65	0.51
Non-obese (n=119)	BA + Clinical panel	**dehydroLCA + DBP + HOMA-IR + 6-ketoLCA**	**0.78**	**0.69**
	Non-invasive diagnostic index	HFS	0.71	0.66
MetS (n=377)	BA + Clinical panel	ALT + TLCA + NorCA/NorDCA + 7-DHCA	0.70	0.59
	Non-invasive diagnostic index	HFS	0.65	0.59
No-MetS (n=173)	BA panel	HDCA/HCA	0.64	0.53
	Non-invasive diagnostic index	FIB-4	0.61	0.59
Stage F2-4 vs. F0-1				
Group	**Panel**	**Content**	**Training AUC**	**Testing AUC**
All (n=550)	BA + Clinical panel	7-DHCA + WBC + TUDCA + BMI	0.64	0.58
	Non-invasive diagnostic index	HFS	0.55	0.60
Men	BA + Clinical panel	UDCA/CDCA + WBC + βUDCA + βUCA + BMI	0.67	0.54

(n=396)	Non-invasive diagnostic index	NFS	0.53	0.37
Women (n=154)	BA panel	**7-DHCA + HbA1c**	**0.75**	**0.65**
	Non-invasive diagnostic index	HFS	0.62	0.56
Obese (n=431)	BA + Clinical panel	WBC + UCA + NorCA + GCA + TCA + GUDCA	0.65	0.51
	Non-invasive diagnostic index	HFS	0.56	0.60
Non-obese (n=119)	BA + Clinical panel	**7-DHCA/CA**	**0.71**	**0.66**
	Non-invasive diagnostic index	FIB-4	0.60	0.43
MetS (n=377)	BA + Clinical panel	WBC + 7-DHCA	0.65	0.59
	Non-invasive diagnostic index	HFS	0.56	0.58
No-MetS (n=173)	BA panel	7-DHCA + CDCA-3Glu/CDCA	0.61	0.59
	Non-invasive diagnostic index	NFS	0.57	0.46

NB: Panel type, contents, and performances in both the training cohorts and testing cohorts of different prediction models are reported. For the sake of clarity, the recommended prediction models with good performances have been marked in bold.

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	6
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	9
Objectives	3	State specific objectives, including any prespecified hypotheses	9
Methods			
Study design	4	Present key elements of study design early in the paper	10
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	10
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	10
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	/
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	*11*
Bias	9	Describe any efforts to address potential sources of bias	/
Study size	10	Explain how the study size was arrived at	10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	12
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	12
		(*b*) Describe any methods used to examine subgroups and interactions	13
		(*c*) Explain how missing data were addressed	12
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	/
		(*e*) Describe any sensitivity analyses	13

Continued on next page

Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	
		(b) Give reasons for non-participation at each stage	
		(c) Consider use of a flow diagram	Fig1
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Table 1
		(b) Indicate number of participants with missing data for each variable of interest	/
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	Table 1
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	*11*
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	16
		(*b*) Report category boundaries when continuous variables were categorized	
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	16
Discussion			
Key results	18	Summarise key results with reference to study objectives	18
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	21
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	20
Generalisability	21	Discuss the generalisability (external validity) of the study results	17
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	4

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.