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ORIGINAL RESEARCH

Association of Serum Bile Acid and Unsaturated Fatty Acid Profiles with the Risk of Diabetic Retinopathy in Type 2 Diabetic Patients

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Aim: We aimed to identify the ability of serum bile acids (BAs) and unsaturated fatty acids (UFAs) profiles to predict the development of diabetic retinopathy (DR) in type 2 diabetes mellitus (T2DM) patients.

Methods: We first used univariate and multivariate analysis to compare 15 serum BA and 11 UFA levels in healthy control (HC) group (n = 82), T2DM patients with DR (n = 58) and T2DM patients without DR (n = 60). Forty T2DM patients were considered for validation. Then, the receiver operating characteristic curve (ROC) and decision curve analysis were used to assess the diagnostic value and clinical benefit of serum biomarkers alone, clinical variables alone or in combination, and the area under the curve (AUC), integrated discrimination improvement (IDI), and net reclassification improvement (NRI) were used to further assess whether the addition of biomarkers significantly improved the predictive ability of the model.

Results: Orthogonal partial least squares-discriminant analysis (OPLS-DA) of serum BAs and UFAs separated the three cohorts including HC, T2DM patients with or without DR. The difference in serum BA and UFA profiles of T2DM patients with or without DR was mainly manifested in the three metabolites of taurolithocholic acid (TLCA), tauroursodeoxycholic acid (TUDCA) and arachidonic acid (AA). Together, they had an AUC of 0.785 (0.918 for validation cohort) for predicting DR in T2DM patients. After adjusting for numerous confounding factors, TLCA, TUDCA, and AA were independent predictors that differentiated T2DM with or without DR. The results of AUC, IDI, and NRI demonstrated that adding these three biomarkers to a model with clinical variables statistically increased their predictive value and were replicated in our independent validation cohort.

Conclusion: These findings highlight the association of three metabolites, TLCA, TUDCA and AA, with DR and may indicate their potential value in the pathogenesis of DR.

Keywords: type 2 diabetes mellitus, diabetic retinopathy, serum bile acid, unsaturated fatty acid

Introduction

Diabetic retinopathy (DR) has become one of the most common microvascular complications of diabetes. A meta-analysis showed that the global prevalence of DR has been as high as 34.6%.[1] The incidence of DR in diabetic population in mainland China was as high as 23.0%.[2] DR increased the risk of all-cause mortality, stroke, heart failure, and depression, seriously affecting the physical and mental health of people with diabetes.[3,4] DR is a highly specific microvascular complication of diabetes, and long duration of diabetes, poor glycemic, lipid, blood pressure control, the blood-retinal barrier, and epigenetic mechanisms may be involved in the development of DR.[5–9] Literature data indicates that microvascular diabetic complications are associated with inflammation. C reactive protein based inflammatory markers, hemogram-derived predictor, serum uric acid, and cytokines such as neuregulin are associated with

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microvascular complications of diabetes.[10–13] Bile acid (BA) and fatty acid[14,15] are also associated with high burden of inflammation. Thus, studying serum BA and unsaturated fatty acid (UFA) profiles in DR makes sense.

BAs are an amphiphilic cholesterol metabolite that promotes lipid digestion and absorption by mid-shaped micelles in the small intestine. In addition, BAs are considered to be important enteroendocrine hormone-like signaling molecules that regulate glucose, lipid, and energy metabolism by acting on cell membranes and nuclear receptors. A family of sodium BA cotransporters (SLC10) that maintain enterohepatic circulation of BAs has been identified in humans.[16] The adult retina also expressed G protein-coupled BA receptor 1 (GPBAR1/TGR5).[17–19] Su et al found significant differences in serum total BAs between DR and NDR.[20] However, the variation in BA composition and its role in human DR remains unclear.

Several studies have reported associations between polyunsaturated fatty acids (PUFAs) and DR, as well as the protective effect of PUFA against DR.[21] Omega-3 PUFA, an important structural component of the retina and protective, reduced the risk of DR and was the main structural lipid of the outer segment membrane of retinal photoreceptors.[22–24] The omega-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have anti-inflammatory effects, were thought to play a protective role in DR development.[22,25] However, Bühler et al found that DR was not associated with omega-3 or −6 PUFA levels in their cohort.[26] Moreover, the results of current studies on fatty acids in DR were inconsistent, and the changes in the levels of fatty acids in DR and the relationship between them and DR were unclear.

Regarding the relationship between BAs and fatty acids, in a double-blinded, randomized controlled trial,[27] PUFA diet decreased lipoprotein subclasses and increased serum BA level in healthy subjects with moderate hypercholesterolemia. Omega-3 PUFA regulates the farnesoid x receptor (FXR), thereby affecting BA metabolism to alleviate atherosclerosis and diabetes.[28] The combination of EPA/DHA+ ursodeoxycholic acid (UDCA) affected the metabolism of BAs by promoting Recombinant Cytochrome P450 7A1 (CYP7A1) gene expression and BA-induced downregulation of caspase 3 activity.[29] Robert found that short-term diet rich in alpha-octadecatrienoic acid (α-C18:3) induces an increase in the abundance of sulfated BA which is poor substrates for the apical sodium-dependent BA transporter (ASBT), thus affecting the passive re-absorption of BAs.[30,31] Moreover, the crosstalk between BAs and UFAs in the DR remains unclear. Given the differences in BA metabolism in mice and humans,[32] the interactions between UFAs and BAs still need to be further explored.

Here, we hypothesized that BAs and UFAs play an important role in the formation of DR in T2DM patients. In this study, High Performance Liquid Chromatography (HPLC) coupled to targeted liquid chromatography with tandem mass spectrometry (LC-MS/MS) was used to quantify 15 BAs and 11 UFAs in T2DM patients with or without DR. We characterized the absolute concentration and composition of serum BAs and UFAs in patients with T2DM and DR and their correlation with clinical indicators, and assessed their correlation with DR risk. Finally, we identified several biomarkers that are highly associated with DR risk and may indicate their potential value in the pathogenesis of DR.

Materials and Methods

Patients

A total of 240 participants were included in the study according to the inclusion and exclusion criteria below. The training cohort was divided into 82 HC, 58 T2DM patients with DR (DR group) and 60 T2DM patients without DR (NDR group). Then, a validation cohort composed of T2DM patients with or without DR (n = 20, n = 20) was used to validate our results. Age, sex, and BMI of the DR and NDR groups were matched well in both cohorts. All patients were enrolled at the Second Affiliated Hospital of Nanjing Medical University, China, from Jan 2019 to Aug 2022. According to the criteria by the World Health Organization in 1999, the diagnosis of DR in the present study was based on history of diabetes and fundus examination.

This study was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Nanjing Medical University and complies with the Declaration of Helsinki. Due to the retrospective and low-risk nature of this study, the ethics committee waived the need for written informed consent.

Inclusion Criteria

Diabetes diagnosis was confirmed following the World Health Organization (WHO) 1999 criteria. The healthy control cohort was selected from the medical examination centre in our hospital. DR diagnosis was based on the fundus examination findings of the ophthalmologist. Hypertension (HT) diagnosis was based on the past medical history of patients or presence of oral antihypertensive medication.

Exclusion Criteria

The exclusion criteria were as follows: (1) type 1 diabetes mellitus; (2) recent infection, inflammatory disorders, heart failure, renal failure, cirrhosis, diabetic ketoacidosis; (3) malignancy or mental disorders; (4) pregnant or lactating women; (5) presence of any other vascular retinopathy other than DR. (6) People who were reluctant to have their BA and UFA levels tested.

Physical Examination

The height, body weight, blood pressure of right upper limb in the supine position, body mass index (BMI, kg/m^2) of the eligible patients were measured.

Laboratory Examination

Blood samples were collected after the patients fasted overnight (≥8 h) on admission. Biochemical parameters, including fasting plasma glucose (FPG), fasting insulin (FINS), Insulin 2 hours postprandial (2h-INS), fasting C-Peptide (FC-P). C-Peptide 2 hours postprandial (2hC-P), lipoprotein A (Lpa), Glycosylated hemoglobin (HbA1c) levels, plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), TC, triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), Uric Acid (UA), blood urea nitrogen (BUN), serum creatinine (SCR) and Urinary albumin to creatinine ratio (UACR) were measured. We advised all participants not to do strenuous exercise before retaining the urine sample. Duration of diabetes was defined as the time interval from the first diagnosis of diabetes to the current admission. Homeostasis model assessment of insulin resistance (HOMA-IR) = fasting insulin (mIU/L) × fasting glucose (mmol/L)/22.5.

BAs and UFAs Measurement and Classification

The peripheral blood samples of all participants were collected under fasting conditions at admission. After centrifugation, the sera were stored at −80°C and thawed until testing. Serum BAs and UFAs were analyzed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS, AB SCIEX Jasper TM HPLC-Triple Quad TM 4500MD).

BAs were classified into four groups based on conjugation degree,[26] including

1. Unconjugated primary BAs (PBAs): cholic acid (CA) and chenodeoxycholic acid (CDCA);
2. Conjugated PBAs: glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), and taurochenodeoxycholic acid (TCDCA);
3. Unconjugated secondary BAs (SBAs): deoxycholic acid (DCA), ursodeoxycholic acid (UDCA), and lithocholic acid (LCA);
4. Conjugated SBAs: glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycolithocholic acid (GLCA), taurolithocholic acid (TLCA), glycoursodeoxycholic acid (GUDCA), and tauroursodeoxycholic acid (TUDCA).

UFAs were classified into three groups, including

1. Omega-3 fatty acid: alpha-octadecatrienoic acid (α-C18:3); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); omega-3 docosapentaenoic acid (ω-3-C22:5);

2. Omega-6 fatty acid: arachidonic acid (AA); linolenic acid (C18:2); gamma linolenic acid (γ-C18:3); Omega-6 docosapentaenoic acid (ω-6-C22:5);
3. Monounsaturated fatty acids: palmitoleic acid (C16:1); octadecenoicacid (C18:1); eicosaenoic acid (C20:1).

Statistical Analysis

First, the data for each group of metabolites were not normally distributed after the test of normality, so we used a combination of univariate analysis (Kruskal–Wallis, Wilcox test and Fold Change (FC)) and multivariable analysis (OPLS-DA). We screened potential biomarkers based on the following three conditions (the first condition is mandatory, the second and third meet one): 1) p. adj (adjusted by FDR method) <0.05, 2) FC value ≥2 or ≤0.5, 3) and variable influence on projection (VIP) ≥1. In the OPLS-DA analysis, we used 9-fold cross-validation to optimize the number of orthogonal components, cross-validation-based metrics (R2X, R2Y, Q2Y values) and 200-fold permutation analysis to evaluate the model. We also explored the correlation of biomarker metabolites with clinical indicators and metabolites with each other using Spearman correlation method. Logistic regression was used to identify and verify BAs and UFAs that could predict DR. Here, we treated the DR predictive model constructed from clinical indicators as model 1, and then built predictive models 2 and 3, respectively, from biomarkers alone or in combination with clinical indicators. Finally, we demonstrated that adding these biomarkers to clinical features can improve the predictive performance of the model. The biomarker and all built models were based on the training cohort and these results were verified in a separate cohort.

The incremental effect of metabolic markers on the prediction of DR risk in patients with T2DM was assessed by ROC curves. Statistically significant improvements in model performance were demonstrated by the values of AUC, IDI and NRI. IDI and NRI were used to assess the differences in prediction effectiveness between models. IDI >0 and NRI >0 represent an improvement in prediction results for the new model compared to the old model. To verify whether the prediction model has good fitting performance and the accuracy of the prediction model, we used 1000 bootstrap resample.

We also assessed the clinical utility and net benefit of the new predictive model using decision curve analysis. x-axis shows the threshold probability of DR risk and y-axis represents the net benefit after treatment with the threshold probability. The “none” and “all” lines indicate no or all DR cases, respectively. The further the model curve is from these two lines, the greater the net return.

All values are shown as the mean ± standard deviation (SD) for normal distribution of continuous variables, median (m) ± interquartile spacing for nonnormal distribution of continuous variables and frequencies for categorical variables. The normal distribution of continuous variables was analyzed using *t*-test, the nonnormal distribution of continuous variables was analyzed using a nonparametric Kruskal−Wallis test (three groups) or Wilcoxon test (two groups), and the categorical variables were analyzed with Fisher’s exact test.

All calculations and multivariate analyses were performed using R software version 4.1.2 and SIMCA software 14.1.0. A p-value of <0.05 (two-tailed) was considered statistically significant.

Results

Clinical and Metabolic Characteristics of T2DM Patients with or Without DR

Age, gender, and BMI of each group did not show any significant statistical difference in Table S1 ($p > 0.05$). However, in both the training and validation cohorts, the duration of diabetes was higher in T2DM patients with DR than in T2DM patients without DR (Table S1) ($p < 0.05$). In addition, in Table S1, the use of the nine hypoglycemic agents was not statistically different between the DR and NDR group ($p > 0.05$).

Univariate Analysis of BAs and UFAs Profiles in T2DM Patients with or Without DKD

Serum concentrations of 15 BAs and 11 UFAs in T2DM patients with or without DR are shown in Figure 1A, C and Table S2. Several BAs and UFAs had significantly different levels in the DR and NDR group, including TLCA, TUDCA, AA, C18:2, C18:1, C20:1, and ω-6-C22:5 ($p < 0.05$). Compared with HC or NDR, the relative abundance levels of

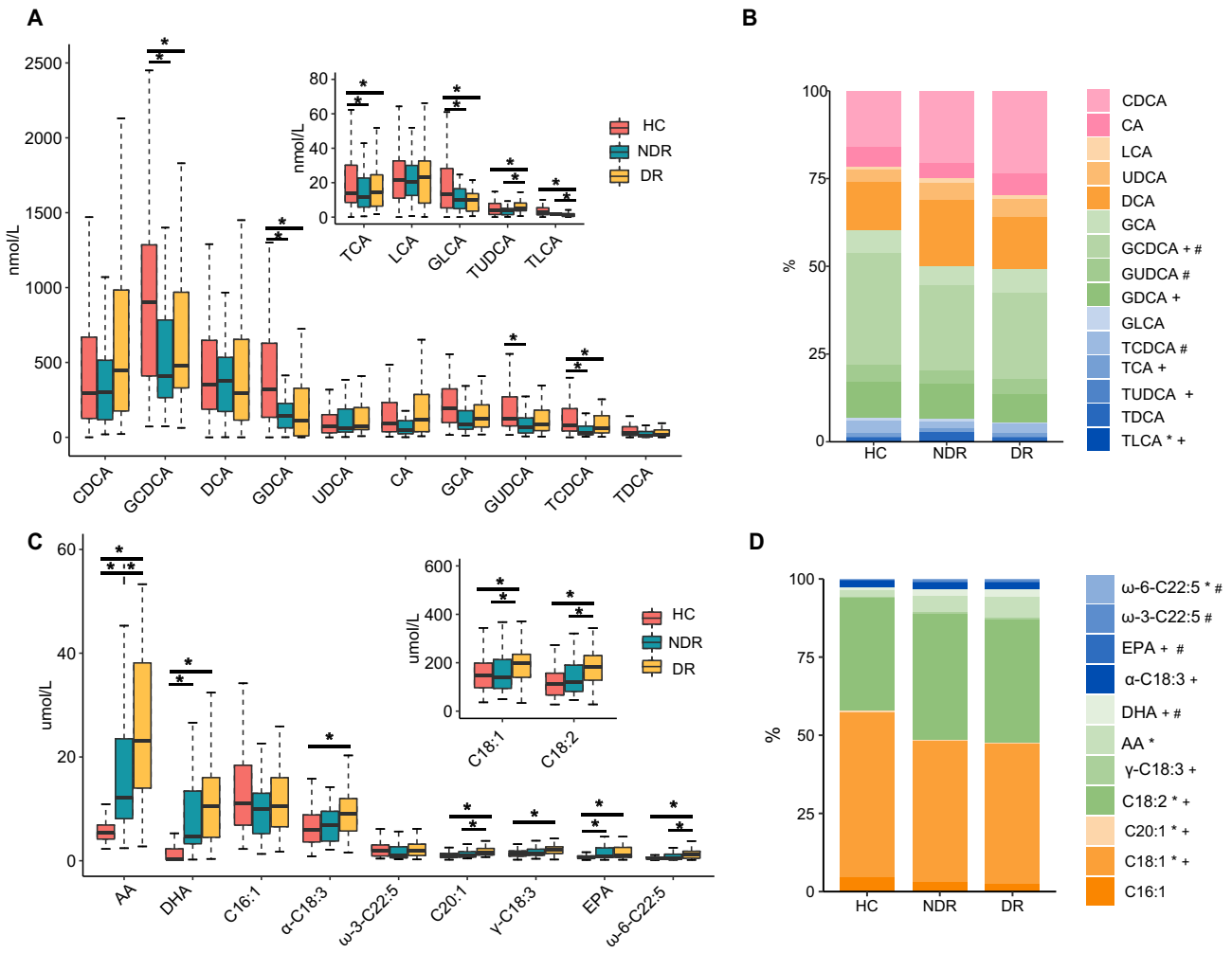

Figure 1 Bile acids and unsaturated fatty acids distribution among Healthy Control (HC), T2DM patients, and Diabetic retinopathy (DR) patients. Serum levels of bile acids and unsaturated fatty acids in three groups were shown in (**B** and **D**). (**A** and **C**) Wilcoxon rank sum test (*p < 0.05). **B** and **D**: Wilcoxon rank sum test (*p < 0.05) when NDR compared with DR, +p < 0.05 when HC compared with DR, #p < 0.05 when HC compared with NDR.

TLCA and C18:1 were decreased in DR group ($p < 0.05$), conversely, TUDCA, AA, C18:2, C20:1, and ω-6-C22:5 were elevated ($p < 0.05$) (Figure 1B, D and Table S2) and FC values of the seven metabolites above are shown in Table S3.

Multivariate Analysis of BAs and UFAs Profiles in T2DM Patients with or Without DKD

First, we conducted an OPLS-DA model to find BAs and UFAs that contributed more to distinguish different groups. Fifteen BAs and eleven UFAs were included in a multivariate analysis, and the results were projected in an OPLS-DA 3D plot (Figure 2). The OPLS-DA score plot clearly showed the difference between the three groups: R2X = 0.468, R2Y = 0.310, and Q2Y = 0.253 (training cohort) (Figure 2A). A similar separation was observed in the multivariate analysis of training cohort (Figure 2C). To verify that the data were not over-fitted, we performed the 200-permutation test in the OPLS-DA model [Q2 = (0.0, −0.098), (0.0, −0.125)] (Figure 2B and D), [Q2 = (0.0, −0.109), (0.0, −0.656)] (Figure S1B and D), since the final ordinate of Q2 was less than 0.05. Based on the conditions for screening potential differential metabolites in statistical analysis and the results of univariate and multivariate (Table S3, Figure S2), we conclude that TLCA ($p = 0.006$; FC = 0.24), TUDCA ($p = 0.002$; VIP = 1.55), and AA ($p = 0.002$; VIP = 1.07) are potential biomarkers for distinguishing DR vs NDR.

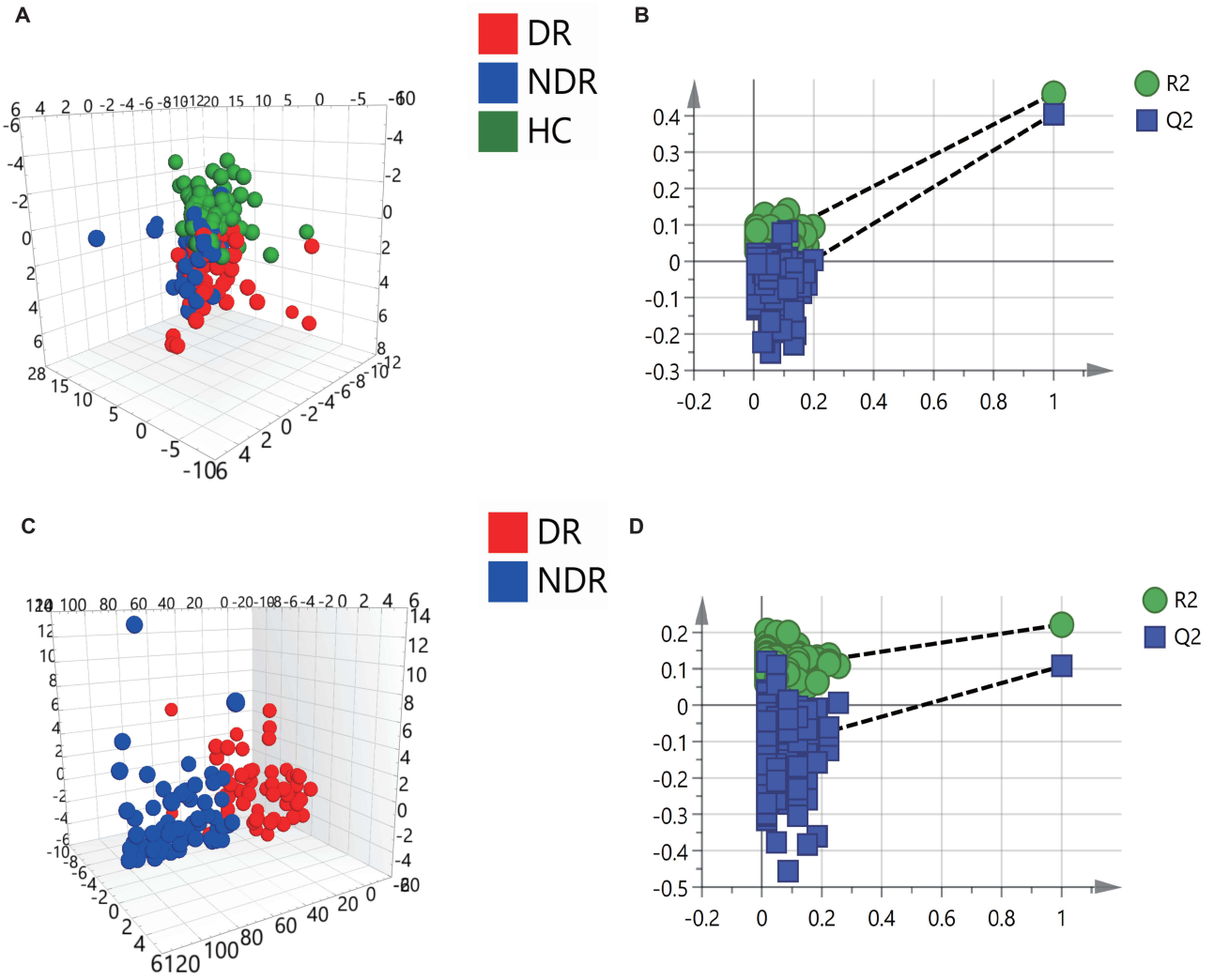

Figure 2 Metabolomic profiling by targeted metabolomics. (**A**) OPLS−DA 3D models among HC, NDR, and DR groups. R2X = 0.468, R2Y = 0.310, and Q2Y = 0.253. (**B**) The 200−permutation test demonstrated no overfitting in the OPLS-DA model [Q2 = (0.0, –0.098)]. (**C**) OPLS−DA 3D models between NDR and DR groups. R2X = 0.404, R2Y = 0.221, and Q2Y = 0.107. (**D**) The 200−permutation test demonstrated no overfitting in the OPLS-DA model [Q2 = (0.0, –0.125)].
Abbreviation: OPLS-DA, orthogonal partial least squares-discriminant analysis.

Association of Metabolites with Clinical Indicators or Metabolites with Each Other in Three Groups

We found the strong positive correlation between BAs and UFAs in the training cohort using Spearman correlation analysis ($p < 0.05$; Figure 3A). A strong correlation was found between TLCA and AA ($r = -0.23$, $p < 0.05$); TLCA and DHA ($r = -0.19$, $p < 0.05$); TLCA and C18:2 ($r = 0.18$, $p < 0.05$); TLCA and γ-C18:3 ($r = 0.20$, $p < 0.05$); TLCA and TUDCA ($r = -0.18$, $p < 0.05$); AA and γ-C18:3 ($r = 0.54$, $p < 0.05$); AA and DHA ($r = 0.85$, $p < 0.05$); AA and C18:2 ($r = 0.67$, $p < 0.05$); AA and EPA ($r = 0.68$, $p < 0.05$) (Figure 3A). Compared to the HC or NDR groups, the ratios of TLCA and γ-C18:3, TLCA and DHA, TLCA and TUDCA, TLCA and AA, TLCA and C18:2 were significantly lower in the DR ($p < 0.05$), and in contrast, the ratios of AA and EPA, AA and DHA were significantly higher (Figure 3B and TableS1). We also further explored the relationship of the conjugated lithocholic acid (conLCA) and GLCA to these fatty acids and the results were generally consistent with TLCA (Table S1 and Figure S3).

The positive correlation among AST, ALT, HOMA-IR and several UFAs (AA, γ-C18:3, C18:2, DHA) was observed in the DR and NDR groups (Figure 3C, Table S4). TLCA and FPG ($r = -0.26$, $p = 0.006$); TUDCA and Lpa ($r = 0.27$, $p = 0.003$); AA and HOMA-IR ($r = 0.22$, $p = 0.018$) (Table S4 and Figure S4).

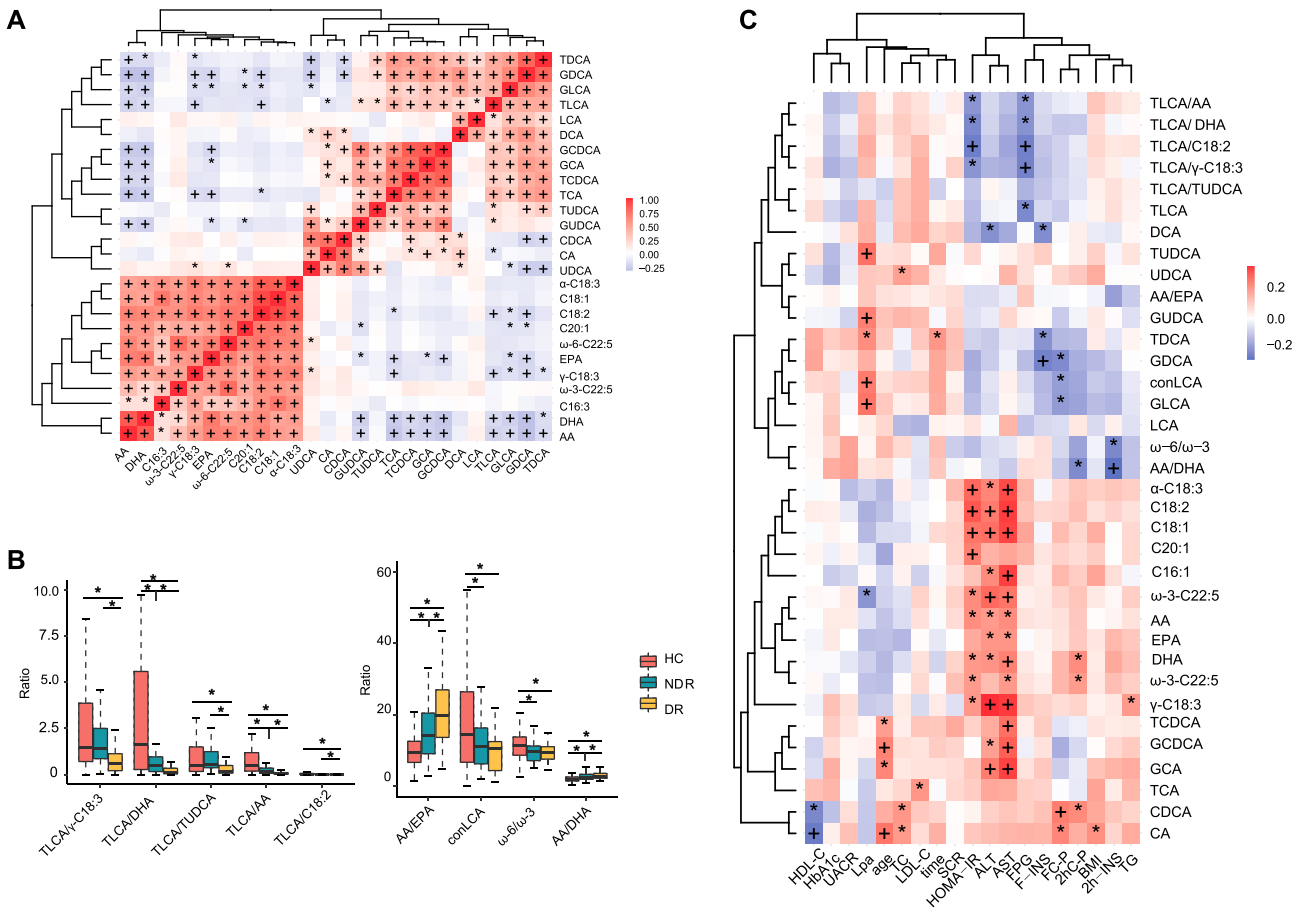

Figure 3 Clustering analysis and levels of serum levels of bile acids and unsaturated fatty acids among Healthy Control (HC), T2DM patients, with Diabetic retinopathy (DR) patients. Bile acids clustering for each patient and each group were shown in (**A**). (**C**) was the Spearman correlation analysis between 15 bile acids and 11 unsaturated fatty acids. (**B**) ratios of bile acids and unsaturated fatty acids distribution between three groups. (**D**) was the Spearman correlation analysis among bile acids, unsaturated fatty acids, ratios with clinical indicators in T2DM and DR patients. (**B**) Wilcoxon rank sum test (*p < 0.05). (**A** and **C**) *p < 0.05, +p < 0.01.

Predictive Ability of Serum BAs and UFAs in T2DM Patients with or Without DR

Univariate and multivariate logistic analyses were performed to identify serum BAs and UFAs that can distinguish T2DM patients with or without DR (Table 1). We performed univariate and multivariate regressions for the three potential biomarkers and the UFAs that were strongly associated with them. After adjusting for numerous confounding factors including sex, duration of diabetes, age, BMI, FPG, HbA1c, FC-P, TG, LDL-C, HDL-C, Lpa, UACR, SCR, anti-diabetic medication and history of hypertension, TLCA, TUDCA, and AA were independent predictors that differentiated T2DM with or without DR.

The AUCs for the metabolites TLCA, TUDCA and AA were 0.660 ($p < 0.05$), 0.694 ($p < 0.05$) and 0.686 ($p < 0.05$), respectively, in Figure S5A, providing evidence that the levels of these metabolic biomarkers had potential predictive capability for DR. Together, risk prediction model 2 constructed from three biomarkers including TLCA, TUDCA, AA, had an AUC of 0.785 (95% CI: 0.704−0.866) ($P < 0.05$) for predicting T2DM with or without DR (Figure S5A). Then, we built predictive model 1 constructed from clinical factors including sex, duration of diabetes, age, BMI, FPG, HbA1c, FC-P, TG, LDL-C, HDL-C, Lpa, UACR, SCR, and model 3 consisted of the elements in model 1 and model 2 together. It showed AUC of model 3 was higher than model 1 (0.925 vs 0.816) ($p < 0.05$) (Figure S5A and Table S5).Table 2 shows that when the identified potential biomarkers were added to the predictive model consisting of the clinical indicators, a statistically significant improvement in the predictive performance of the model was observed.

The decision curve analysis showed the apparent net benefit of potential biomarkers when combined with clinical indicators, which demonstrated the extent of their benefit in clinical applications (Figure S5B). For example, assuming we

Table 1 Univariate and Multivariate Analysis of Bile Acids and Fatty Acids for Differentiating T2DM with or Without DR

Variable	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
TLCA	0.91 [0.80, 0.98]	0.052	0.88 [0.77, 0.97]	**0.037***
TUDCA	1.14 [1.05, 1.26]	**0.005***	1.19 [1.07, 1.35]	**0.005***
AA	1.03 [1.01, 1.06]	**0.002***	1.06 [1.03, 1.10]	**<0.001***
γ-C18:3	1.16 [0.90, 1.51]	0.260	1.58 [1.09, 2.38]	**0.038***
C18:2	1.00 [1.00, 1.01]	0.064	1.01 [1.00, 1.01]	**0.010***
DHA	1.05 [1.00, 1.10]	**0.044***	1.12 [1.05, 1.21]	**0.003***
Ratio				
TLCA/γ-C18:3	0.94 [0.87, 1.01]	0.093	0.92 [0.83, 0.98]	**0.018***
TLCA/AA	0.57 [0.30, 1.06]	0.074	0.51 [0.23, 0.85]	**0.021***
TLCA/C18:2	0.00 [0.00, 0.19]	0.073	0.00 [0.00, 0.08]	**0.030***
TLCA/DHA	0.95 [0.88, 0.99]	0.076	0.71 [0.52, 0.88]	**0.006***
TLCA/TUDCA	0.50 [0.28, 0.79]	**0.010***	0.30 [0.13, 0.61]	**0.007***
AA/EPA	1.05 [1.01, 1.09]	**0.011***	1.05 [1.01, 1.10]	**0.022***
AA/DHA	1.41 [1.02, 2.01]	**0.044***	1.47 [1.02, 2.21]	0.093

Notes: Multivariate Analysis adjusted sex, duration of diabetes, age, body mass index, Fasting blood sugar, hemoglobin A1c, Fasting c-peptide, Triglycerides, Low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, lipoprotein a, Urinary protein to creatinine ratio, Serum creatinine, anti-diabetic medication and history of hypertension. Data in asterisk and bolded are statistically significant (*p < 0.05).

Abbreviations: T2DM, type 2 diabetes mellitus; DR, diabetic retinopathy; OR, odds ratio; CI, confidence interval; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; conLCA, conjugated lithocholic acid; AA, alpha-linoleic acid; γ-C18:3, γ linolenic acid; C18:2, octadecadienoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

Table 2 Discriminatory Capacity of Models with Clinical Variables Alone or with Biomarkers

Comparison	Index	Data Set	Estimate	Ci95low	CI95up	p value
Model 3 vs model 1	Difference in AUC	Training	0.109	0.044	0.173	**0.001***
		Validation	0.198	0.062	0.333	**0.004 ***
	NRI	Training	0.982	0.667	1.296	**<0.001***
		Validation	2.000	2.000	2.000	**<0.001***
	IDI	Training	0.252	0.172	0.333	**<0.001***
		Validation	0.698	0.548	0.849	**<0.001***

Notes: Risk prediction model 1 for type 2 diabetic retinopathy constructed from clinical factors including sex, duration of diabetes, age, body mass index, Fasting blood sugar, hemoglobin A1c, Fasting c-peptide, Triglycerides, Low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, lipoprotein a, Urinary protein to creatinine ratio, Serum creatinine. Risk prediction model 3 for type 2 diabetic retinopathy constructed from clinical factors plus biomarker metabolites including taurolithocholic acid, tauroursodeoxycholic acid, and Alpha-linoleic acid. Data in asterisk and bolded are statistically significant (*p < 0.05).

Abbreviations: CI, confidence interval; AUC, area under the ROC curve; IDI, integrated discrimination improvement; NRI, net reclassification improvement.

choose a predictive probability of 60% to diagnose DR and treat it, 35 out of 100 patients using model 3 will benefit from it without harming others (Figure S5B).

Validation Study of Three Potential Metabolic Biomarkers

We used an independent validation cohort to demonstrate the predictive performance of the model constructed with the training cohort. Differences of BA and UFA profiles in 3 groups in validation cohort are shown in Table S6. The OPLS-

DA score plot showed that the metabolites separated the three groups well: R2X = 0.447, R2Y = 0.354, and Q2Y = 0.179 (Figure S1A), with better performance in two groups: R2X = 0.593, R2Y = 0.652, and Q2Y = 0.214 (Figure S1C).

The AUC values of AA, TLCA, TUDCA, Model 1, Model 2, and Model 3 were 0.724, 0.718, 0.666, 0.802, 0.918 and 1.000, respectively. In the validation cohort, similarly, model 3 had better predictive performance than model 1 (Tables S4, S5, and Figure S5C-D). These results further confirmed that the inclusion of screened potential biomarkers improved the predictive performance of the clinical model for DR risk in T2DM patients.

Discussion

Increasing number of studies have shown that diabetic retinopathy is not only a microvascular problem but also a predictor of macrovascular complications, such as ischaemic coronary heart disease and stroke.[33–35] Therefore, preventing the development of DR is crucial. In addition, screening for potential biomarkers of DR in patients with T2DM is imperative to provide methods for disease prediction, screening, mechanisms and exploration of therapeutic targets. The role of BAs and UFAs as biological signalling molecules is increasingly being explored and applied by scholars. However, up now, there are no reports of systematic analysis of serum BAs and UFAs in patients with DR.

In this study, we quantified serum BAs and UFAs in T2DM patients. Then we defined two serum BAs and one UFA associated with DR and combined them with traditional clinical indicators to predict DR risk in T2DM patients, which, interestingly, improved the performance of the DR risk prediction model.

Our results showed that AA and TUDCA may be capable of impairing UFAs and BAs flow and inducing DR, and TLCA may play a protective role. However, in contrast to CA, CDCA and their conjugates, which are the major components of BA levels, serum concentration of TLCA was very low (Figure 1 and Table S2). It is likely that TLCA plays a key regulatory role in the development of DR in patients with T2DM. In multivariate regression analysis, we also found that TLCA, AA, and TUDCA were all independent predictors that differentiated DR patients from NDR patients. Our results suggested that changes in BAs and UFAs may be associated with the risk of DR in patients with T2DM, whereas higher level of TLCA was generally protective.

BAs have been shown to reduce markers of oxidative stress damage in the retina.[36] Li et al found that patients with T2DM and obesity had significantly lower LCA species levels, especially TLCA concentrations.[37] This also reflects the beneficial role of TLCA in metabolic diseases at a certain level. The adult retina also expressed the BA receptor TGR5,[17–19] and TLCA is one of the most obvious agonists of TGR5. Interestingly, in our study, TLCA showed a decreasing trend between the three groups and the difference was statistically significant. We speculated that TLCA species play a protective role against the progression of DR. UDCA and TUDCA have been shown to be protective against DR in a number of animal models.[38–42] However, in our present human study, the role of TUDCA seems to contradict this. In our study, TUDCA was high in DR patients and TLCA, a protective BA, was low, probably because of dysbiosis of the gut microbiota in DR patients, resulting in the inability of specific gut microbiota to convert TUDCA to TLCA. In addition, the total BA level of DR patients was higher. Thus, the role of TUDCA in our DR predictive model is likely driven by high overall BAs levels and does not necessarily suggest a negative effect of TUDCA or UDCA.

Oxylipins in the retina, formed by high levels of omega-3 PUFAs, had anti-inflammatory and anti-angiogenic effects.[43] G protein-coupled receptor 120 (GPR120), a receptor for omega-3 PUFAs, when activated by agonists, is involved in stimulating potent anti-inflammatory effects by blocking the signaling of several pro-inflammatory mediators and increasing insulin sensitivity.[44] The difference in omega-3 PUFAs found in our study was not very striking, possibly because omega-3 PUFAs is more prominent in its neovascularization prevention role, and since the vast majority of this controlled study was NPDR patients, the effect of these metabolites on DR progression could not be further explored. EPA and DHA, which are omega-3 PUFAs, prevented diabetic retinal neovascularization by inhibiting vascular endothelial growth factor-induced endothelial cell proliferation, migration and tube formation.[45,46] In order to further explore the relationship between the pro-inflammatory AA and anti-inflammatory mediators (EPA and DHA) in DR patients, here we also explored the differences in ratio of AA: DHA and AA: EPA between the three groups. Our results showed that AA is a risk factor for DR, but AA shows a high positive correlation with DHA and EPA. Given that derivatives of AA produced by lipoxygenase and cytochrome p450 have been shown to be implicated with retinal pro-angiogenesis,[47] so increased AA may contribute to DR progression. However, the relationship among AA, EPA and DHA needs to be further explored.

Lei et al found that dihomo-γ-Linolenic acid (DGLA) was highly negatively correlated with DCA species, and the ratio of them could be used to distinguish T2DM patients with or without obesity.[48] This is one of the few clinical studies currently on the relationship between BAs and UFAs in T2DM patients. In our study, the levels of TLCA were negatively correlated with AA, ω-6-C22:5 and DHA, the reason remains unclear. AA is an important inflammatory marker of risk of metabolic syndrome, and increased AA levels may explain the inflammatory phenotype in some DR patients. AA belongs to omega-6 PUFA. The reduction of the ratio of omega-6: omega-3 PUFA is beneficial to the regulation of intestinal homeostasis and microbiota.[49] Both conjugated and unconjugated forms of LCA activated TGR5.[50] Guo et al found that BAs exert anti-inflammatory effects via TGR5 signaling and ameliorate high-fat diet-induced metabolic disorders in mice,[51] perhaps explaining the correlation between TLCA and AA, considering DR as an inflammatory disease.

Retinal neuropathy is an early event in the pathogenesis of DR, and diabetes causes structural and functional alterations in almost all types of retinal nerve cells.[5,52] BAs are important signalling molecules for metabolic pathways and their interactions with specific receptors are regulated by the gut microbiota. With a growing number of studies linking neurodegenerative diseases to the status of the microbiota, we hypothesize that alterations in BAs may induce or exacerbate neurodegenerative processes, particularly retinopathy. EPA-enriched phosphoethanolamine plasmalogens alleviated atherosclerosis by remodeling gut microbiota to regulate BA metabolism in LDLR−/−Mice.[53] Intestinal FXRα1 overexpression protects against omega-6 PUFAs-induced accumulation of human-specific primary BAs in the cecum.[54] Although some studies have reported on the relationship between BAs and UFAs, many have been in animal studies and there are few studies in human DR. More importantly, there were many conflicting findings and the relationship between BAs and fatty acids still needs to be further explored.

However, our study had no information on other confounding factors that may affect the concentrations of BAs and UFAs, such as the use of insulin and statins. Therefore, there is a need to explore the changes in BA and UFA profiles in DR using animal models and in vitro cellular experiments. In addition, attention should be paid to the study of the mechanism of action of TLCA in DR.

Conclusion

In summary, this is the first study on the association between BA and UFA profiles in DR risk in patients with T2DM. We found that the risk of DR in T2DM patients was associated with disturbances in the metabolism of BAs and UFAs. Emerging serum biomarkers help provide methods for predicting, screening, and exploring therapeutic targets for DR.

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Disclosure

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

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