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The ratio of dihomo-γ-linolenic acid to deoxycholic acid species is a potential biomarker for the metabolic abnormalities in obesity

Sha Lei,* Fengjie Huang,* Aihua Zhao,* Tianlu Chen,* Wenlian Chen,† Guoxiang Xie,† Xiaojiao Zheng,* Yunjing Zhang,* Haoyong Yu,‡,§,¶ Pin Zhang,‖ Cynthia Rajani,† Yuqian Bao,‡,§,¶ Weiping Jia,*,‡,§,¶ and Wei Jia*,†,1

*Shanghai Key Laboratory of Diabetes Mellitus and Center for Translational Medicine, ‡Department of Endocrinology and Metabolism, §Shanghai Diabetes Institute, Shanghai Clinical Center of Diabetes, ¶Shanghai Key Clinical Center for Metabolic Disease, and ‖Department of General Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China; and †University of Hawaii Cancer Center, Honolulu, Hawaii, USA

ABSTRACT: Bile acid (BA) signaling regulates fatty acid metabolism. BA dysregulation plays an important role in the development of metabolic disease. However, BAs in relation to fatty acids have not been fully investigated in obesity (OB)-related metabolic disorders. A targeted metabolomic measurement of serum BA and free fatty acid profiles was applied to sera of 381 individuals in 2 independent studies. The results showed that the ratio of dihomo-γ-linolenic acid (DGLA) to DCA species (DCAs) was significantly increased in OB individuals with type 2 diabetes (T2DM) from a case–control study and decreased in the remission group of OB subjects with T2DM after metabolic surgery. The changes were closely associated with their metabolic status. These results were consistently confirmed in both serum and liver of mice with diet-induced OB, implying that such a metabolic alteration in circulation reflects changes occurring in the liver. *In vitro* studies of human liver L-02 cell lines under BA treatment revealed that DCA and its conjugated form, TDCA, significantly inhibited mRNA expression of fatty acid transport protein 5 in the presence of DGLA, which was involved in hepatocyte DGLA uptake. Thus, the DGLA: DCAs ratio may be a promising biomarker for metabolic abnormalities in OB.—Lei, S., Huang, F., Zhao, A., Chen, T., Chen, W., Xie, G., Zheng, X., Zhang, Y., Yu, H., Zhang, P., Rajani, C., Bao, Y., Jia, W., Jia, W. The ratio of dihomo-γ-linolenic acid to deoxycholic acid species is a potential biomarker for the metabolic abnormalities in obesity. FASEB J. 31, 000–000 (2017). www.fasebj.org

KEY WORDS: bile acid · free fatty acid · targeted metabolomics · type 2 diabetes

OB results from the accumulation of excess adipose tissue and is a major risk factor for type 2 diabetes mellitus (T2DM), cardiovascular disease, nonalcoholic fatty liver disease, and cancer. Recent studies have shown that bile acids (BAs) modulate dysfunction of lipid metabolism *via* their signaling effect on the pregnane X receptor. Activation of this receptor has been shown to increase expression of the hepatic fatty acid (FA) transporter FA translocase (CD36) and induce expression of peroxisome proliferator–activated receptor (PPAR)-γ in transgenic mice, both resulting in enhanced lipogenesis and repressed FA oxidation, ultimately leading to OB and hepatic steatosis (1). On the other hand, BA activation of the TGR5 receptor expressed in brown adipose tissue in mice with diet-induced OB has an anti-OB effect because of its ability to up-regulate expression of type 2 deiodinase and subsequent conversion of thyroxine (T4) to active 3,5,3′-triiodothyronine (T3), which in turn, stimulates mitochondrial oxidative phosphorylation and energy expenditure (2). BA activation of the farnesoid X receptor (FXR) is known to alter the transcription of genes involved in FA and triglyceride synthesis and lipoprotein metabolism. In mice, FXR agonists reduce plasma triglyceride synthesis and lipoprotein metabolism *via* repression of the lipogenic genes sterol regulatory element–binding protein-1c and FA synthase in liver.

ABBREVIATIONS: BA, bile acid; BMI, body mass index; CPT1A, carnitine palmitoyltransferase 1A; DCAs, deoxycholic acid species; DGLA, dihomo-γ-linolenic acid; FA, fatty acid; FATP, fatty acid transport protein; FFA, free fatty acid; FINS, fasting insulin; FXR, farnesoid X receptor; GDCA, glycodeoxycholate acid; GLP, glucagon-like peptide; HFD, high-fat diet; HOMA-IR, homeostasis model assessment-insulin resistance; ITT, insulin tolerance test; LCFA, long chain fatty acid; PPAR, peroxisome proliferator activated receptor; OB, obese, obesity; OGTT, oral glucose tolerance test; OW, overweight; PUFA, polyunsaturated fatty acid; ROC AUC, receiver operating characteristic area under the curve; RYGB, Roux-en-Y gastric bypass; T2DM, type 2 diabetes mellitus; T3, 3,5,3′-triiodothyronine; T4, thyroxine; TBA, total bile acid; TC, cholesterol; TDCA, taurine-deoxycholic acid; TG, triglyceride; TSH, thyroid-stimulating hormone; UDCA, ursodeoxycholic acid; UPLC, ultra-performance liquid chromatography

[1] Correspondence: University of Hawaii Cancer Center, 701 Ilalo St., Honolulu, HI 96813, USA. E-mail: wjia@cc.hawaii.edu

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FXR activation also leads to induced expression of PPAR-α, a nuclear receptor that promotes lipid β-oxidation (3).

One study has suggested that the increase in free FA (FFA) is a cause of insulin resistance and a pathogenic factor in the development of T2DM, atherosclerosis, and nonalcoholic fatty liver disease (4). Our group has recently reported that several circulating unsaturated FFAs are predictive of the development of metabolic syndrome in a group of OB subjects, especially DGLA (5). In addition, some studies have found that a high DGLA level in serum is associated with a high risk of development of T2DM and hepatic steatosis (6–8).

OB is associated with dysregulation of BA fluctuations (3). Altered BA metabolism is critical in establishing the metabolic complications of OB. After Roux-en-Y gastric bypass (RYGB) , increased volumes of BAs are delivered to the distal gut, along with increased enterohepatic circulation and plasma BAs with different postsurgical compositions that contribute to the beneficial effects of bariatric surgery (9).

Recently, a novel link between BAs and FFAs has been identified—that is, specific BAs, such as DCA and ursodeoxycholic acid (UCDA), are able to inhibit hepatic long chain FA (LCFA) uptake (10). Although BAs as important metabolic modulators *via* their signaling capabilities on various receptors have effects on FFA metabolism, how specific BAs correlate with FFAs in association with metabolic disorders, and the potential value of them to be clinical biomarkers, is largely unknown.

In the present study, we investigated the correlation between BAs and FFAs by analyzing serum BA and FFA profile changes in 2 different groups of human individuals and in HFD mice. Our hypothesis for this study was that changes in BAs important in metabolic signaling would correlate with changes in specific FFAs in patients with T2DM. The purpose of this study was to identify specific correlations between a set of specific BAs and a set of specific FFAs by using a targeted metabolomics approach and evaluate their potential value as biomarkers for metabolic abnormalities in OB subjects.

MATERIALS AND METHODS

Study participants

All participants provided written informed consent. Studies were approved by the ethics committee of Shanghai University of Traditional Chinese Medicine and Shanghai Jiao Tong University Affiliated Sixth People's Hospital and all protocols were compliant with the Declaration of Helsinki.

Two cohorts were used in this study. A total of 343 subjects were recruited for a case–control study from the Shuguang Hospital affiliated with Shanghai University of Traditional Chinese Medicine. The study included 199 healthy subjects, 78 OW or OB subjects (OW/OB), and 66 OW/OB subjects with T2DM (OW/OB DM). The second cohort was a metabolic surgery intervention study with enrollment of 38 OB subjects who had undergone RYGB. Participants were studied at different time points, including baseline and at 1, 3, 6, 12, and 24 mo after surgery. Exclusion criteria included malignancy, acute infection or injury, type 1 DM, secondary diabetes, debilitating disease, unresolved psychiatric illness, a history of open abdominal surgery, gallstones with or without cholecystectomy, and the use of medications known to affect BA and FA metabolism.

Anthropometric measurements (*e.g.*, height and weight), age, sex, personal health history, and current medications were recorded. Fasting blood samples were collected from all individuals and returned on ice to the laboratories within 2 h for blood component separation and storage at −80°C until analysis. Clinical characteristics and routine biochemical tests were examined for the participants in the 2 independent studies (11, 12).

A diagnosis of T2DM was based on the 1999 World Health Organization criteria (13). According to Chinese standard guidelines for normal weight, OW, and OB were defined as body mass index (BMI) $< 24\ kg/m^2$, $24\ kg/m^2 \leq BMI < 28\ kg/m^2$, and $BMI \geq 28\ kg/m^2$, respectively (14). Diabetes remission was defined as hemoglobin A1c level <6.5% and a fasting glucose concentration <7.0 mM for 1 yr or more without active pharmacological intervention (15).

Animal study

Animal experiments were performed in accordance with the ethics committee of Shanghai Jiao Tong University. Three-wk-old male C57BL/6J mice were purchased from Shanghai Laboratory Animal Co., Ltd. (SLAC, Shanghai, China), and given 1 wk of acclimatization. The mice were housed under specific pathogen-free conditions in a temperature-controlled (23°C) facility with a 12-h light–dark cycle and were given free access to food and water. Mice were fed chow diets (10% fat, 71% carbohydrate, and 19% protein) or HFD diets (45% fat, 36% carbohydrate, and 19% protein) for 26 wk.

The mice were unfed 8 h before harvesting blood for subsequent blood measurements. Liver samples and fat tissues were collected immediately after euthanasia. All samples were stored at −80°C until analysis. The levels of total cholesterol (TC), triglyceride (TG), HDL, and LDL cholesterol (LDL), glucose and nonesterified FA were measured using an automatic biochemical analyzer (TBA-40FR; Toshiba, Tokyo, Japan) (16).

Metabolic assays on mice

Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed on mice after 16 wk of HFD. For OGTT, after 6 h unfed, blood was drawn and then mice were gavaged with glucose at a dose of 1 g/kg. For ITT, after food had been withheld for 4 h, blood was drawn and then mice were injected intraperitoneally with insulin using a dose of 0.75 U/kg body weight. Blood samples were taken from the tail vein at 0, 15, 30, 60, and 120 min after glucose gavage and insulin injection, and glucose levels were measured with a glucometer (Johnson & Johnson, New Brunswick, NJ, USA).

Cell culture and treatment

The normal human liver L-02 cell lines were cultured in DMEM supplemented with 10% charcoal-stripped fetal bovine serum (Omega Scientific, Tarzana, CA, USA) and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cells were exposed to DMSO (control), 50 μM DCA, 100 μM TDCA, and 100 μM GDCA, with or without DGLA (100 μM). After 5 d of treatment, the cells were harvested for RNA extraction and gene expression assay using quantitative PCR (17). The assay was performed with SYBR Premix Ex Taq (Takara, Otsu, Japan) on a 7900 Real-Time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA). Primers of the genes analyzed in this study are listed in Supplemental Table 1. Actin was used as the internal reference. Targeted gene levels were normalized to actin levels, and the relative expression was expressed as fold change relative to those of control group.

BAs and FFAs analysis

All the serum and liver samples were stored at −80°C until analyzed. BAs were quantified using ultra-performance liquid chromatography (UPLC)–triple quadrupole mass spectrometry and FFAs were quantified using UPLC quadrupole time-of-flight mass spectrometry (Waters Corp., Milford, MA, USA), according to our previously reported protocol (5, 16, 18, 19). For the measurements of FFAs in liver tissue, the extraction method had a minor correction. For more details, please refer to the Supplemental Data. Data acquisition and quantification were performed using the TargetLynx v.4.1 applications manager (Waters Corp.). All the reference standards were mixed at an appropriate concentration and run after every 10 samples for quality control.

Statistical analysis

Data are expressed as means ± SEM or median (95% CI). All continuous variables were tested with the Shapiro-Wilk test to examine the distribution. Differences in parameters before and after RYGB were evaluated using the paired t test after logarithmic transformation. Differences in serum parameters between groups and the DGLA:DCAs ratio were compared using the Mann-Whitney U test. The gene expression between groups *in vitro* was evaluated using the Student's t test. Spearman correlation was used to evaluate the relationships between BAs, metabolic variables, and FFAs. Receiver operating characteristic (ROC) area under the curves (AUCs) of FFAs were compared between OW/OB subjects and OW/OB subjects with T2DM. The logistic regression analysis was performed to investigate whether the DGLA:DCAs ratio is independent of well-known factors. A 2-sided value of $P < 0.05$ was considered statistically significant. All statistical analyses were calculated with Prism 6.0 (GraphPad Software, La Jolla, CA, USA) and SPSS 17.0 (IBM SPSS, Chicago, IL, USA).

RESULTS

The DGLA:DCAs ratio was significantly increased in OW/OB individuals with DM, relative to normal weight and healthy OW/OB controls

A brief overview of patients in the case–control study can be found in **Table 1**. As expected, BMI and fasting glucose, TC, and TG levels were significantly higher in the OW/OB subjects with DM (Table 1).

Our metabolic analysis showed that subjects with T2DM had quite different FFA profiles and BA composition relative to the controls. We observed that subjects in the OW/OB DM group were well separated from those in the control and OW/OB groups (Supplemental Fig 1*A*). Furthermore, most of the polyunsaturated FAs (PUFAs) had strong and positive correlations with metabolic markers (Supplemental Fig. 1*B*). To determine which individual FFA discriminates OB subjects with T2DM from healthy OB ones, we further compared ROC areas and P values. We found that DGLA (C20:3 n6c) had the highest ROC value (0.993; 95% CI, 0.986–0.999) and the smallest P value ($P = 7.02 \times 10^{-25}$; **Fig. 1*A***). Also the Spearman correlation analysis showed that the level of DGLA correlated positively with BMI, blood glucose, TC, and TG (Supplemental Fig. 1*B*). On the other hand, the major BA species of serum analysis revealed that cholic acid and chenodeoxycholic acid species were increased in OW/OB patients; however, DCAs were greatly decreased in subjects with T2DM relative to controls and OW/OB subjects (Fig. 1*B*). DCAs levels stood out because they correlated more closely to the metabolic markers, such as BMI, TC, and TG (Fig. 1*C*).

BA metabolism is tightly associated with FFA metabolism. To evaluate whether DCAs had a correlation with FFAs, especially DGLA, we performed a Spearman analysis. The level of DCAs correlated negatively with that of DGLA ($r = -0.24$, $P < 0.0001$; Fig. 1*D*). To identify whether DCAs together with DGLA reflected the metabolic status of the participants, we compared the DGLA:DCAs ratio for all patients in 3 groups. We found that this ratio was significantly higher in the OB T2DM group than in both the normal weight control and the OW/OB group (Fig. 1*E*). Also the ratio had strong and positive correlations with the metabolic markers, BMI, glucose, TC, and TG ($r = 0.22$–0.37; $P < 0.05$; **Table 2**). Finally, the logistic regression model indicated that the ratio was a positive predictor of OB-related diabetes after adjustment for gender, age, and BMI. The odds ratio was 1.025 (95% CI, 1.01–1.04).

TABLE 1. *The clinical characteristics of subjects in the case–control study*

Variable	N	OW/OB	OW/OB DM	N *vs.* OW/OB P1	N *vs.* OW/OB DM P2	OW/OB *vs.* OW/OB DM P3
Male/female	104/95	47/31	35/31			
Age (yr)	37.74 ± 0.84	39.09 ± 1.32	57.41 ± 0.85	2.49E-01	3.51E-23	9.55E-16
BMI (kg/m^2)	21.11 ± 0.13	26.72 ± 0.25	27.82 ± 0.33	2.55E-38	4.38E-34	1.45E-03
GLU (mM)	5.26 ± 0.03	5.37 ± 0.13	7.97 ± 0.31	6.75E-02	2.43E-23	4.80E-13
TC (mM)	5.08 ± 0.07	5.51 ± 0.11	5.43 ± 0.12	8.76E-04	4.86E-03	9.10E-01
TG (mM)	1.38 ± 0.06	1.92 ± 0.1	2.42 ± 0.19	7.06E-08	3.35E-10	1.08E-01
ALT (U/l)	26.12 ± 0.91	38.01 ± 2.1	22.73 ± 1.27	4.77E-08	4.40E-02	1.07E-08
AST (U/l)	20.87 ± 0.41	23.46 ± 0.75	20.58 ± 0.71	2.43E-03	7.06E-01	6.14E-03
γ-GGT (U/l)	13.85 ± 0.71	20.1 ± 1.42	37.97 ± 2.75	2.09E-05	2.09E-24	1.05E-09

Data represent means ± SEM. P values are calculated with the Mann-Whitney U test to compare the statistical significance of metabolic markers between normal (N), OW/OB, and OW/OB DM, and are underlined if $P < 0.05$. γ-GGT, γ-glutamyltranspeptidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GLU, glucose.

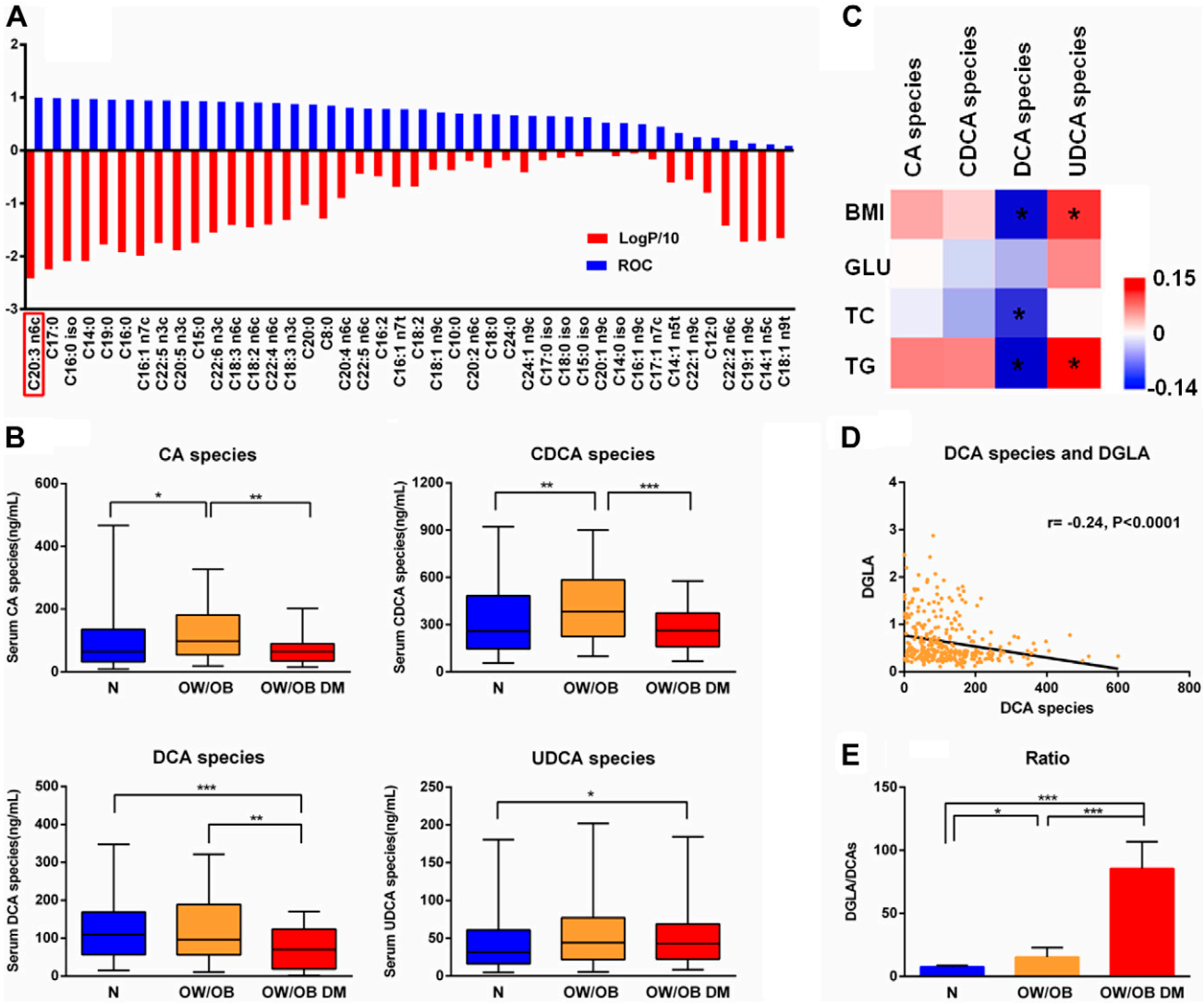

Figure 1. Serum FFAs, BAs, and the DGLA:DCAs ratio analysis in the case–control study. *A*) Bar plots of the ROC AUCs of serum FFAs and *P* values (LogP/10) calculated between the OW/OB and OW/OB DM groups. *B*) Major BA species in the case–control study. BA species include unconjugated, taurine-conjugated, and glycine-conjugated BAs. *C*) Heat map of correlation coefficients between BA species and metabolic markers. $^*P < 0.05$ (Spearman). *D*) The correlation between DCAs and DGLA in the 3 groups. *E*) Analysis of DGLA:DCAs ratio in the 3 groups. CA, cholic acid; CDCA, chenodeoxycholic acid. Data are expressed as median (95% CI) or means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The DGLA:DCAs ratio decreased dramatically in the OB group with T2DM with remission after metabolic surgery

RYGB surgery is one of the most efficient procedures for the treatment of morbid OB. So to examine the changes of the DGLA:DCAs ratio after metabolic surgery, we observed 38 OB subjects with T2DM from the metabolic surgery follow-up study. The clinical characteristics of the participants are summarized in Supplemental Table 2. Compared with baseline values, BMI, glucose tolerance, insulin sensitivity, and lipid profiles were significantly improved 12 mo after RYGB surgery.

The results of the second year follow-up examination indicated that 26 of 38 patients achieved remission of T2DM (68.4%). Therefore, the DGLA and BAs in the remission group at baseline and 12 mo after RYGB were re-examined. The level of DGLA was lower at 12 mo after RYGB in the remission group compared with baseline (**Fig. 2*A***). We found that serum total bile acid (TBA) concentration was increased from 1828.99 ± 214.64 ng/ml at baseline to 2451.24 ± 422.48 ng/ml 12 mo after RYGB in remission group ($P > 0.05$). To address which BA species contributed to the increase, we analyzed individual, major fasting serum BA species and found that the level of DCAs was a major contributor to the observed increase in TBAs (Fig. 2*B*).

Similarly, correlation analysis of the remission group showed that the level of DCAs correlated negatively with DGLA in patients with T2DM who underwent RYGB ($r = -0.29$, $P = 0.03$; Fig. 2*C*). Twelve months after RYGB, the DGLA:DCAs ratio was significantly lower than baseline for those who achieved remission (Fig. 2*D*). This ratio was positively associated with BMI, TG,

TABLE 2. *Spearman correlation analysis of the DGLA:DCAs ratio with metabolic markers in human and animal studies*

Human study						Animal study					
Case-control study	*R*	*P*	MS	*R*	*P*	Serum	*R*	*P*	Liver	*R*	*P*
BMI	0.37	2.80E-12	BMI	0.46	1.09E-03	Body weight	0.73	1.31E-03	Body weight	0.87	2.46E-04
GLU	0.33	1.62E-07	TG	0.44	1.79E-03	Liver weight	0.71	1.98E-03	Liver weight	0.81	1.36E-03
TC	0.22	1.11E-04	TC	0.25	8.12E-02	VAT	0.72	1.75E-03	VAT	0.85	5.21E-04
TG	0.33	4.07E-09	HDL-C	−0.31	3.02E-02	TG	0.74	1.09E-03	TG	0.66	1.96E-02
ALT	−0.02	6.65E-01	LDL-C	0.20	1.76E-01	TC	0.77	5.51E-04	TC	0.80	1.84E-03
AST	0.07	2.43E-01	FPG	0.25	8.07E-02	HDL-C	0.83	7.32E-05	HDL-C	0.89	1.14E-04
γ-GGT	0.45	1.68E-17	2hPG	0.27	6.38E-02	LDL-C	0.75	8.78E-04	LDL-C	0.68	1.58E-02
			FINS	0.35	1.50E-02	FFA	0.66	5.70E-03	FFA	0.43	1.62E-01
			2hINS	0.33	2.11E-02	GLU	0.63	9.22E-03	GLU	0.74	6.22E-03
			HOMA-IR	0.37	8.73E-03	ALT	0.76	6.51E-04	ALT	0.78	2.83E-03
			HOMA-β	0.17	2.53E-01	AST	0.51	4.59E-02	AST	0.47	1.21E-01

HOMA-IR is calculated to reflect insulin resistance by the formula: [FPG (millimolar) × FINS (microunits/milliliter)]/22.5. HOMA-β is calculated to assess insulin sensitivity by the formula: [20 × FINS (microunits/milliliter)]%/[FPG (millimolar) −3.5]. Values of $P < 0.05$ are underlined. γ-GGT, γ-glutamyltranspeptidase; 2hPG, 2h postprandial plasma glucose; ALT, alanine aminotransferase; FPG, fasting plasma glucose; GLU, glucose; VAT, visceral adipose tissue.

fasting insulin (FINS) level, insulin level at 2 h (2hINS), and homeostasis model assessment-insulin resistance (HOMA-IR) (r = 0.33–0.46, $P < 0.05$; Table 2). These results confirmed that the DGLA:DCAs ratio was a potential biomarker for defining the metabolic status of OB subjects.

In mice with HFD-induced OB with impaired glucose tolerance, the DGLA:DCAs ratio was greatly increased in both serum and liver

To determine whether the serum DGLA:DCAs ratio reflects the metabolic status of the liver, we examined and compared BAs and FFAs in both serum and liver of HFD mice. After 26 wk of HFD, the average body weight and liver weight of mice were significantly increased (**Fig. 3*A***). The HFD group displayed significantly increased levels of lipid profiles, fasting blood glucose, and nonesterified FA (Supplemental Fig. 2). To explore the role of HFD in OB-related glucose homoeostasis, OGTT and ITT were performed. OGTT revealed that the HFD group had significantly increased blood glucose levels after glucose loading (Fig. 3*B*). ITT demonstrated that the insulin sensitivity also decreased significantly in the HFD group (Fig. 3*C*). These results verified that HFD induced OB and impaired glucose tolerance (IGT). Detailed analysis of major BA species,

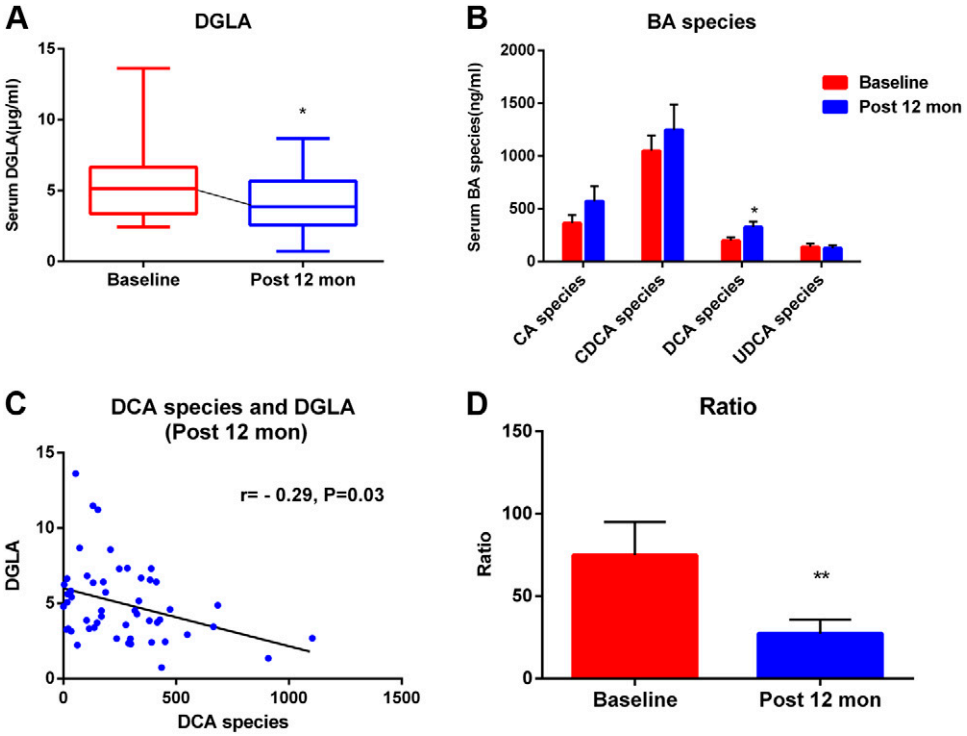

Figure 2. The DGLA:DCAs ratio analysis for the remission group in the metabolic surgery study. *A*) Serum DGLA concentrations at baseline and 12 mo after RYGB. *B*) The concentrations of serum major BA species at preoperative and 12 mo after RYGB. *C*) Correlation between DCAs and DGLA at 12 mo after RYGB. *D*) The ratio analysis of DGLA to DCAs at baseline and 12 mo after RYGB. Data are expressed as means ± SEM. $*P <$ 0.05, $**P < 0.01$, $***P < 0.001$.

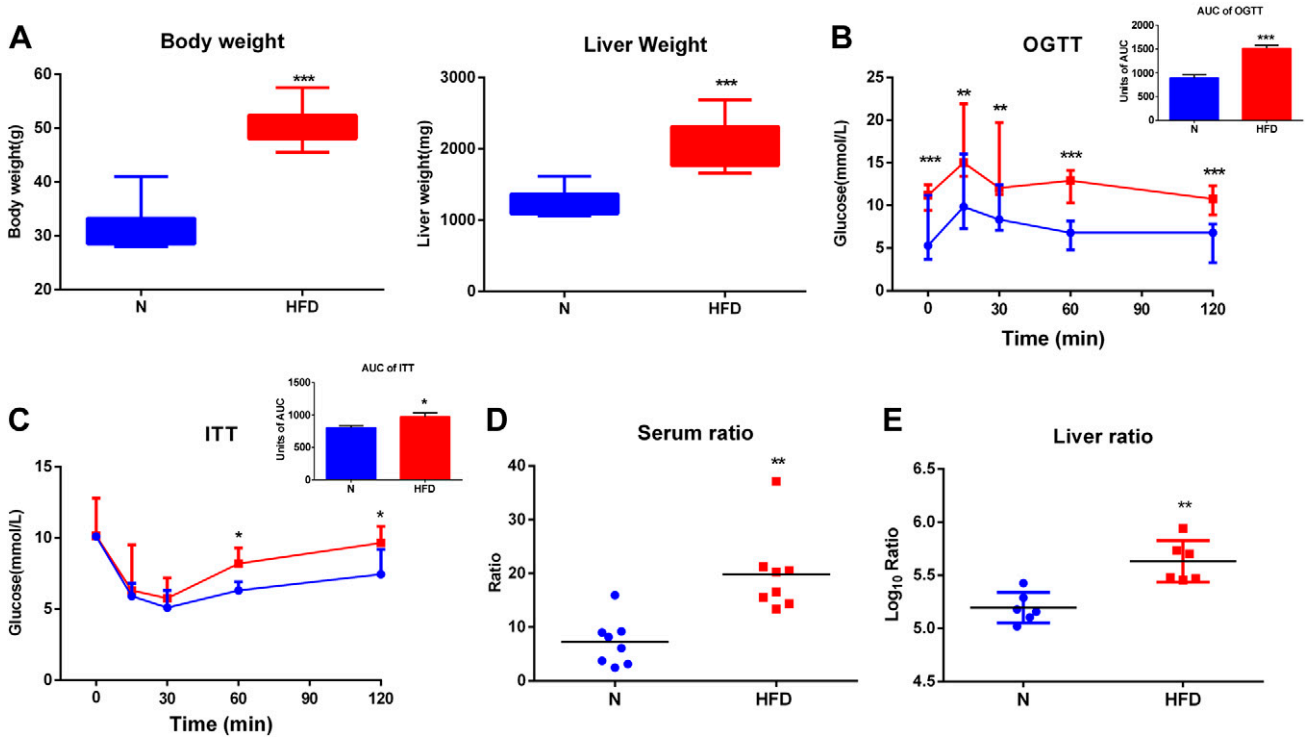

Figure 3. HFD-induced OB and IGT and DGLA:DCAs ratio analysis in mice. *A*) Body and liver weights of C57BL/6J mice after 26 wk of a chow diet and HFD. *B*) Glucose levels during an OGTT and the ROC AUCs in mice fed the different diets for 16 wk. *C*) Glucose levels during an ITT and the ROC AUCs in mice fed the different diets for 16 wk. *D*) The ratio of DGLA:DCAs analysis in serum after 26 wk of chow diet and HFD. *E*) DGLA:DCAs ratio analysis in liver after 26 wk of chow diet and HFD (n = 5–8). Data are expressed as means ± SEM. *$P < 0.05$, *$P < 0.01$, ***$P < 0.001$ *vs.* HFD.

DGLA, and their correlations in both serum and liver were performed (Supplemental Fig. 2). Results showed that the DGLA:DCAs ratio was greatly increased in both serum and liver of HFD mice (Fig. 3*D*, *E*) and had close correlation with biochemical markers (r = 0.51–0.89, $P < 0.05$; Table 2).

DCA and TDCA significantly inhibited the mRNA expression of FATP-5, PPAR-α, and carnitine palmitoyltransferase 1A, in the presence of DGLA

To further demonstrate whether the changes in levels of DCAs and DGLA in circulation were derived from changes occurring in the liver, the most important organ for BA and FFA metabolism, we measured the mRNA expression level of key enzymes related to hepatic FA metabolism in normal L-02 hepatocytes under treatment with BAs, with or without DGLA. In the presence of DGLA, gene expression of FATP-5 was markedly inhibited in the DCA and TDCA treatment groups, whereas its expression was not significantly changed in the GDCA group (**Fig. 4**). Two important factors involved in FA oxidation, the transcription factor PPARα and the enzyme, carnitine palmitoyltransferase 1A (CPT1A) had significantly decreased mRNA levels under DCA treatment that was accompanied by decreased hepatocyte uptake of DGLA. Other mRNA levels involved in FA transformation and synthesis remained similar in the BA treatment groups compared with the control. In addition, genes for FA uptake and oxidation in the BA treatment groups did not show significant alteration of mRNA levels relative to control when DGLA was not present.

DISCUSSION

In the present study, the concentrations of both BAs and FFAs in OW/OB DM patients were determined, and statistical analysis of the data revealed that dysregulated BA and FFA metabolism contributed to OB-related metabolic disorders. We found that the levels of DCAs correlated inversely with DGLA and that the DGLA:DCAs ratio was a potential biomarker to identify metabolic abnormalities in OW/OB subjects.

Numerous studies attempt to explore the link between serum BA levels and different metabolic phenotypes associated with OB and its complications. The existing literature is ambiguous regarding serum TBA concentrations in T2DM, OB, and healthy control subjects. Some have reported that TBA concentrations are higher in T2DM than in normal glucose–tolerant patients before RYGB surgery (20), whereas others found comparable TBA concentrations between preoperative T2DM patients and healthy control (21). Another recent study showed that basal serum BA levels were significantly lower in OB DM patients when compared to healthy control subjects (22), which was consistent with our results. It has been shown that high serum insulin levels or diabetic status repressed cytochrome P450 family 7 subfamily A member 1 transcription and BA biosynthesis (23), leading to impairment of BA metabolism. In

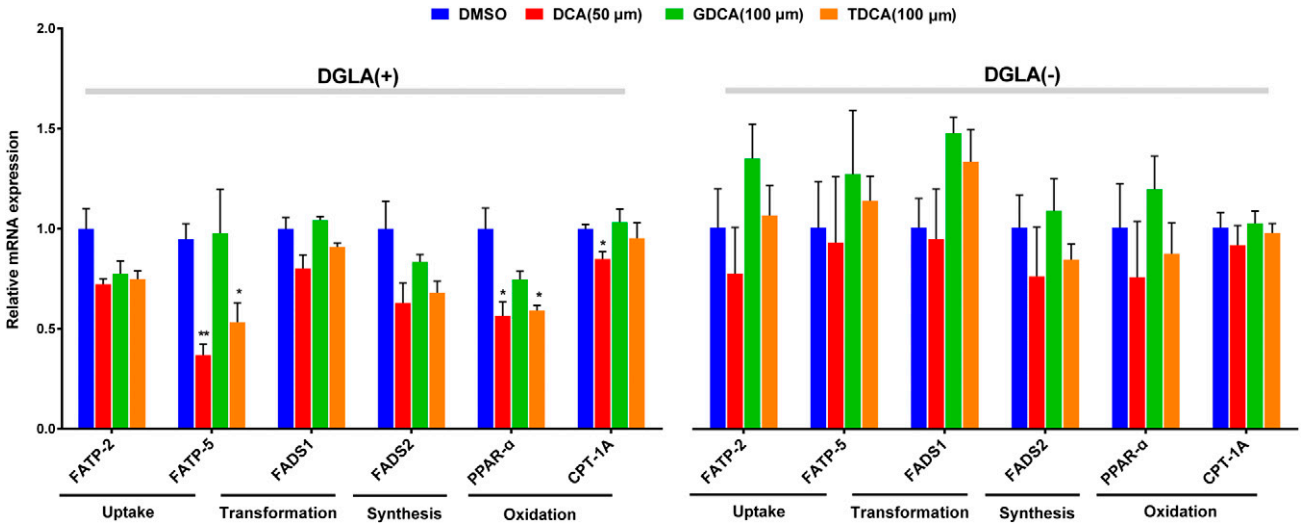

Figure 4. Gene expression analysis in L-02 cells exposed to DMSO, DCA, GDCA, and TDCA, with or without DGLA added. Data are expressed as means ± SEM. $*P < 0.05$, $**P < 0.01$ (2-tailed Student's *t* test) *vs.* DMSO.

our study, we observed low-serum BA levels in OB patients with T2DM that may be caused by metabolic changes, particularly by hyperinsulinemia. These findings highlight the possibility that alteration in BA synthesis may affect pathophysiology of T2DM.

From the examination of specific BA species, we determined that serum levels of DCAs were able to distinguish OB diabetes *vs.* those without diabetes. In the metabolic surgery study, TBAs were increased, and levels of DCAs were changed in parallel with TBAs at 12 mo after RYGB. Prior studies showed that plasma TBA concentrations were significantly increased after RYGB (24, 25), similar to our results. Albaugh *et al.* (26) reported the longitudinal changes in BAs after RYGB, showing the early improvements at 1 mo after RYGB associated with surges in UDCA and its conjugates and the late changes associated with increased DCA and its glycine conjugate at 24 mo, the results of which were similar to ours. But they did not distinguish T2DM with nondiabetic subjects at the time of surgery (26). It is known that the secondary BAs, DCAs, are converted by intestinal 7α-dehydroxylation bacteria, particularly the phylum of *Clostridium* cluster XIVa. Another study indicated that the proportions of the phylum Firmicutes and class Clostridia were significantly reduced in diabetes (27). Taken together, these findings suggest that BAs, especially DCAs play an important role in OB-related T2DM.

Increased plasma FFA levels are an important cause of insulin resistance in OW/OB DM patients (4). In our study, most PUFAs were more closely correlated with metabolic disorders in OB subjects. It is well known that n-6 PUFAs are one of the major classes of PUFAs, which are required for optimal human health. Linoleic acid is the parent FA through elongase and desaturase to synthesis of the n-6 series of FAs in mammals (28). Eicosanoids derived from n-6 PUFAs such as arachidonic acid have proinflammatory and immunoactive functions (29). In the EPIC-InterAct case–cohort study, Forouhi *et al.* (30) found that some n-6 PUFAs were associated with higher T2DM incidence. DGLA, as the precursor of arachidonic acid, is a key inflammation marker in predicting the risk of developing metabolic syndrome and has strong correlation with inflammation markers in OB, such as IL-6 and soluble ICAM-1 (31, 32). Our findings showed that DGLA was significantly increased in OW/OB DM individuals, and markers of low-grade inflammation, such as DGLA and C-reactive protein, were decreased after RYGB, consistent with the findings by Cho *et al.* (33) and Netto *et al.* (34). These results suggest that the increased level of DGLA contributes to the inflammatory phenotype in T2DM.

The reason that the levels of DCAs were negatively correlated with DGLA remains unclear. Our results demonstrated that DCA and TDCA inhibited FATP-5, PPARα, and CPT1A mRNA expression in the presence of DGLA. Previous results obtained by Nie *et al.* (10) confirmed that DCA had an inhibitory effect on LCFA uptake in a FATP-5-dependent manner by using nonradioactive hepatic LCFA uptake assay *in vitro* and *in vivo*; however, they did not measure mRNA levels of FATP-5 or levels of PPARα and CPT1A. FATP-5 has been shown to be a multifunctional protein. *In vivo*, FATP-5 not only increases uptake of LCFA, but also acts as a bile-CoA ligase (35). BAs that can serve as substrates for enzymatic activation by FATP-5 could inhibit LCFA uptake (10). Another study found that decreased intrahepatic lipids in FATP-5-deletion mice with HFD may have a decreased FA oxidation because of a trend toward decreased ketone bodies (36). These findings begin to shed light on the complex interrelationships in hepatic BA and lipid metabolism. However, the underlying structure–function relationships between these genes and DCAs and DGLA warrant further investigation.

Multiple BAs that activate TGR5 include conjugated and unconjugated forms of DCA (37). A newly published study demonstrated that BAs inhibited NLR family pyrin domain containing 3 inflammasome activation *via* TGR5 signaling, leading to the control of inflammation in LPS-induced sepsis and improvements in metabolic disorders induced by mice eating an HFD (38), perhaps explaining the correlation between DCAs and DGLA in T2DM as an inflammatory disease.

After metabolic surgeries, raised circulating BA levels are positively correlated with increased glucagon-like peptide (GLP)-1 in both humans and rats (25, 39, 40). GLP-1 and its analog have additional antidiabetic effects and also could reduce expression of lipogenic transcription factors, PPARγ, and CCAAT/enhancer binding protein-α in both db/db mice and rats with diet-induced OB (41–43). On the other hand, to better characterize the net effects of RYGB surgery, Hao *et al.* (44, 45) found that energy expenditure was significantly increased after bariatric surgery in a new mouse RYGB model which closely replicated human RYGB. Furthermore, it has been demonstrated that BAs could trigger TGR5-cAMP- type 2 deiodinase signaling pathway and subsequent conversion of T4 to T3 to increase energy expenditure (46). Therefore, we assessed serum thyroid hormone levels after RYGB to confirm the role of DCAs in energy expenditure. Our findings were that free T4, T3, and TSH were in normal range, but the DCAs level after 12 mo of RYGB showed a strong inverse correlation with TSH in the remission group (Supplemental Fig. 3), in accordance with the study by Kohli *et al.* (24), which demonstrated that increased BAs after RYGB were associated with GLP-1 secretion and thyroid hormone function *via* TGR5-mediated mechanisms. Furthermore, it has been reported that the increase in TBA accompanied by a decrease in conjugated BAs after RYGB correlated with decreased glucose oxidation and increased lipid oxidation (47). Taken together, these observations may indirectly contribute to the explanation for our observed decline in the DGLA levels in OB DM patients who underwent RYGB.

There are several limitations in this study. First, we did not detect serum gut peptides, such as GLP-1, and therefore, lack mechanistic links between peptides and the BAs and FFAs. Second, BA excretion and gut microbiota composition analysis were not evaluated in feces. In addition, the population size of the case–control study and metabolic surgery intervention study may also be a limitation. Therefore, our results should be regarded as preliminary. Further studies are necessary to fully characterize the mechanistic link between DCAs and DGLA in metabolic diseases.

In summary, we revealed a novel link between secondary BA, DCAs, and a specific FFA, DGLA. The present study showed that the DGLA:DCAs ratio was elevated in OW/OB DM individuals and decreased after weight loss, which correlated closely with changes of metabolic status in OB patients. The underlying reason may be that DCAs inhibited mRNA levels of important genes involved in lipid metabolism. The DGLA:DCAs ratio may therefore be useful as a biomarker for metabolic abnormalities in OB subjects. FJ

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AUTHOR CONTRIBUTIONS

W. Jia and W. Jia designed the research; F. Huang and Y. Zhang collected the clinical samples in the case–control study; H. Yu, P. Zhang, and Y. Bao provided the samples in the metabolic surgery study; F. Huang and Y. Zhang performed the mouse study; W. Chen performed the cell experiment; S. Lei, A. Zhao, F. Huang, and X. Zheng performed BAs and FFAs targeted metabolomics analysis; S. Lei, T. Chen, and G. Xie analyzed the data; S. Lei performed the statistical analysis and wrote the manuscript; and W. Jia and C. Rajani revised the manuscript.

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Sha Lei, Fengjie Huang, Aihua Zhao, et al.

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