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# Potential biomarker in serum for predicting susceptibility to type 2 diabetes mellitus: Free fatty acid 22:6

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**Keywords**
C22:6, Obesity, Type 2 diabetes mellitus

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## ABSTRACT

**Aims/Introduction:** Type 2 diabetes mellitus is closely linked to increased levels of free fatty acids (FFAs) in obese individuals, although which FFA is most associated with type 2 diabetes mellitus is unclear. This study aimed to identify the specific FFAs that best predict the occurrence of type 2 diabetes mellitus in obese individuals, and assess their potential application value.

**Materials and Methods:** Participants were divided into three groups: a normal weight group ($n$ = 20), an obese group ($n$ = 10) and a type 2 diabetes mellitus group ($n$ = 10). FFAs in serum samples were determined by ultra-high-pressure liquid chromatography–mass spectrometry, and orthogonal partial least squares discriminant analysis models were used to study the FFA profile among the three groups.

**Results:** Compared with the normal weight group, 14 FFAs (C8:0/10:0/14:0/16:1/18:1/20:2/ 20:3 /20:4/ 20:5/ 22:6/7:0/9:0/11:0 and C13:0) were significantly increased in the obese group, and nine FFAs (C14:0, C18:1, C20:1, C 18:2, C20:2, C20:3, C18:3, C20:5 and C22:6) were significantly increased in the type 2 diabetes mellitus group. Subsequently, the Venn diagram results showed that six FFAs (C14:0, C18:1, C20:2, C20:3, C20:5 and C22:6) were significantly increased in both the obese and type 2 diabetes mellitus groups. Among these six, C22:6 was finally identified as an independent risk factor for type 2 diabetes mellitus, and had a great potential to predict the susceptibility to type 2 diabetes mellitus (area under the curve 0.803).

**Conclusions:** C22:6 can be an independent risk factor for type 2 diabetes mellitus, and it has a great potential to predict the susceptibility to type 2 diabetes mellitus.

## INTRODUCTION

As one of the most common chronic diseases in the world, type 2 diabetes mellitus is a persistent and universal threat to human health and global medical care. In 2019, 463 million people had type 2 diabetes mellitus, and it is estimated that by 2045, the number of patients with type 2 diabetes mellitus will reach 700 million[1]. Obesity is the most common cause of type 2 diabetes mellitus[2,3]. According to the latest data in the National Diabetes Statistics Report (2017), 87.5% of adults with diabetes are overweight/obese[4]. Unfortunately, the specific mechanism of obesity leading to type 2 diabetes mellitus is still unclear.

Obese persons are typically characterized by dyslipidemia, which is reported to be a key risk factor for obesity leading to type 2 diabetes mellitus[5,6]. As a result, some lipid-lowering agents have been used as treatment strategies to alleviate hyperlipidemia in type 2 diabetes mellitus patients. However, there are some inevitable drawbacks to this treatment strategy, such as the side-effects of these drugs and the failure of some type 2 diabetes mellitus patients to effectively reduce excessive blood glucose levels, even after blood lipids return to normal levels[7–9]. That is to say, the normalization of triglyceride (TG), total cholesterol (TC) and other blood lipid related biochemical

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indicators does not mean that the patient has achieved a good blood lipids homeostasis, which can effectively reduce the blood glucose level of the patients[10]. Previous studies showed that obesity can also cause increased concentrations of free fatty acids (FFAs) content in serum, eventually leading to lipid metabolism disorders[11,12]. The increase in FFAs concentrations in obese (OB) people can lead to insulin resistance (IR) and cell dysfunction, which are two main characteristics of type 2 diabetes mellitus[13–15]. This research shows that disordered lipid metabolism, especially changes in FFAs concentrations, has been found to be a main factor for obesity-induced type 2 diabetes mellitus.

According to the length and spatial structure of the carbon chain, FFAs in the body consist of many subtypes, and different types play different roles[16,17]. Current research shows that not only the changes observed in serum total FFAs, but also changes in different types of FFAs in diseases are important. Our previous studies showed that compared with the normal weight (NW) individuals, there were significant changes in different FFAs in the serum of OB individuals (for example, 10 FFAs, such as C10:0, C11:0 and C14:0, were significantly increased in OB individuals)[18]. Some studies found that compared with healthy non-obese individuals, the serum FFA profile of type 2 diabetes mellitus individuals had also changed; some FFAs were increased (e.g., saturated fatty acids, such as C16:0 and C18:0; and monounsaturated fatty acids, such as C16:1 and C18:1)[19], and that of some FFAs were decreased (e.g., ω-6 polyunsaturated fatty acids [PUFAs], such as C20:3; and ω-3 PUFAs, such as C18:3)[20]. These studies all provide evidence for the etiology of type 2 diabetes mellitus and follow-up treatment, but the aforementioned studies overlooked obesity as an important risk factor for type 2 diabetes mellitus, and did not pay attention to the specific changes in FFA profiles in the dynamic change process from NW to obesity, and finally to type 2 diabetes mellitus. Therefore, screening which kinds of FFA are altered in OB individuals and type 2 diabetes mellitus patients to determine the key FFAs that link obesity and type 2 diabetes mellitus, and whether these differential FFAs can be used as early diagnosis biomarkers of type 2 diabetes mellitus or as a drug target in the future, became our focus.

In the present study, the FFA profile in the serum of 20 NW individuals, 10 OB individuals and 10 type 2 diabetes mellitus patients were analyzed by using the ultra-high-pressure liquid chromatography–mass spectrometry metabolic platform. We aimed to identify the differences in FFAs among the three groups. Then, we assessed some of these biomarkers for the prediction of type 2 diabetes mellitus susceptibility.

## METHODS

### Participants

From June to October 2018 in the First Affiliated Hospital of Shihezi University, Shihezi, China, we enrolled 40 participants aged 30–60 years. The participants were divided into three groups: the NW group ($n$ = 20), the OB group ($n$ = 10) and the type 2 diabetes mellitus group ($n$ = 10).

The inclusion criteria were as follows: (i) the participants in the NW group met the following conditions: 18.5 ≤ body mass index (BMI) < 24 (kg/m$^2$), TG ≤1.7 mmol/L, TC ≤5.2 mmol/L, low-density lipoprotein cholesterol (LDL-C) ≤3.4 mmol/L, high-density lipoprotein cholesterol (HDL-C) ≥1.0 mmol/L and fasting blood glucose (FPG) <6.1 mmol/L; (ii) the participants in the OB group met the following conditions: BMI ≥28 (kg/m$^2$) and FPG <7.0mmol/ L; and (iii) the participants in the type 2 diabetes mellitus group met the following conditions: FPG >7.0 mmol/L or 2-h postprandial glucose >11.1 mmoL/L for two consecutive days. Type 2 diabetes mellitus was diagnosed according to the 1999 criteria of the World Health Organization: FPG >7.0 mmol/L or 2-h postprandial glucose >11.1 mmoL/L. Normal glucose tolerance was defined as FPG <6.1 mmol/L.

The exclusion criteria were: (i) liver, kidney, gastrointestinal, blood or endocrine diseases; (ii) history of surgery or emergency treatments; (iii) pregnant or lactating women; (iv) recent history of taking antibiotics; (v) history of mental illness or substance abuse; or (vi) currently receiving medical treatment or taking any medication, including eating medication, and doing exercise/controlling diet to treat the disease.

### Informed consent and study ethics

The study was approved by the Ethics Review Committee of the First Affiliated Hospital, Shihezi University School of Medicine (Approval Number: 2018-057-01). All participants gave their informed consent for inclusion before they participated in the study.

### General data and biochemical index

General data, such as height, weight, BMI and waist circumference (WC), were collected. BMI was calculated using the formula BMI = weight (kg) / height (m)$^2$. Blood samples were taken from 08.00 to 11.00 hours. after an overnight fast of at least 8 h. The venous blood was centrifuged in a separate gel accelerating tube to isolate the serum (4,400 $g$ × 10 min). Part of the serum was used to detect the relevant biochemical indicators. The remaining serum was immediately stored at −80°C. The levels of FPG, TC, TG, LDL-C and HDL-C were determined by using an automatic biochemical analyzer.

### Ultra-high-pressure liquid chromatography–mass spectrometry analysis

FFAs in the serum samples were determined by ultra-high-pressure liquid chromatography–mass spectrometry on the Tsinghua University metabolomics platform. A 30-μL sample was extracted, to which 70 μL acetonitrile was added. The solution was then mixed and kept static for 15 min at 4°C, followed by centrifugation at 13,200 $g$ for 15–20 min and subsequent removal of the supernatant. Derivatives (20 μL) were added to the supernatant to improve the degree of

separation and the detection sensitivity of the mixture. Then, 20 μL 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide was added to improve the coupling efficiency. After mixing, the mixture was briefly centrifuged, followed by incubation at 40°C for 30 min and centrifugation at 12,000 rpm for 15–20 min. The supernatant (20 μL), plus 20 μL internal standard, was removed, bottled and analyzed by using an UltiMate 3000 HPLC system.

### Statistical analysis

The data were statistically analyzed by using the SPSS statistical software package version 20.0 (SPSS Inc., Chicago, IL, USA). First, the Shapiro–Wilk normality test was carried out to evaluate the normality of the data. The non-parametric rank sum test was used to compare the characteristics of participants among the NW group, the OB group and the type 2 diabetes mellitus group. The independent samples *t*-test was used to study the differences in FFAs levels between the NW and OB groups, and between the NW and type 2 diabetes mellitus groups. A *P*-value <0.05 was considered statistically significant. All metabolomic analysis plots were carried out by using the SIMCA 14.1 (Sartorius, Göttingen, Germany), GraphPad Prism 8.0 (GraphPad, La Jolla, CA, USA) and the MetaboAnalyst 4.0 software (Metrics, Stockholm, Sweden). To evaluate the similarities or differences in FFA profiles between the NW and OB groups, and between the NW and type 2 diabetes mellitus groups in the cross-sectional analysis, a supervised multivariate model called the orthogonal partial least squares discriminant analysis (OPLS-DA) was carried out based on the overall metabolic profile. The correlation of FFAs and the biochemical indices (TC, TG, LDL-C, HDL-C and FPG) was tested by Spearman correlation analysis. Then, risk factors associated with dyslipidemia and hyperglycemia were analyzed by binary logistic regression analysis. The receiver operating characteristics (ROC) curve combined with a $\chi^2$-test were used to assess the ability of FFAs to predict type 2 diabetes mellitus in the cross-sectional study.

## RESULTS

### Participant characteristics

Table 1 shows the participants' characteristics. The results showed that the bodyweight, BMI and WC of the participants in the OB group were significantly higher than those in the NW group and the type 2 diabetes mellitus group ($P < 0.01$), and the levels of TG in the OB group were significantly higher than those in the NW group ($P < 0.05$), but not significantly different from the type 2 diabetes mellitus group. The FPG levels of type 2 diabetes mellitus patients were significantly higher than those of the NW group and the OB group ($P < 0.001$); in addition, the bodyweight, BMI, WC and the levels of TG in the type 2 diabetes mellitus group were not significantly different from those of the NW group. There was no significant difference in TC, LDL-C and HDL-C levels among the three groups.

**Table 1** | Comparison of clinical characteristics and biochemical parameters among participants with normal weight, obesity and type 2 diabetes mellitus

| Index | NW | OB | Type 2 diabetes mellitus |
|---|---|---|---|
| Total no. cases | 20 | 10 | 10 |
| Age (years) | 7.00 | 9.00 | 12.75* |
| Height (cm) | 12.75 | 15.25 | 16.25 |
| Weight (kg) | 12.00 | 22.25*** | 17.50$^{\#\#}$ |
| BMI (kg/m$^2$) | 2.71 | 3.27*** | 3.46$^{\#\#\#}$ |
| WC (cm) | 13.50 | 11.50*** | 13.75 $^{\#\#\#}$ |
| FPG (mmol/L) | 0.97 | 1.87 | 2.77***, $^{\#\#}$ |
| TC (mmol/L) | 0.98 | 1.47 | 1.53 |
| TG (mmol/L) | 0.55 | 0.78* | 0.79 |
| LDL-C (mmol/L) | 0.84 | 0.96 | 1.42 |
| HDL-C (mmol/L) | 0.31 | 0.24 | 0.53 |

The values represent the interquartile range. The values represent non-parametric rank-sum test compared with normal weight (NW) participants, *$P < 0.05$, ***$P < 0.001$; compared with obese (OB) participants, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. BMI, body mass index; FPG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; WC, waist circumference.

### OPLS-DA model construction and validation

First, to determine whether there was a difference in the FFA profile between the NW and the OB groups, we carried out an OPLS-DA model. As can be seen from the results in the OPLS-DA score chart, there was significant separation without overlapping between the NW and the OB group, showing that there was a significant metabolic difference in the FFA profile between the two groups (Figure 1a). Second, to ensure the robustness of the OPLS-DA model, we used sevenfold cross-validation to calculate the goodness of fit; this was 0.999, and the goodness of prediction was 0.952. The 200-item permutation test results showed that the model was not overfitted (Figure 1b). The same method was used to explore whether there were metabolic differences between the NW group and the type 2 diabetes mellitus group. From the results of Figure 1c,d, there was obvious separation between the NW and the type 2 diabetes mellitus group. The goodness of fit and goodness of prediction values obtained from the model were 0.981 and 0.952, which show that the model provided a high degree of goodness of fit and goodness of prediction, respectively. These results showed that the FFA profile differences have promise as a predictive method of linking obesity and type 2 diabetes mellitus patients.

### Differential FFAs identification

#### *Identification of differences in FFAs between the NW group and the OB groups*

From the performed model, we constructed an S-plot in which the *x*- and *y*-axes represented the contribution and confidence

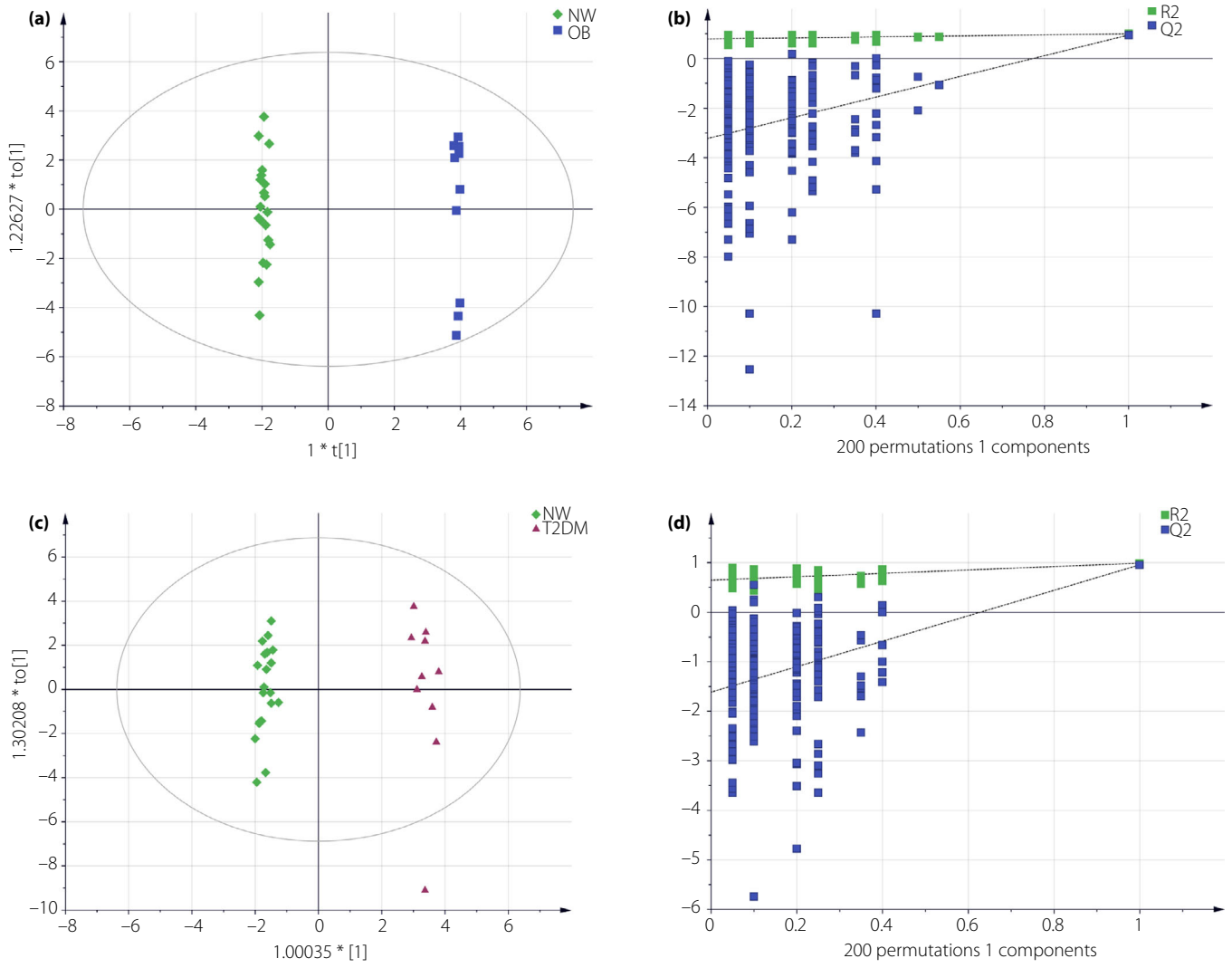


**Figure 1** | Orthogonal partial least squares discriminant analysis for the different groups. (a) The score plot of the orthogonal partial least squares discriminant analysis model shows a clear discrimination between the 20 normal weight (NW) participants (green diamond) and 10 obese (OB) participants (blue square). (b) Permutation test with a permutation number of 200 between the NW and OB groups. (c) The score plot of the orthogonal partial least squares discriminant analysis model shows a clear discrimination between the 20 NW participants and 10 type 2 diabetes mellitus participants (T2DM; purple triangle). (d) Permutation test with a permutation number of 200 between the NW and type 2 diabetes mellitus groups. R2, goodness of fit ; Q2, goodness of prediction.

of each variable, respectively. The S-Plot demonstrated that 14 different FFAs (C8:0, C10:0, C14:0, C16:1, C18:1, C20:2, C20:3, C20:4, C20:5, C22:6, C7:0, C9:0, C11:0 and C13:0) were the FFAs that contributed most to the separation of the NW and the OB groups in the OPLS-DA score plot (Figure 2a). Combined with the results of volcano plot analysis, we found that the concentrations of these 14 FFAs in the OB group were higher than those in the NW group, and the fold change of these FFAs were all >1.50 (Figure 2b). The heatmap showed that these FFAs could effectively discriminate between the two groups (Figure 2c).

For 34 FFAs whose concentrations had normal distributions, we used the independent samples *t*-test to confirm the 14 differentiated FFAs screened by the OPLS-DA model. $P < 0.05$ was taken as the standard to show a significant difference between the two groups (NW vs OB). Our analysis found that the 14 differentiated FFAs screened by the OPLS-DA model still showed significant changes in statistical analysis. Thus, it was determined that these 14 different FFAs manifested significant differences between the NW group and the OB group. The detailed information of these differential FFAs is described in Tables 2 and S1.

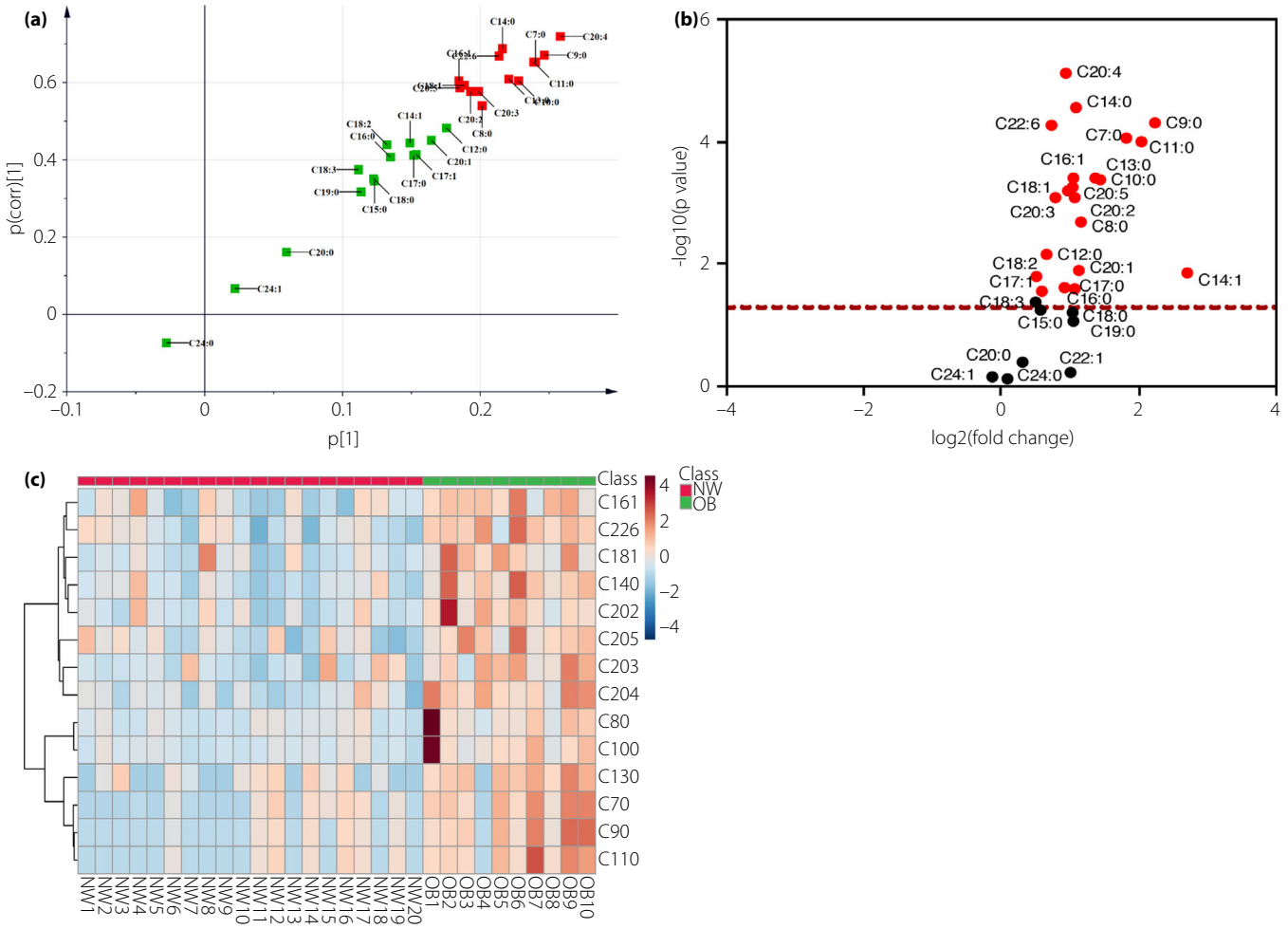


**Figure 2** | Screening free fatty acids (FFAs) with different concentrations between normal weight (NW) and obese (OB) groups. (a) S-plot of the NW and OB groups. FFAs are highlighted in red to show different regions in the S-plot. (b) Volcano plot showing a significant increase (red) in FFAs concentration for participants in the OB group. (c) Heat map visualization based on the content of FFAs in the NW (red) and OB (green) groups.

*Identification of differences in FFAs between the NW group and the type 2 diabetes mellitus group*

The S-Plot showed 11 different FFAs (C14:0, C16:0, C18:1, C20:1, C22:1, C18:2, C20:2, C20:3, C18:3, C20:5 and C22:6) were the FFAs that contributed most to the separation of the NW and the type 2 diabetes mellitus groups in the OPLS-DA score plot (Figure 3a). The volcano plot analysis showed that the concentrations of these 11 kinds of FFAs (with fold change >1.50) in type 2 diabetes mellitus group were higher than those in the NW group (Figure 3b). The heatmap also showed that these 11 FFAs could effectively discriminate between the two groups (Figure 3c).

The results of independent sample *t*-test analysis showed that nine of the 11 FFAs screened in the OPLS-DA model met the conditions of $P < 0.05$. Therefore, we determined that these nine different FFAs (C14:0, C18:1, C20:1, C18:2, C20:2, C20:3, C18:3, C20:5 and C22:6) showed significant differences between the NW and the type 2 diabetes mellitus groups. Tables 3 and S2 shows the results.

*FFAs with significant changes in both the OB and type 2 diabetes mellitus groups*

From the aforementioned results, compared with the NW group, the concentrations of 14 FFAs in the OB group were significantly increased, and the concentrations of nine FFAs in the type 2 diabetes mellitus group were also significantly increased. Interestingly, we found that the concentrations of six kinds of FFAs (C14:0, C18:1, C20:2, C20:3, C20:5 and C22:6) in both the OB group and the type 2 diabetes mellitus group were significantly higher than that in the NW group, and the concentrations of these six FFAs were highest in the type 2 diabetes mellitus group (Figure 4a,b).

**Table 2** | Significant differences in free fatty acids concentrations detected by ultra-high-pressure liquid chromatography–mass spectrometry and analyzed by orthogonal partial least squares discriminant analysis and independent samples *t*-test between the normal weight and obese groups

| Category | Free fatty acid | Mean ± SD | | VIP† | *P*-value‡ | FC§ |
|---|---|---|---|---|---|---|
| | | NW[20] | OB[10] | | | |
| SFAs | C8:0 | 0.47 ± 0.15 | 1.05 ± 0.75 | 1.10* | 0.037* | 2.23* |
| | C10:0 | 0.16 ± 0.06 | 0.44 ± 0.30 | 1.25* | 0.018* | 2.75* |
| | C14:0 | 3.06 ± 1.54 | 6.53 ± 2.24 | 1.19* | <0.001* | 2.13* |
| MUFAs | C16:1 | 7.65 ± 5.53 | 15.83 ± 4.60 | 1.01* | <0.001* | 2.07* |
| | C18:1 | 80.70 ± 52.09 | 165.94 ± 64.81 | 1.03* | 0.001* | 2.06* |
| ω-6 PUFAs | C20:2 | 2.24 ± 1.35 | 4.71 ± 2.26 | 1.06* | 0.001* | 2.10* |
| | C20:3 | 0.78 ± 0.38 | 1.35 ± 0.41 | 1.09* | 0.001* | 1.73* |
| | C20:4 | 2.18 ± 0.85 | 4.17 ± 1.11 | 1.41* | <0.001* | 1.91* |
| ω-3 PUFAs | C20:5 | 0.28 ± 0.18 | 0.55 ± 0.18 | 1.02* | 0.001* | 1.96* |
| | C22:6 | 1.77 ± 0.62 | 2.93 ± 0.66 | 1.17* | <0.001* | 1.66* |
| OCFAs | C7:0 | 0.17 ± 0.20 | 0.59 ± 0.30 | 1.32* | <0.001* | 3.47* |
| | C9:0 | 1.60 ± 2.28 | 7.52 ± 4.55 | 1.35* | 0.002* | 4.70* |
| | C11:0 | 0.06 ± 0.08 | 0.24 ± 0.14 | 1.31* | <0.001* | 4.00* |
| | C13:0 | 0.04 ± 0.04 | 0.10 ± 0.04 | 1.21* | <0.001* | 2.50* |

†The variable importance in the projection (VIP) was obtained in the orthogonal partial least squares discriminant analysis. *VIP >1. ‡The *P*-values were calculated from the independent sample *t*-test. **P* < 0.05. §The fold changes (FCs) were calculated from the intragroup means of the free fatty acids levels, with a positive value indicating a relatively higher concentration in the obese (OB) group and a negative value indicating a relatively lower concentration compared with the normal weight (NW) group. *The absolute fold change (FC) value is >1.5. MUFAs, monounsaturated fatty acids; OCFAs, odd-chain fatty acids; SFAs, saturated fatty acids; VIP, Variable importance in the projection; ω-3 PUFAs, ω-3 polyunsaturated fatty acids; ω-6 PUFAs, ω-6 polyunsaturated fatty acids.

## Potential FFAs biomarkers

### *Differential FFAs are related to many metabolic indicators*

These six differential FFAs showed a significant positive correlation with FPG levels; the correlation coefficients of C14:0, C18:1, C20:2 and C22:6 were all >0.5. In addition, these FFAs were correlated with other metabolic indicators. For example, C14:0, C18:1, C20:2 and C20:3 were positively correlated with BMI. C14:0, C18:1 and C20:5 were also significantly positively correlated with TG, C18:1 was positively correlated with LDL-C, and C22:6 was positively correlated with bodyweight. Table 4 shows the results.

### *Areas under the ROC curve for the differential FFAs*

Based on the results of correlation analysis, we used ROC curve analysis to examine whether these six FFAs have the potential to predict type 2 diabetes mellitus. Our results showed that four of the six FFAs could predict type 2 diabetes mellitus.

C14:0 and C18:1 produced an area under the ROC curve (AUC) of 0.727 (95% confidence interval [CI] 0.55–0.903) and 0.743 (95% CI 0.588–0.899), respectively (Figure 5a,b). The AUCs of C20:3 and C22:6 were 0.720 (95% CI 0.549–0.891) and 0.803 (95% CI 0.644–0.963; Figure 5d-e). The Youden Index of these four FFAs (C14:0, C18:1, C20:3 and C22:6) was higher than 0.4 (0.467, 0.500, 0.467 and 0.533, respectively). The results showed that all the four FFAs could predict type 2 diabetes mellitus. Surprisingly, C22:6 had the highest predictive ability among the four kinds of FFAs, and the corresponding cut-off value was 2.843 μmol/μL. The AUC, sensitivity, specificity, Youden Index and cut-off value are presented in Table S3.

### *Logistic regression analysis of differential FFAs*

We used binary logistic regression analysis to further determine the risk factors for type 2 diabetes mellitus. The present results showed that only C22:6 was a positive risk factor for type 2 diabetes mellitus (β = 1.311, 95% CI 1.328–10.371) after adjusting for confounding variables (age, height, weight, BMI and WC). Table 5 shows the results.

### *$\chi^2$-test analysis of the potential biomarker: C22:6*

Based on the aforementioned study results, participants with C22:6 concentrations above the cut-off value were preliminarily defined as type 2 diabetes mellitus patients, and all participants were analyzed using the $\chi^2$-test. Using C22:6 as the diagnostic criteria, our analysis showed that the serum C22:6 concentration was above the cut-off level (≥2.843 mol/L) in 12 of the 40 participants, including seven confirmed type 2 diabetes mellitus patients and five healthy participants (non-type 2 diabetes mellitus participants). Interestingly, the five non-type 2 diabetes mellitus participants were all in the OB group, and three of them had FPG levels >6.1 mmol/L (6.25, 6.4 and 6.64 mmol/L, respectively). Meanwhile, we found that C22:6 was highly sensitive (70%) and specific (83.30%) in predicting type 2 diabetes mellitus ($P$ = 0.001; Table 6). These results suggest that C22:6

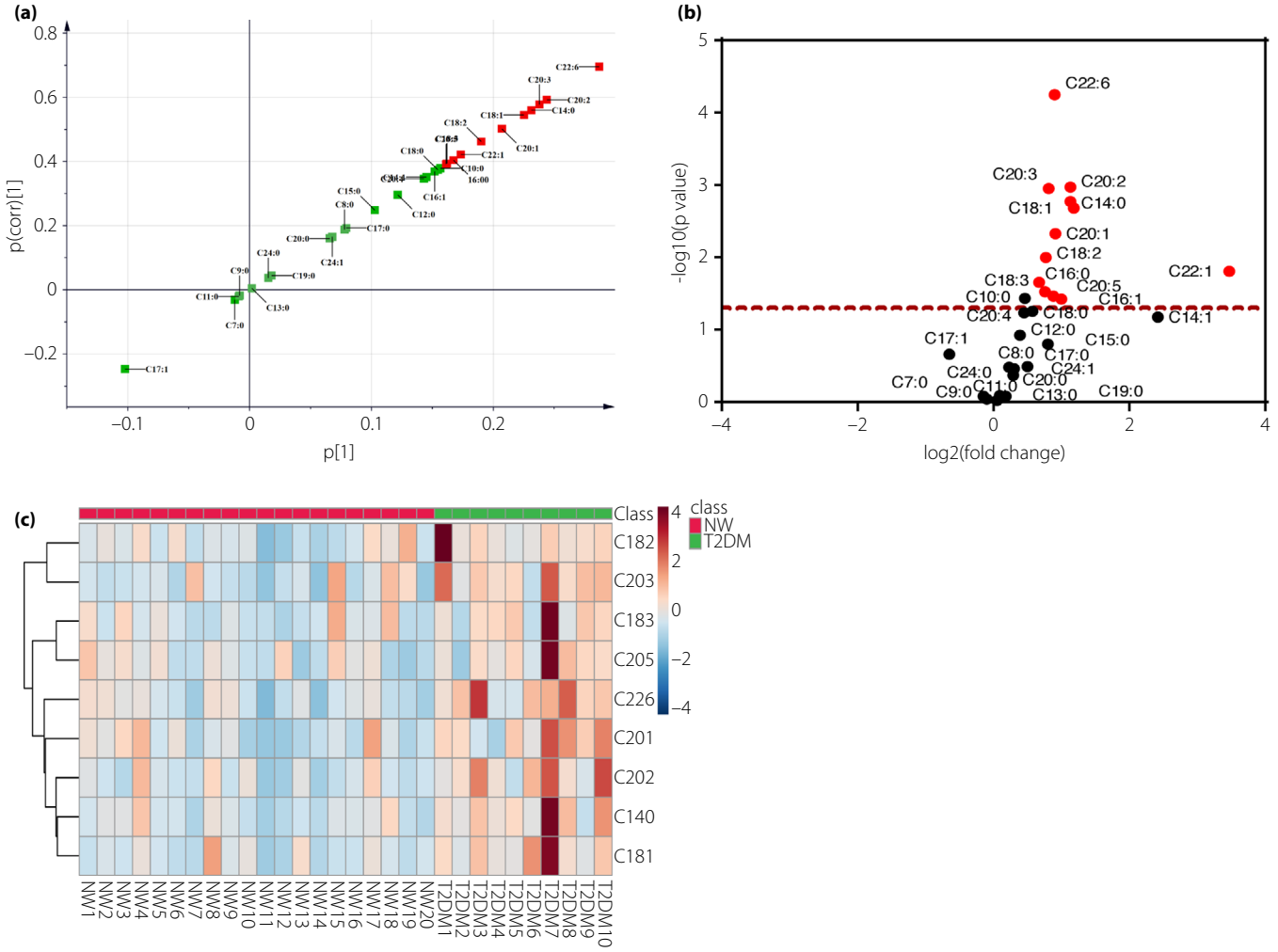


**Figure 3** | Screening free fatty acids (FFAs) with different concentrations between normal weight (NW) and type 2 diabetes mellitus (T2DM) groups. (a) S-plot of the NW and type 2 diabetes mellitus groups. FFAs are highlighted by red to show different regions in the S-plot. (b) Volcano plot showing a significant increase (red) in FFAs concentrations for participants in the type 2 diabetes mellitus group. (c) Heat map visualization based on the content of FFAs in the NW (red) and type 2 diabetes mellitus (green) groups.

can be a good biomarker for predicting type 2 diabetes mellitus and prediabetes. Table 6 shows the results.

## DISCUSSION

Several disorders are related to obesity. Obesity leads to excess fat storage and dysregulation of adipocyte signaling, which leads to disorders of lipid metabolism[5,21,22]. This disordered lipid metabolism in obese individuals is an important risk factor for type 2 diabetes mellitus[11,12], but the research on what the key substances are in obese individuals that lead to type 2 diabetes mellitus is still equivocal. Dyslipidemia has been observed to be prevalent in obesity, and this dyslipidemia is usually referred to as hyperlipidemia (hypertriglyceridemia, hypercholesterolemia, hyper-LDL cholesterolemia and hypo-HDL cholesterolemia)[6,23]. A number of studies showed that obesity is accompanied by mixed dyslipidemia, which increases the risk of type 2 diabetes mellitus[24,25]. Unlike the previous studies, the present study found that, except for the increased TG content in the OB group (although the TG content did not reach the standard of hypertriglyceridemia), there were no significant differences in other lipids levels compared with the NW group, and there were no differences in the serum lipids levels between the OB group and type 2 diabetes mellitus group. Similarly, there was no difference in serum lipid level between the OB group and type 2 diabetes mellitus group. This suggests that even if there is no hyperlipidemia in obese individuals, there might be more important lipid molecules associated with the occurrence of type 2 diabetes mellitus.

FFAs, as key lipid substances in the synthesis and decomposition of TG, increase in most obese individuals[11,26]. In

**Table 3** | Significant differences in free fatty acids content detected by ultra-high-pressure liquid chromatography–mass spectrometry and analyzed by orthogonal partial least squares discriminant analysis and independent samples *t*-test between the normal weight and type 2 diabetes mellitus groups

| Category | Free fatty acid | Mean ± SD | | VIP† | *P*-value‡ | FC§ |
|---|---|---|---|---|---|---|
| | | NW[20] | Type 2 diabetes mellitus[10] | | | |
| SFAs | C14:0 | 3.06 ± 1.54 | 6.66 ± 4.16 | 1.46* | 0.024* | 2.18* |
| MUFAs | C18:1 | 80.70 ± 52.09 | 181.23 ± 111.76 | 1.44* | 0.002* | 2.25* |
| | C20:1 | 0.96 ± 0.59 | 1.79 ± 0.88 | 1.06* | 0.005* | 1.86* |
| ω-6 PUFAs | C18:2 | 72.99 ± 33.13 | 123.64 ± 68.36 | 1.27* | 0.010* | 1.69* |
| | C20:2 | 2.24 ± 1.35 | 4.89 ± 2.66 | 1.50* | 0.012* | 2.18* |
| | C20:3 | 0.78 ± 0.38 | 1.37 ± 0.48 | 1.51* | 0.001* | 1.76* |
| ω-3 PUFAs | C18:3 | 1.30 ± 0.66 | 2.19 ± 1.47 | 1.08* | 0.030* | 1.68* |
| | C20:5 | 0.28 ± 0.18 | 0.52 ± 0.40 | 1.05* | 0.034* | 1.86* |
| | C22:6 | 1.77 ± 0.62 | 3.29 ± 1.15 | 1.85* | <0.001* | 1.86* |

†The variable importance in the projection (VIP) was obtained in the orthogonal partial least squares discriminant analysis. *VIP >1. ‡The *P*-values were calculated from the independent sample *t*-test. **P* < 0.05. §The fold changes (FCs) were calculated from the intra-group means of the free fatty acids levels, with a positive value indicating a relatively higher concentration in the obese (OB) group, and a negative value indicating a relatively lower concentration compared with the normal weight (NW) group. *The absolute FC value is >1.5. MUFAs, monounsaturated fatty acids; OCFAs, odd-chain fatty acids; SFAs, saturated fatty acids; VIP, Variable importance in the projection; ω-3 PUFAs, ω-3 polyunsaturated fatty acids; ω-6 PUFAs, ω-6 polyunsaturated fatty acids.

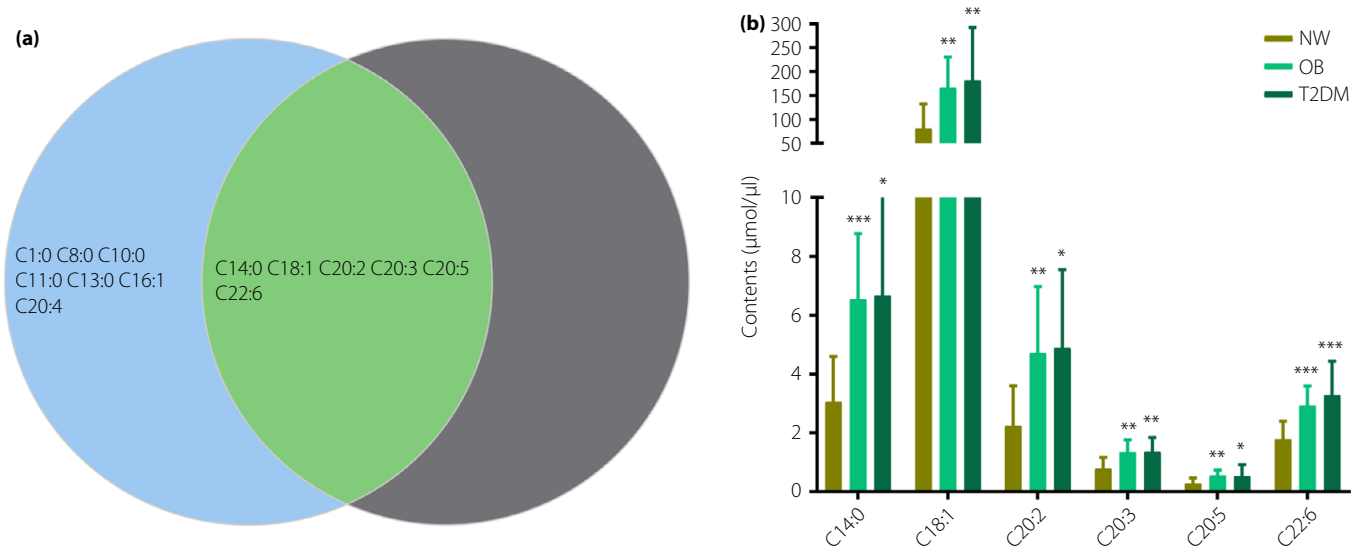


**Figure 4** | Screening free fatty acids (FFAs) concentrations with significant changes in both the obese (OB) and type 2 diabetes mellitus (T2DM) groups. (a) The traditional Venn diagram is used to observe the generality differences and overlap of FFAs between OB and type 2 diabetes mellitus group. (b) Concentrations of six FFAs in the three groups, **P* < 0.05, ***P* < 0.01, ****P* < 0.001; compared with the normal weight (NW) group.

addition, a great deal of studies have shown that the increased concentrations of FFAs plays an important role in IR, β-cell dysfunction and the pathogenesis of type 2 diabetes mellitus, and are considered to be an important risk factor of obesity leading to type 2 diabetes mellitus[13,27]. The increased FFAs concentrations in serum can reduce glucose consumption under the stimulation of insulin, and also damage the function of β-cells[28,29]. Furthermore, it has also been found that FFAs levels in the serum of type 2 diabetes mellitus patients with a new diagnosis and long-term drug control increased significantly[30]. Many studies have observed that in addition to the changes of total FFAs in serum, there are also significant differences in the concentrations of different types of FFAs in type 2 diabetes mellitus patients, and diverse kinds of FFAs play different roles

**Table 4** | Correlation analyses between free fatty acids and the metabolic indicators of all participants

| | C14:0 | C18:1 | C20:2 | C20:3 | C20:5 | C22:6 |
|---|---|---|---|---|---|---|
| Weight | 0.285 | 0.187 | 0.289 | 0.224 | 0.169 | 0.350* |
| BMI | 0.382* | 0.318* | 0.348* | 0.363* | 0.244 | 0.277 |
| WC | 0.286 | 0.213 | 0.199 | 0.275 | 0.24 | 0.245 |
| FPG | 0.558*** | 0.524** | 0.585*** | 0.482** | 0.374* | 0.622*** |
| TC | 0.191 | 0.261 | 0.046 | 0.189 | 0.270 | −0.108 |
| TG | 0.535*** | 0.419** | 0.286 | 0.305 | 0.467** | 0.084 |
| LDL-C | 0.286 | 0.371* | 0.225 | 0.269 | 0.278 | 0.170 |
| HDL-C | −0.122 | 0.109 | −0.056 | −0.153 | −0.085 | −0.190 |

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. BMI, body mass index; FPG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; WC, waist circumference.

in the pathogenesis of type 2 diabetes mellitus. Previous studies on different types of FFAs have found that most of the saturated fatty acids levels in type 2 diabetes mellitus patients are significantly higher (such as, C14:0, C16:0 and C18:0), and high levels of palmitic acid (C16:0) and stearic acid (C18:0) can aggravate obesity and liver steatosis, leading to mitochondrial dysfunction, inflammation and oxidative stress, thus increasing the risk of IR and type 2 diabetes mellitus[31,32]. Unsaturated fatty acids usually plays a role in improving IR and preventing type 2 diabetes mellitus, but different kinds of unsaturated fatty acids also show different effects. For example, in type 2 diabetes mellitus patients, the content of C14:1 usually decreases, whereas the content of C18:1 usually increases[20,33,34]. The aforementioned studies simply observed the changes in serum FFA profiles in patients with type 2 diabetes mellitus, and did not identify the key risk factors leading to the occurrence of type 2 diabetes mellitus from the source. This might also be one of the reasons for the lack of effective strategies for the treatment of type 2 diabetes mellitus. It is worth noting that obesity is the main risk factor for type 2 diabetes mellitus, so the increase of FFAs in obese individuals is likely to be the key substance leading to the occurrence of type 2 diabetes mellitus.

The results of the present study show that there were significant differences in serum FFA profiles between the OB group and the type 2 diabetes mellitus group compared with the NW group. Specifically, 14 kinds of FFAs in the OB group were significantly higher than that in the NW group, and nine FFAs in the type 2 diabetes mellitus group were significantly higher than those in the NW group. We further found that the concentrations of six FFAs were increased significantly in both the OB and the type 2 diabetes mellitus groups, and showed a trend of increasing among the three groups (NW, OB and type 2 diabetes mellitus groups), among which four FFAs (C14:0, C18:1, C20:3 and C22:6) had the ability to predict the occurrence of type 2 diabetes mellitus. It is suggested that these four FFAs are potential risk factors of obesity leading to type 2 diabetes mellitus. To further screen the key FFAs for type 2 diabetes mellitus associated with obesity, we used logistic regression analysis to identify the independent risk factors of type 2 diabetes mellitus. The present results show that among the four kinds of FFAs screened, only C22:6 can be used as an independent risk factor for type 2 diabetes mellitus, and it also has the highest potential to predict the susceptibility to type 2 diabetes mellitus (AUC 0.803). Therefore, our results suggest that C22:6 might be the key lipid substance for type 2 diabetes mellitus induced by obesity.

The present finding of C22:6 as a key risk factor for obesity-induced type 2 diabetes mellitus was found to be controversial in previous studies. ω-3 PUFAs usually enjoy a wide reputation as healthy FFAs[35]. As a common ω-3 PUFA, C22:6 also plays a beneficial role in type 2 diabetes mellitus. Some studies have observed that the level of C22:6 decreases in type 2 diabetes mellitus patients[20], and the review report by Arnoldussen *et al.*[36] also showed that C22:6 reduces excessive bodyweight, visceral fat content and the prevalence of type 2 diabetes mellitus in patients, which means that the concentration of C22:6 is negatively correlated with the risk of type 2 diabetes mellitus. More specific studies have found that C22:6 can play an anti-inflammatory role by activating the activity of peroxisome proliferator-activated receptor-γ and other receptors to reduce the risk of type 2 diabetes mellitus, and some studies reported that C22:6 can be used as a treatment strategy to improve the complications of type 2 diabetes mellitus[37,38]. Although a large number of studies reported the positive role of C22:6 in the pathogenesis of type 2 diabetes mellitus, some studies differed. A recent study showed that the concentrations of C22:6 were significantly higher in type 2 diabetes mellitus patients than in healthy individuals. In addition, Kaushik *et al.* showed that the intake of ω-3 PUFAs (C22:6) can moderately increase the blood glucose and decreased insulin sensitivity in type 2 diabetes mellitus patients, and another study found that the consumption of ω-3 PUFAs actually increased the risk of type 2 diabetes mellitus in men and women in the USA[20,34,39]. These studies also support the present findings.

Previous studies showed that various FFAs play different roles in the pathogenesis of type 2 diabetes mellitus. The present results show that C22:6 is closely related to type 2 diabetes mellitus induced by obesity, which is an independent risk factor

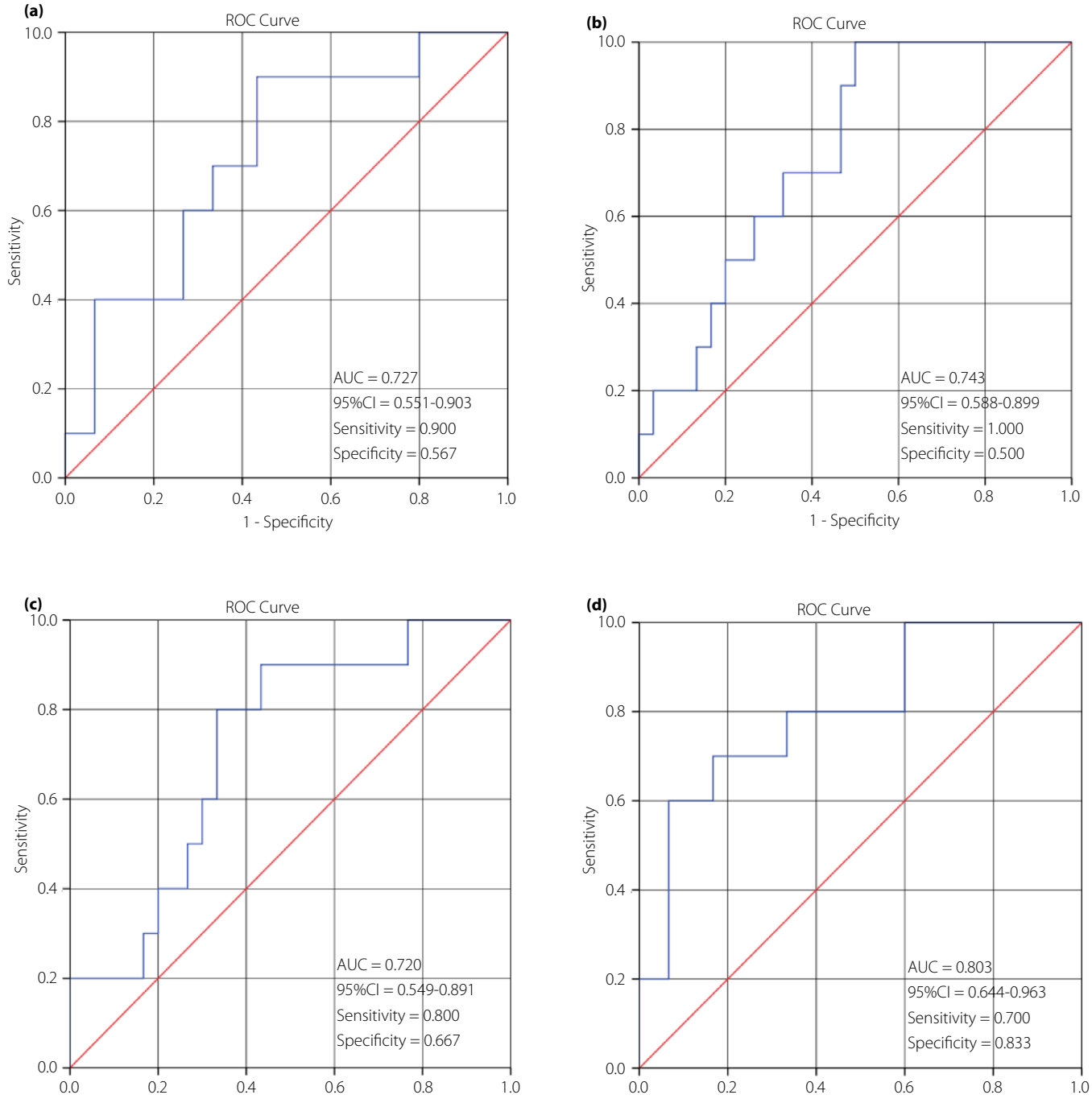


**Figure 5** | The diagnostic performance of targeted metabolomics in type 2 diabetes mellitus; area under the receiver operating characteristic curve (AUC) analysis. (a) the diagnostic accuracy of C14:0 to predict type 2 diabetes mellitus, measured as the AUC, was 0.727 (95% confidence interval [CI] 0.551–0.903). (b) The diagnostic accuracy of C18:1 to predict type 2 diabetes mellitus, measured as the AUC, was 0.743 (95% CI 0.588–0.899). (c) The diagnostic accuracy of C20:3 to predict type 2 diabetes mellitus, measured as the AC curve, was 0.720 (95% CI 0.549–0.891). (d) The diagnostic accuracy C22:6 to predict type 2 diabetes mellitus, measured as the AUC curve, was 0.803 (95% CI 0.644–0.963).

for type 2 diabetes mellitus and has a great potential to predict the susceptibility to type 2 diabetes mellitus. This suggests that even in the absence of hyperlipidemia, the increased C22:6 concentrations might be an important risk factor for type 2 diabetes mellitus caused by obesity. Therefore, C22:6 could be used as a biomarker for early screening of type 2 diabetes

**Table 5** | Binary logistic regression analysis

| Dependent variable | *B* | Wald | *P* | Exp(*B*) 95% CI | SE |
|---|---|---|---|---|---|
| Constant | –4.594 | 9.196 | 0.002 | 0.010 | 1.515 |
| C22:6 | 1.311 | 6.251 | 0.012 | 3.710 (1.328–10.371) | 0.524 |

The binary logistic regression analysis excluded the effects of age, height, weight, body mass index and waist circumference on the model. *B*, partial regression coefficient; CI, confidence interval; *P*, *P*-value; SE, standard error.

**Table 6** | $\chi^2$-test analysis of cases of C22:6 contents and type 2 diabetes mellitus

| | Type 2 diabetes mellitus (+) ($n$ = 10) | Type 2 diabetes mellitus (–) ($n$ = 30) | Total |
|---|---|---|---|
| C22:6 ≥2.843 μmol/L/μL | 7 | 5 | 12 |
| C22:6 <2.843 μmol/L/μL | 3 | 25 | 28 |
| Total | 10 | 30 | 40 |
| Sensitivity | 70% | | |
| Specificity | 83.30% | | |

*P*-value from $\chi^2$-test, $P$ = 0.001.

mellitus susceptibility and as a drug treatment target for type 2 diabetes mellitus in the future, depending on further confirmation of the present findings.

In conclusion, the serum FFA profiles of the OB and the type 2 diabetes mellitus groups were significantly different from that of the NW group, but the changes in the FFA profile of the OB and the type 2 diabetes mellitus individuals had some similarities, which might be an important risk factor for obesity associated with type 2 diabetes mellitus. In the present study, we identified that C22:6 has a great potential to predict the susceptibility to type 2 diabetes mellitus, and might be a key risk factor for type 2 diabetes mellitus associated with obesity. This could provide multiple theoretical bases for studying the specific mechanism of obesity-induced type 2 diabetes mellitus, as well as a new drug target for the prognosis and treatment of type 2 diabetes mellitus. In the follow-up research, we need to expand the sample size and verify the specific role of C22:6 in the process of obesity leading to type 2 diabetes mellitus *in vitro* and *in vivo* to improve its potential clinical application value.

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## DISCLOSURE

The authors declare no conflict of interest.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1** | Significant differences in free fatty acids concentrations detected by ultra-high-pressure liquid chromatography–mass spectrometry and analyzed by orthogonal partial least squares discriminant analysis and independent samples *t*-test between the normal weight and obese groups.

**Table S2** | Significant differences in free fatty acids concentrations detected by ultra-high-pressure liquid chromatography–mass spectrometry and analyzed by orthogonal partial least squares discriminant analysis and independent samples *t*-test between the normal weight and type 2 diabetes mellitus groups.

**Table S3** | Area under the curve, sensitivity, specificity and Youden Index of free fatty acids to predict type 2 diabetes mellitus.