BACKGROUND: Isolated postchallenge diabetes (IPD), a subtype of type 2 diabetes mellitus (T2DM) defined as 2-h postprandial plasma glucose ≥200 mg/dL (≥11.1 mmol/L) and fasting plasma glucose (FPG) <108 mg/dL (<6.0 mmol/L), is often overlooked during screening for diabetes on the basis of FPG concentrations. A key challenge is early identification of IPD by the use of fasting serum, which is critical for large-scale diabetes screening.

METHODS: We applied a nontargeted metabolomic approach using ultra-high-performance liquid chromatography–quadrupole TOF–mass spectrometry (UPLC-QTOF-MS) to analyze serum samples from 51 patients with IPD, 52 with newly diagnosed T2DM, and 49 healthy individuals. We processed metabolite profiles by multivariate analysis to identify potential metabolites, which were further confirmed by tandem MS (MS/MS). We also used GC-MS and ELISA methods to detect potentially important metabolites. A number of independent samples were selected to validate the identified candidates.

RESULTS: We selected 15 metabolites with a view to distinguishing patients with IPD, whereas 11 were identified with an authentic standard. The selected metabolites included linoleic acid, oleic acid, phospholipids, and dehydroepiandrosterone sulfate (DHEA-S). In IPD samples, significantly higher linoleic and oleic acid (P < 0.001) and lower DHEA-S (P < 0.001) concentrations were observed, compared with controls. The area under the curve from a combination of linoleic acid, oleic acid, and DHEA-S in the validation study was 0.849 for the IPD group.

CONCLUSIONS: The current study provides useful information to bridge the gaps in our understanding of the metabolic alterations associated with IPD and might facilitate the characterization of patients with IPD by the use of fasting serum.

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Fasting Serum Lipid and Dehydroepiandrosterone Sulfate as Important Metabolites for Detecting Isolated Postchallenge Diabetes: Serum Metabolomics via Ultra-High-Performance LC-MS

Liyan Liu,1 Maoqing Wang,1 Xue Yang,1 Mingxin Bi,1 Lixin Na,1 Yucun Niu,1 Ying Li,1* Changhao Sun1*

1 Department of Nutrition and Food Hygiene, Public Health College, Harbin Medical University, Harbin, P. R. China.
* Address correspondence to these authors at: Department of Nutrition and Food Hygiene, Public Health College, Harbin Medical University, 157 Baqian Road, Nangang District, Harbin, P. R. China, 150086. Fax: +86-(0)451-87502885; e-mail changhao2002sun@yahoo.com or liying_helen@163.com.

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of high-content methods of analysis, such as genomics, proteomics, and metabolomics. In the current investigation, we focused on using metabolomics for the detection of isolated postchallenge diabetes.

The field of metabolomics provides a dynamic portrait of metabolic status. Metabolic profiles are particularly effective indicators of the phenotype or tissue physiology of an organism (3) and provide information that is not accessible through other “omics” approaches. To date, metabolomics has been successfully applied to several fields, including disease diagnosis (4), biomarker screening (5, 6), characterization of biological pathways (7), and nutrition research (8). Recently, the ultra-high-performance LC-MS (UPLC-MS)–based metabolomics approach has been increasingly applied to several fields, including disease diagnosis (4), biomarker screening (5, 6), characterization of biological pathways (7), and nutrition research (8).

We used UPLC and quadrupole TOF (QTOF)/MS–based metabolomic platforms to profile metabolites in fasting serum from IPD and T2DM patients as well as healthy individuals. Initially, the repeatability of FFAs was validated. Subsequently, we attempted to identify the metabolites significantly associated with IPD that may be effectively used for distinguishing variants of T2DM (9–12). Until now, however, the majority of studies have focused on individuals with FPG >126 mg/dL (7.0 mmol/L) and limited attention has been paid to IPD patients. On the basis of the findings of earlier studies, we hypothesized that considerable metabolic variation exists in the fasting serum glucose concentrations of patients with IPD, supporting the possibility of identifying clinically significant metabolites to facilitate IPD diagnosis. In keeping with this theory, our group recently showed that free fatty acid (FFA) profiles in fasting serum of patients with diabetes are useful for IPD diagnosis in large populations (13). Notably, FFA profiles represent only a small proportion of the metabolite fingerprint and comprehensively characterized patients with IPD using a metabolomics approach.

Materials and Methods

STUDY SAMPLES

The study was approved by the Ethics Committee of Harbin Medical University and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant.

All subjects participated in a population survey to establish the prevalence and risk factors of diabetes in Harbin (PRFD-Harbin). Subjects were recruited in 2008 from communities in Harbin from the Heilongjiang Province in northern China. Fifty-two patients with newly diagnosed T2DM, 51 with IPD, and 49 healthy individuals were included in the study. T2DM was diagnosed according to the 1997 American Diabetes Association criteria (14), defined as FPG >126 mg/dL (7.0 mmol/L) and 2 h-PG >200 mg/dL (11.1 mmol/L). The cutoff values for FPG and 2 h-PG used to diagnose IPD were 108 mg/dL (6.0 mmol/L) and 200 mg/dL (11.1 mmol/L), respectively. The patient samples in the current study were different from those used previously (13). Enrolled patients were not on any medication before sample collection. A 10-mL blood sample was collected into a serum separator tube (red-top Vacutainer) in the morning before breakfast. These fasting blood samples were immediately centrifuged at 3000g for 10 min at room temperature and stored at −80 °C until analysis.

We further validated our findings in independent samples (400 individuals). Individuals in the latter sample were recruited in 2012 from the Hexing and Yixing districts in Harbin to establish the prevalence and risk factors of chronic noncommunicable diseases. Mean (SD) values for age and glycosylated hemoglobin (Hb A1c) in the different groups were as follows: IPD, n = 133; age = 50.5 (5.5) years, Hb A1c = 6.4% (0.5%); newly diagnosed T2DM, n = 137, age = 49.2 (5.4) years, Hb A1c = 7.9% (1.2%); and healthy controls, n = 130; age = 51.1 (5.4) years, Hb A1c = 5.4% (0.3%).

SAMPLE PREPARATION

A 200-μL aliquot of each serum sample was used for metabolite extraction before UPLC-QTOF-MS analysis. We carried out the extraction procedure after addition of 1.0 mL methanol (chromatographic grade, Honeywell Burdick & Jackson) to serum. The mixture was vortex-mixed for 1 min and incubated at room temperature for 10 min, followed by centrifugation at 14 000g for 10 min at 4 °C. The supernatant was evaporated to dryness under N2, and 200 μL of a mixture of acetonitrile (chromatographic grade, Honeywell Burdick & Jackson) and water (3:1) was added to each tube. The solution was filtered through a syringe filter (0.22 μm) and placed into a 2-mL glass vial pending UPLC-QTOF-MS analysis.

UPLC-QTOF-MS ANALYSIS

UPLC-MS analysis was performed with a Waters Acquity UPLC system coupled to a Waters Micromass Q-TOF micro™ mass spectrometer with electrospray ionization (ESI) in the positive and negative modes. A 2-μL aliquot of the sample solution was injected into an Acquity UPLC™ BEH C18 column (50 × 2.1 mm; i.d. 1.7 μm; Waters Corp.). The flow rate of the mobile phase was 350 μL/min. Analytes were eluted from the column under a gradient (solvent A, 0.1% formic acid in water, and solvent B, acetonitrile). The optimal con-
ditions for UPLC separation and ESI-TOF-MS detection are shown in online Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue9. All analyses were acquired with a lock spray to ensure accuracy and reproducibility. A lock mass of leucine enkephalin for positive ([M+H]⁺ = 556.2771) and negative ([M+H]⁻ = 554.2615) ion modes was applied via a lock spray interface. Lock spray frequency was set at 0.48 s, and lock mass data were averaged over 10 scans for correction.

We used QC samples to assess reproducibility and reliability of the UPLC-MS system. The QC samples were prepared by mixing equal volumes of different individual serum samples (10 healthy controls, 10 patients with IPD, and 10 with T2DM). One QC sample was injected at the start of the analytical batch, followed by analysis of 1 QC sample at every fifth sample injection throughout the analytical workflow. The reproducibility of the method was determined with principal component analysis (PCA), and reliability was assessed by extracting 6 ions from chromatographic peaks of the QC samples.

PCA performed on QC and other groups revealed that QC samples were clustered in the PCA scores plot (see online Supplemental Fig. 1). Six ions extracted from the chromatographic peaks (m/z 100.07, 330.33, 496.34, 524.37, 566.43, and 792.59 in the positive ion mode) were selected for method reliability. The relative standard deviations (CV) of peak intensity, retention times, and m/z were estimated as 3.13%–15.24%, 0%–0.051%, and 0%–0.005%, respectively. These results collectively indicated good repeatability, reliability, and stability of this method for metabolite analysis.

UPLC-QTOF-MS raw data were analyzed with MarkerLynx Application Manager 4.1 (Waters Corp.). The online Supplemental Methods contains a description of the data analysis. The matrix from UPLC-QTOF-MS was introduced into SIMCA-P 11.0 software (Umetrics) and standardized to a mean of 0 and variance of 1, according to the formula \(X_{i} = \frac{X_{i} - \text{mean}(X)}{\text{std}(X)}\), for multivariate statistical analysis. Initially, we used unsupervised PCA to demonstrate general separation in all samples. Next, we performed orthogonal partial least-squares discriminant analysis (OPLS-DA). The parameters of the modeling (PCA and OPLS-DA), R² and Q², were used to evaluate the model. R² was the fraction of the variation of the variables (X or Y) explained by the model, and Q² represented the fraction of the variation of the variables (X or Y) predicted by the model. Furthermore, R²cum and Q²cum represented the accumulated values of R² and Q² from several principal components in the model. When the values of R²cum and Q²cum were >0.5, the model was considered successful. To avoid model overfitting, supervised models were validated with a permutation test. A description of the multivariate statistical analysis can be found in the online Supplemental Methods.

IDENTIFICATION OF SIGNIFICANT METABOLITES

We selected the serum differential variables associated with IPD and T2DM on the basis of a threshold of variable importance in the projection (VIP) value (VIP >1.0) from the OPLS-DA model (15). The VIP values reflect the importance of terms in the model both with respect to Y (its correlation to all the responses), and with respect to X (the projection). VIP values are computed, by default, from all extracted components. One can compare the VIP of 1 term to the others. Terms with large VIP, >1, are the most relevant for explaining Y (15).

In parallel, we validated these differential metabolites from the OPLS-DA model at a univariate level with the Student t-test. The critical P value of the test was set to 0.05. Metabolites were identified on the basis of the accurate mass, retention time, and matching MS spectra of the unknown with standard model compounds. To support the identification process, we used Metlin (http://metlin.scripps.edu/) and a human metabolite database (http://www.hmdb.ca/). Finally, metabolites were confirmed by comparison of retention time and fragmentation pattern with authentic standards.

QUANTITATIVE ANALYSIS OF 3 METABOLITES

Three important metabolites identified in UPLC-QTOF-MS experiments were further quantified for confirmation purposes with the addition method. Quantification of linoleic acid and oleic acid was performed with GC-MS, as previously described (13). Dehydroepiandrosterone sulfate (DHEA-S) was measured with a commercial ELISA kit (IBL Corp.).

STATISTICAL ANALYSIS

Serum biochemical indicators were expressed as mean (SD). We analyzed the differences between the 2 groups with independent sample t-tests. The significance of linoleic acid, oleic acid, and DHEA-S was assessed with analysis of covariance (ANCOVA) by adjusting for potential covariates [body mass index (BMI), TG, total cholesterol (TC), 2-h PG, blood pressure, and insulin]. Data were considered statistically significant at \(P < 0.05\). We performed ROC curve analysis (16) on the basis of a logistic regression model to determine the area under the curve (AUC) as a measure for comparing the predictive ability of metabolites (linoleic acid, oleic acid, and DHEA-S) and known risk factors for diabetes (age, sex, weight, height, BMI, TG, TC, waist circumference, and blood pressure). We performed t-tests and ANCOVA with SPSS software (ver-
Results

BIOCHEMICAL CHARACTERISTICS

In comparison with the control group, significant differences in insulin and homeostasis model assessment for insulin sensitivity (HOMA-IS) and for insulin resistance (HOMA-IR) values were observed in the IPD and T2DM groups (Table 1). Notably, BMI, 2-h PG, TG, TC, and systolic and diastolic blood pressure were not found to be significantly different between the IPD and T2DM groups.

UPLC-QTOF-MS DATA ANALYSIS

The PCA scores plot from all samples in ESI+ revealed separation between T2DM patients and the 2 other groups, whereas IPD samples were dispersed among those of the control group (see online Supplemental Fig. 2A). In ESI−, we observed a separation between control and other groups (see online Supplemental Fig. 2B), whereby IPD samples were dispersed among those of T2DM. In general, there may be an overlap in classes owing to Q2 values (PCA) < 0.4–0.6, and the model may lead to multiple misclassifications. To manage this issue, 3 subcomparisons were performed by use of PCA to compare control and IPD patients, control and T2DM patients, and IPD and T2DM patients. The results obtained indicated clear separations in the 3 subcomparison groups (Fig. 1).

To further identify the significant serum metabolites that effectively distinguish IPD from the other groups, 3 subcomparisons were performed by use of OPLS-DA analyses, which revealed distinct separation between the 3 groups (see online Supplemental Fig. 3). On the basis of the permutation test, all R2cum and Q2cum values were lower than the original values in the validation plot (see online Supplemental Fig. 4). The subcomparison results confirmed the validity of the OPLS-DA model.

IDENTIFICATION OF POTENTIAL METABOLITES

We calculated VIP values in the OPLS-DA model. The metabolite ions with VIP values > 1.0 were initially selected as distinguishing factors, and the most significant variables contributing to class separation were selected (Table 2). We identified 11 metabolites using commercial standards. Four metabolites were identified by use of tandem MS (MS/MS) analysis or comparison of the extract mass. The concentrations of specific metabolites were significantly higher in IPD and T2DM patients, compared with the control group, including linoleic acid, oleic acid, docosanoic acid, cholesteryl-β-D-glucoside, and 1,2-distearoyl phosphatidyl serine, whereas other metabolites, such as lysophosphatidylcholine (lysoPC), lysophosphatidyl ethanolamine (lysoPE), DHEA-S, and 5-hydroxykynurenine,

---

**Table 1. Demographic and clinical chemistry characteristics of participants in the PRFD-Harbin study.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IPD</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>49</td>
<td>51</td>
<td>52</td>
</tr>
<tr>
<td>Age, years</td>
<td>52.3 (6.8)</td>
<td>53.7 (7.4)</td>
<td>52.2 (6.5)</td>
</tr>
<tr>
<td>Sex, F/M</td>
<td>35/24</td>
<td>31/20</td>
<td>31/21</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.0 (1.4)</td>
<td>26.9 (3.0)b</td>
<td>26.6 (3.3)b</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>68.3 (6.3)</td>
<td>86.2 (13.7)b</td>
<td>85.0 (10.5)b</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>112.8 (6.3)</td>
<td>142.1 (25.7)b</td>
<td>142.8 (24.0)b</td>
</tr>
<tr>
<td>FPG, mg/dL</td>
<td>77.4 (9.5)</td>
<td>91.7 (11.4)</td>
<td>194.8 (50.6)b</td>
</tr>
<tr>
<td>2-h PG, mg/dL</td>
<td>81.2 (16.4)</td>
<td>255.7 (81.4)b</td>
<td>338.6 (85.1)b</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>80 (27)</td>
<td>177 (97)b</td>
<td>213 (97)b</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>162.4 (27.1)</td>
<td>201.1 (50.3)b</td>
<td>209.9 (39.6)</td>
</tr>
<tr>
<td>Insulin, mU/L</td>
<td>6.04 (2.26)</td>
<td>6.72 (3.73)b</td>
<td>8.27 (4.47)b</td>
</tr>
<tr>
<td>Hb A1c, %</td>
<td>5.3 (0.4)</td>
<td>6.2 (0.5)b</td>
<td>7.6 (1.2)b</td>
</tr>
<tr>
<td>HOMA-IS</td>
<td>226.65 (136.17)</td>
<td>92.59 (47.62)b</td>
<td>19.09 (9.69)b</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.25 (0.56)</td>
<td>1.85 (0.77)b</td>
<td>4.01 (1.70)b</td>
</tr>
</tbody>
</table>

a Data are mean (SD). To convert glucose concentrations in mg/dL to mmol/L, multiply by 0.05551; to convert total cholesterol concentrations in mg/dL to mmol/L, multiply by 0.02586; to convert triglycerides concentrations in mg/dL to mmol/L, multiply by 0.01129.

b P < 0.05 compared with healthy controls.
were significantly lower in these groups. Bar plots based on the relative intensities of differential metabolites [lysoPC(16:0), lysoPC(18:0), linoleic acid, oleic acid, DHEA-S, and 5-hydroxykynurenine] from different metabolic pathways are depicted in Fig. 2.

**PREDICTION OF CRITICAL METABOLITES FOR IPD**

To establish the optimal serum metabolites that can be effectively used to discriminate IPD patients, we generated an OPLS-DA model using UPLC-QTOF-MS data as the X-matrix and relative intensity of several metabolites in the differential metabolic pathways as the Y-matrix. The \( t \)-predicted scores plot (see online Supplemental Fig. 5) indicates that linoleic acid, oleic acid, and DHEA-S are more significant than lysoPC, since more individuals in the control group are localized below the baseline, whereas the majority of patients are correctly assigned to the IPD group.

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**Fig. 1. PCA score plots between 3 subgroups.**

(A), Healthy control and IPD groups (ESI\(^{+}\), 5 components, \( R_{X_{cum}}^{2} = 0.578 \), \( Q_{cum}^{2} = 0.609 \)). (B), Healthy control and T2DM groups (ESI\(^{+}\), 5 components, \( R_{X_{cum}}^{2} = 0.561 \), \( Q_{cum}^{2} = 0.639 \)). (C), IPD and T2DM groups (ESI\(^{-}\), 5 components, \( R_{X_{cum}}^{2} = 0.501 \), \( Q_{cum}^{2} = 0.598 \)). (D), Healthy control and IPD groups (ESI\(^{-}\), 5 components, \( R_{X_{cum}}^{2} = 0.512 \), \( Q_{cum}^{2} = 0.607 \)). (E), Healthy control and T2DM groups (ESI\(^{-}\), 5 components, \( R_{X_{cum}}^{2} = 0.743 \), \( Q_{cum}^{2} = 0.612 \)). (F), Healthy control and IPD groups (ESI\(^{-}\), 5 components, \( R_{X_{cum}}^{2} = 0.853 \), \( Q_{cum}^{2} = 0.650 \)). ▲, Healthy control; ■ IPD; ▼ T2DM. \( t[1] \), \( t[component 1] \) (the scores of the first component); \( t[2] \), \( t[component 2] \) (the scores of the second component).
Table 2. Important metabolites in UPLC/MS positive and negative ion modes (ESI⁺ and ESI⁻).

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Ion mode</th>
<th>m/z</th>
<th>IPD vs control</th>
<th>T2DM vs control</th>
<th>IPD vs T2DM</th>
<th>Related metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>VIPᵃ</td>
<td>Pᵇ</td>
<td>Trendᶜ</td>
<td>VIP</td>
</tr>
<tr>
<td>LysoPC(16:0)</td>
<td>ESI⁺</td>
<td>496.3445</td>
<td>22.47 1.31 × 10⁻⁶ ↓</td>
<td>29.79 2.53 × 10⁻²³ ↓</td>
<td>5.69 1.75 × 10⁻¹⁵ ↓</td>
<td>Phospholipid metabolism</td>
</tr>
<tr>
<td>LysoPC(18:0)</td>
<td>ESI⁺</td>
<td>520.3426</td>
<td>19.64 3.12 × 10⁻⁶ ↓</td>
<td>27.56 4.16 × 10⁻²³ ↓</td>
<td>5.04 4.45 × 10⁻⁵ ↓</td>
<td>Phospholipid metabolism</td>
</tr>
<tr>
<td>LysoPC(18:0)</td>
<td>ESI⁺</td>
<td>524.3774</td>
<td>10.66 2.67 × 10⁻⁶ ↓</td>
<td>25.99 2.61 × 10⁻²³ ↓</td>
<td>4.86 3.06 × 10⁻⁵ ↓</td>
<td>Phospholipid metabolism</td>
</tr>
<tr>
<td>Docosanoic acid</td>
<td>ESI⁺</td>
<td>358.3689</td>
<td>8.27 4.12 × 10⁻¹⁵ ↑</td>
<td>6.43 4.36 × 10⁻³¹ ↑</td>
<td>2.17 1.67 × 10⁻¹¹ ↑</td>
<td>FFA biosynthesis</td>
</tr>
<tr>
<td>Cholesteryl-β-D-glucoside</td>
<td>ESI⁺</td>
<td>566.4370</td>
<td>4.56 0.0023 ↑</td>
<td>19.09 1.26 × 10⁻⁸ ↑</td>
<td>1.24 2.08 × 10⁻¹⁶ ↑</td>
<td>Cholesterol metabolism</td>
</tr>
<tr>
<td>Cholesteryl-β-D-glucoside</td>
<td>ESI⁺</td>
<td>341.2445</td>
<td>3.05 2.91 × 10⁻⁸ ↑</td>
<td>3.52 4.75 × 10⁻²³ ↑</td>
<td>1.19 1.37 × 10⁻⁴ ↑</td>
<td>Cholesterol metabolism</td>
</tr>
<tr>
<td>1,2-Distearoyl phosphatidyl</td>
<td>ESI⁺</td>
<td>792.5965</td>
<td>2.53 2.23 × 10⁻⁴ ↑</td>
<td>3.67 4.38 × 10⁻¹⁸ ↑</td>
<td>2.08 3.61 × 10⁻⁵ ↑</td>
<td>Glycine, serine, and threonine</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>ESI⁻</td>
<td>279.2358</td>
<td>44.41 2.65 × 10⁻⁸ ↑</td>
<td>41.95 1.93 × 10⁻⁸ ↑</td>
<td>6.75 0.047 ↑</td>
<td>FFA metabolism</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>ESI⁻</td>
<td>281.2491</td>
<td>9.02 3.10 × 10⁻⁵ ↑</td>
<td>12.55 1.71 × 10⁻⁷ ↑</td>
<td>5.14 0.0017 ↑</td>
<td>FFA metabolism</td>
</tr>
<tr>
<td>DHEA-S</td>
<td>ESI⁻</td>
<td>367.1608</td>
<td>14.44 0.042 ↓</td>
<td>20.05 0.0078 ↓</td>
<td>6.17 0.014 ↓</td>
<td>Steroid hormone biosynthesis</td>
</tr>
<tr>
<td>LysoPE(20:1/0:0)</td>
<td>ESI⁻</td>
<td>506.3279</td>
<td>1.08 5.17 × 10⁻⁴ ↓</td>
<td>2.34 9.63 × 10⁻¹⁸ ↓</td>
<td>1.45 1.18 × 10⁻⁶ ↓</td>
<td>Phospholipid metabolism</td>
</tr>
<tr>
<td>LysoPE(20:2/0:0)</td>
<td>ESI⁻</td>
<td>504.3194</td>
<td>1.22 0.0025 ↓</td>
<td>2.29 8.27 × 10⁻¹¹ ↓</td>
<td>2.34 2.17 × 10⁻⁵ ↓</td>
<td>Phospholipid metabolism</td>
</tr>
<tr>
<td>LysoPE(20:0/0:0)</td>
<td>ESI⁻</td>
<td>508.3342</td>
<td>1.24 7.55 × 10⁻⁵ ↓</td>
<td>1.88 5.14 × 10⁻¹⁴ ↓</td>
<td>1.58 3.37 × 10⁻¹² ↓</td>
<td>Phospholipid metabolism</td>
</tr>
<tr>
<td>LysoPC(18:0)</td>
<td>ESI⁻</td>
<td>568.3619</td>
<td>2.07 0.0083 ↓</td>
<td>2.89 1.81 × 10⁻⁷ ↓</td>
<td>2.34 4.59 × 10⁻⁴ ↓</td>
<td>Phospholipid metabolism</td>
</tr>
<tr>
<td>5-Hydroxykynurenine</td>
<td>ESI⁻</td>
<td>223.0672</td>
<td>1.32 0.0088 ↓</td>
<td>1.73 5.43 × 10⁻⁴ ↓</td>
<td>1.24 0.0038 ↓</td>
<td>Tryptophan catabolism</td>
</tr>
</tbody>
</table>

ᵃ Variable importance in the projection (VIP) was obtained from OPLS-DA with a threshold of 1.0.
ᵇ P values calculated from Student t-test.
ᶜ Fold change calculated from the arithmetic mean values of each group. Trend ↑, higher than control levels; ↓, lower than control levels.
ᵈ Identified based on retention time and MSⁿ spectrum of an authentic standard.
ᵉ Identified by the accurate mass and observed MSⁿ fragments acquired from UFLC-QTOF/MS analysis and the online database.
QUALIFICATION OF THE 3 METABOLITES AND ROC ANALYSIS

We measured the absolute concentrations of linoleic acid, oleic acid, and DHEA-S in the 3 groups (Figs. 3A–C). Compared with the control group, DHEA-S concentrations were significantly lower in IPD ($P < 0.001$) and T2DM groups ($P < 0.001$), whereas linoleic and oleic acid concentrations were significantly higher in the 2 patient groups ($P < 0.001$). Moreover, we observed significantly higher amounts of DHEA-S ($P = 0.024$) and lower concentrations of linoleic acid ($P = 0.047$) and oleic acid ($P < 0.01$) in the IPD group, compared with the T2DM group.

AUCs for linoleic acid, oleic acid, and DHEA-S are shown in online Supplemental Table 2. AUCs (95% CI) for the 3 parameters were 0.987, 0.926, and 0.772 between control and IPD patients, and 0.990, 0.924, and 0.876 between control and T2DM patients, respectively. AUCs of the combined diabetes risk factors (DRFs) were 0.881 between control and IPD patients and 0.886 between control and T2DM patients (see online Supplemental Table 2). The diagnostic sensitivity and specificity of prediction from the ROC curve was >72%.

VALIDATION OF THE METABOLITES FOR IPD

We quantitatively measured the 3 metabolites in independent samples from IPD and non-IPD subjects (including controls and T2DM patients), as shown in Fig. 3, D–F. Trends similar to those obtained with the metabolite study were observed for all 3 compounds.

In ROC analysis (Fig. 4), AUC values for the 3 metabolites were 0.821, 0.780, and 0.739 for IPD and non-IPD subjects, and that of the combined DRFs was 0.746. To optimize the diagnostic ability of the 3 metabolites, we performed ROC analysis of the 3 com-

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Fig. 2. Bar plots of the relative intensity of typical differential metabolites obtained from serum samples in the PRFD-Harbin study.

(A), LysoPC(16:0). (B), LysoPC(18:0). (C), Linoleic acid. (D), Oleic acid. (E), DHEA-S. (F), 5-Hydroxykynurenine. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $P$ values were calculated by comparing IPD or T2DM samples with controls.
combined metabolites. AUC for discrimination between IPD and non-IPD subjects was 0.849 (Fig. 4). Comparison of ROC curve areas disclosed statistically significant differences between the combined metabolites and DRFs ($P < 0.0382$), and no differences among linoleic acid, oleic acid, DHEA-S, and DRFs. Furthermore, we observed significant differences between combined metabolites and DHEA-S ($P < 0.0349$), but not among linoleic acid, oleic acid, and combined metabolites ($P > 0.05$). These results indicate that a combination of the three metabolites can be effectively used for distinguishing IPD in patients.

**Discussion**

Our results suggest distinct ESI$^+$ and ESI$^-$ modes between the IPD and T2DM groups (Fig. 1). There are different pathogenic mechanisms underlying IPD and T2DM (FPG $\geq 126$ mg/dL [$\geq 7.0$ mmol/L] and 2-h PG $\geq 200$ mg/dL [$\geq 11.1$ mmol/L]) (17). IPD is characterized by increased peripheral insulin resistance and reduced first-phase insulin secretion, and T2DM by progressive increase in peripheral and hepatic insulin resistance and reduced basal and first-phase insulin secretion (17), signifying that T2DM is a more serious...
metabolic disturbance than IPD. We detected higher insulin concentrations in T2DM than IPD cases (Table 1), which may be the basis for more serious disorders of lipid, steroid hormone, and glucose metabolism in these patients. Conversely, metabolic disorders (e.g., imbalances in FFAs and DHEA-S) may lead to increased islet damage that contributes to IPD and T2DM development. Our experiments revealed significantly higher DHEA-S and lower linoleic and oleic acid concentrations in the IPD group, compared with T2DM. The high DHEA-S concentration should aid in improving insulin resistance, and lower linoleic acid and oleic acid concentrations in reducing islet damage. These results collectively demonstrate metabolic differences between IPD and T2DM, among which 3 metabolites (DHEA-S, linoleic acid, and oleic acid) are of significant benefit in distinguishing between IPD and T2DM.

Recently, metabolic profiling technology has been used to analyze significant metabolic variations in the sera of patients with prediabetes and diabetes. Wang-Sattler et al. (18) reported marked alterations in the concentrations of lysoPC (18:2) and acetyl carnitine in patients with prediabetes. Furthermore, Floegel et al. (19) reported that glucose metabolites, amino acids, and choline-containing phospholipids are associated with higher risk of T2DM in the early stages. Consistent with this finding, Wang et al. (20) reported that branched-chain amino acids and aromatic amino acids could be effectively used to predict T2DM in a prospective cohort study. In our experiments, 15 metabolites involved in different biochemical metabolic pathways, such as phospholipids, FFAs, and steroid hormones, were distinguishable between the healthy control and 2 patient groups. Earlier studies have demonstrated that diabetes is intimately associated with metabolic disorders of lipids, such as phospholipids (21, 22). Phospholipids are important constituents of biomembranes that participate in various biological pathways (23, 24) and are therefore a particular focus of attention in metabolomic studies. Our results suggest that 7 metabolites belong to the phospholipid group, mainly lysoPC and lysoPE.

Notably, lysoPC(16:0), lysoPC(18:2), and lysoPC(18:0) were found to be important phospholipid metabolites, owing to their high VIP values. LysoPC, an endogenous phospholipid, contributes to pathophysiological changes in humans (25). Various species of lysoPC, with different physical and biological properties, have been identified on the basis of FFA chain length and degree of saturation (26). Our research showed that 3 lysoPC species are downregulated in the IPD and T2DM groups, possibly owing to low activity of phospholipase A2, which catalyzes PC hydrolysis to lysoPC. Alternatively, these changes are related to deficiency of lecithin-cholesterol acyltransferase (27). Consistent with our findings, recent studies have shown that decreased concentrations of lysoPC(18:2) are associated with increased risk of T2DM (12, 19). Furthermore, lysoPC(18:2) was significantly altered in patients with impaired glucose tolerance (IGT) and was identified as an IGT-specific marker (18). IPD is a more serious form of diabetes than IGT in terms of onset and development. Therefore, low concentrations of lysoPC (18:2) may predict the risk of IPD. However, imbalance of lysoPC appears to be involved in several other metabolic disorders, and 3 lysoPC metabolites have been identified as markers of disorders such as chronic renal failure and Alzheimer disease (28, 29). Hence, despite our finding that 3 lysoPC metabolites are critical for identifying IPD patients, the specificity of these metabolites may limit application in the clinical diagnosis of IPD.

Another important lipid change in IPD and T2DM patients is the dramatic increase in FFAs, which leads to accumulation of FFAs in blood. Although an acute increase of blood FFAs is necessary for insulin secretion, chronic exposure to high concentrations leads to apoptosis of pancreatic islet β-cells and aggravation of insulin resistance (30, 31), as well as presenting a major risk factor for cardiovascular disease and sudden death in patients with insulin resistance (32). In our study, the concentrations of 3 different FFAs (linoleic, oleic, and docosanoic acids) were increased in the IPD and
T2DM groups. By use of GC-MS and UPLC-MS, linoleic and oleic acids were identified as the crucial metabolites for distinguishing IPD. In a previous study by our group (13), linoleic and oleic acids have been identified as important metabolites of IPD via detection of FFA profiles in patients by use of GC-MS. The utility of linoleic acid and oleic acids was validated, as both analytical platforms revealed the same pattern of alteration (upregulation) in different studies. Validation by use of different instruments and populations facilitates greater confidence of measurements for metabolite biomarker identification. Our ROC results confirmed that linoleic and oleic acids are effective in predicting IPD. Linoleic acid is an essential FFA in the body affected by diet. In agreement with our results, Li et al. (33) reported that linoleic acid is an important metabolite in diabetes. However, those earlier studies did not consider the influence of diet. Therefore, it is not clear whether changes in linoleic acid are the result or cause of IPD, and the utility and specificity of linoleic acid in the diagnosis of IPD require further confirmation. Interestingly, a combination of metabolites (Fig. 4) appears more beneficial in improving specificity and ability of IPD diagnosis.

One highlight of our study is the discovery of the previously unidentified metabolite, DHEA-S, present at low concentrations in IPD and T2DM patients. DHEA-S, which is converted to the active form, dehydroepiandrosterone (DHEA) in a linear manner, represents the circulating hormonal pool of DHEA and presents a marker for DHEA availability. DHEA and DHEA-S, the most abundant circulating adrenal steroids in humans, have been shown to increase glucose uptake rates and affect glucose transporters in cell experiments (34). Epidemiologic studies have shown that low serum DHEA-S concentrations contribute to insulin resistance (35), whereas DHEA supplementation appears to improve insulin sensitivity and retard T2DM progression (36). Moreover, high insulin concentrations in IPD and T2DM patients may suppress the concentration of DHEA-S by inhibiting production and promoting clearance (37). In the current report, serum DHEA-S was identified as an important metabolite in IPD patients for the first time. The ρ-predicted scores plot indicated that IPD can be predicted with DHEA-S. To facilitate the clinical application of DHEA-S, its absolute concentration in fasting serum was detected by use of ELISA. The results indicate lower concentrations of DHEA-S in IPD and T2DM patients than healthy controls, consistent with metabolomic data. Moreover, ROC analyses in 2 separate studies confirmed the utility of DHEA-S in fasting serum for predicting IPD.

Repeated quantitative analyses of the 3 metabolites were additionally performed. Interestingly, although the separate metabolites could be used to diagnose IPD, specificity of diagnosis was not high. It must be noted that these metabolites are present in other diseases and do not demonstrate a comprehensive response to metabolic alterations in the body. However, a combination of the metabolites facilitated more accurate assessment of IPD, with a high AUC value (Fig. 4). Therefore, a combination of the linoleic acid, oleic acid, and DHEA-S metabolites in fasting serum might be helpful for application to the clinical identification of patients with IPD.

In summary, data obtained with our metabolomics approach reveal significant metabolic differences between IPD patients and healthy controls. From a panel, several significant metabolites in IPD were identified, including linoleic and oleic acids and DHEA-S, indicative of lipid and steroid hormone metabolic disorders in IPD patients. In particular, linoleic acid, oleic acid, and DHEA-S were associated with greater confidence in the specificity and accuracy of IPD identification in patients. On the basis of the collective findings, we propose that measurement of these metabolites in fasting serum effectively aids in the clinical identification of patients with IPD.
References


