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Plasma metabolic fingerprinting of childhood obesity by GC/MS in conjunction with multivariate statistical analysis

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ABSTRACT

Metabolic fingerprinting is a powerful tool for exploring systemic metabolic perturbations and potential biomarkers, thus may shed light on the pathophysiological mechanism of diseases. In this work, a new strategy of metabolic fingerprinting was proposed to exploit the disturbances of metabolic patterns and biomarker candidates of childhood obesity. Plasma samples from children with normal weight, overweight and obesity were first profiled by GC/MS. ULDA (uncorrelated linear discriminant analysis) then revealed that the metabolic patterns of the three groups were different. Furthermore, several metabolites, say isoleucine, glyceric acid, serine, 2,3,4-trihydroxybutyric acid and phenylalanine were screened as potential biomarkers of childhood obesity by both ULDA and CCA (canonical correlation analysis). CCA also shows satisfactory correlation between the metabolic patterns and clinical parameters, and the results further suggest that WHR (waist–hip ratio) together with TG (total triglycerides), TC (total cholesterol), HDL (high density lipoprotein) and LDL (low density lipoprotein) were the most important parameters which are associated closely with the metabolic perturbations of childhood obesity, so as to be paid more attention for dealing with metabolic disturbances of childhood obesity in clinical practice rather than regularly monitored BMI (body-mass index). The results have demonstrated that the proposed metabolic fingerprinting approach may be a useful tool for discovering metabolic abnormalities and possible biomarkers for childhood obesity.

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1. Introduction

The prevalence of childhood obesity has risen greatly all over the world [1]. Considering the consequences of wide range of related serious complications such as type 2 diabetes mellitus and cardiovascular diseases, childhood obesity has become a public-health crisis during the past two decades [2]. So screening, prevention and early treatment of childhood obesity are very important [3]. For these purposes, it may be of great importance for elucidating the pathophysiological process of childhood obesity. Metabolic pattern discrimination and biomarker screening for childhood obesity may be effective for this issue. Since metabolic fingerprinting has proved to be a powerful tool for exploring systemic metabolic changes and biomarker candidates of diseases [4], it may shed light on the pathophysiological process of childhood obesity. BMI (body-mass index) is routinely measured for dealing with childhood obesity [5], and sometimes blood glucose, TG (total triglycerides), TCH (total cholesterol), HDL (high density lipoprotein) and LDL (low density lipoprotein) are further measured for monitoring related complications [6]. Metabolomic studies of obesity have been reported before [7–9], however, to the best of our knowledge, metabolomics, especially metabolic fingerprinting of childhood obesity has not yet been conducted.

For metabolic fingerprinting research, information-rich analytical technique is required. Due to high efficiency of chromatographic separation and subsequent sensitive detection of separated components, GC/MS has been widely applied to metabolic fingerprinting research in conjunction with multivariate statistical analysis [10]. Multivariate statistical analyses such as PCA (principal component analysis) [11], PLS-DA (partial least squares-discriminant analysis) [12] and OPLS-DA (orthogonal partial least squares-discriminant analysis) [13] were widely employed in metabolic fingerprinting research for biomarker discovery.

In this study, a new strategy of metabolic fingerprinting by GC/MS combined with multivariate statistical analysis was developed to explore the disturbances of metabolic patterns and possible

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biomarkers of childhood obesity. Plasma samples from normal weight, overweight and obesity children groups were first profiled by GC/MS. ULDA (uncorrelated linear discriminant analysis), which finds new variables with optimal discriminatory ability and small redundancy [14], as well as CCA (canonical correlation analysis) were employed to explore perturbations of metabolic patterns and potential biomarkers of childhood obesity. Furthermore, CCA was performed to explore the relationships between metabolic patterns and clinical parameters, so as to try to find some useful information for dealing with metabolic abnormalities of childhood obesity.

2. Materials and methods

2.1. Chemicals and reagents

Heptadecanoic acid (98%) and BSTFA (*N*,*O*-bis(trimethylsilyl)-trifluoroacetamide) with 1% TMCS (trimethylsilyl chloride) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Chloroform, n-hexane and acetonitrile were of analytical grade and purchased from the Hanbang Chemical Corporation (Zhenjiang, China).

2.2. Sample collection

Childhood obesity was staged according to the standards proposed by the Group of China Obesity Task Force [15]. Children with a BMI from 85th to 95th percentile for age and sex were diagnosed as overweight, while the ones with a BMI above 95th percentile were diagnosed as obesity. The puberty age is 12–16 years old for boys and 10–14 years old for girls in China. In this study, all the children were checked to be with no spermatorrhea for boys and no menses for girls, so as to confirm that all of them are clearly pre-puberty. Blood plasma samples were collected from 65 children in Xiangya Hospital of Central South University in Changsha, China. They were all from 6 to 12 years old, and 18 of them were diagnosed as normal weight, 13 as overweight and 34 as obesity. Aliquots of plasma were stored at −80 °C until required for GC/MS analysis. All clinical experiments were approved by Xiangya Institutional Human Subjects Committee.

2.3. Sample preparation

Plasma samples were prepared according to our former work [16,17]. The plasma sample (100 μL) was first added with 20 μL heptadecanoic acid solution (1 mg mL^{-1} in chloroform) as internal standard and acetonitrile (500 μL) to precipitate the proteins. Then the mixture was vigorously vortexed for 1 min and centrifuged for 15 min at 16 000 rpm (17 800 × *g*) at 4 °C, and the supernatant (500 μL) was evaporated to dryness in a vacuum chamber. After adding 50 μL n-hexane as solvent, the sample was derivatized using 50 μL *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylsilyl chloride (TMCS) at 70 °C for 30 min.

2.4. GC/MS analysis

All analyses were performed on a Shimadzu GCMS-QP2010 gas chromatography quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). 1 μL of the derivatized sample was injected at a split ratio of 1:10 into a DB-1 column (30 m × 250 μm × 0.25 μm) with helium as the carrier gas at a flow of 1.0 mL min^{-1}. The temperature of the ion source and injector were set to 200 and 280 °C, respectively. The column temperature was initially maintained at 100 °C for 3 min, and increased at a rate of 8 °C min^{-1} to 300 °C and held for 2 min. Electron impact energy was operated at 70 eV. The data acquisition was performed in the full scan mode from *m/z* 35 to 800 with a scan time of 0.2 s. The detector voltage was set at 0.9 kV, and solvent cut time and data acquisition start time were 3.5 and 4.0 min, respectively.

2.5. Data preprocessing

All the collected data were used for the analysis. The automated mass spectral deconvolution and identification system (AMDIS, National Institute of Standards and Technology, Gaithersburg, MD) was first employed to support peak finding and deconvolution. Tentative identification of structures of the interesting peaks was supported by similarity search in NIST/EPA/NIH Mass Spectra Library (NIST 05) containing 190 825 EI spectra for 163 198 compounds using NIST Mass Spectral Search Program Version 2.0d and the characteristic ions according to the literatures [18,19]. Among the detected peaks of all the 65 chromatograms, 30 peaks were considered as endogenous metabolites excluded glucose and the peak areas were then extracted by our custom scripts to generate a data matrix. The rows and columns of the matrix represent the observations (samples) and variables (normalized peak areas), respectively. As for normalization in this paper, the variables of corresponding metabolites were expressed as the ratio of its peak area to that of the internal standard on the same chromatogram. To check the clinical significance of our measurements, 100 times of the values of the variables were employed for univariate *t*-test. The resulting data matrix was imported into our custom scripts in MATLAB 7.0 (The MathWorks, Inc., USA) for subsequent multivariate statistical analyses.

2.6. Multivariate statistical analyses

For metabolic fingerprinting, PCA was usually employed for visualizing the trends of samples. Furthermore, the supervised method PLS-DA was regularly utilized to explore differences between two groups of samples. However, these two methods sometimes may not obtain satisfactory results for certain dataset. Thus, in this study, a new method, say ULDA, was introduced to explore the metabolic disturbances and potential biomarkers of childhood obesity. This method finds the optimal linear combination of original variables under the so-called S-orthogonal constraint. The original data matrix **X** in high-dimension space was projected into the low-dimension space as **Z** by transformation matrix **G**, that is **Z** = **XG**.

The new variables, say UDVs (uncorrelated discriminant vectors), are mutually orthogonal with each other [20,21].

CCA [22] was performed to exploit the relationships between metabolic patterns and clinical parameters, furthermore, to assist clinical practice for handling with metabolic abnormalities for childhood obesity. Consider **X** and **Y** as the matrices of metabolic patterns and clinical parameters, the corresponding rows and the columns of **X** and **Y** represent observations and variables, respectively. The two matrices were first preprocessed by autoscaling, which can make the standard deviation to be unit and the mean to be zero.

$$\mathbf{C} = \begin{bmatrix} \mathbf{C}_{\mathbf{xx}} & \mathbf{C}_{\mathbf{xy}} \\ \mathbf{C}_{\mathbf{yx}} & \mathbf{C}_{\mathbf{yy}} \end{bmatrix} = \mathbf{E}\left[\begin{pmatrix} \mathbf{x} \\ \mathbf{y} \end{pmatrix}\begin{pmatrix} \mathbf{x} \\ \mathbf{y} \end{pmatrix}^{\mathrm{T}}\right]$$

is a block matrix where $\mathbf{C}_{\mathbf{xx}}$ and $\mathbf{C}_{\mathbf{yy}}$ are the within-sets covariance matrices of **X** and **Y**, respectively. $\boldsymbol{C}_{\boldsymbol{xy}} = \boldsymbol{C}_{\boldsymbol{yx}}^{\mathrm{T}}$ is the between-sets covariance matrix. The canonical correlations between **X** and **Y** can be found by solving the eigenvalue equations

$$\begin{cases} \boldsymbol{C}_{\boldsymbol{xx}}^{-1}\boldsymbol{C}_{\boldsymbol{xy}}\boldsymbol{C}_{\boldsymbol{yy}}^{-1}\boldsymbol{C}_{\boldsymbol{yx}}\hat{\boldsymbol{a}}_{\boldsymbol{x}} = \rho^2\hat{\boldsymbol{a}}_{\boldsymbol{x}} \\ \boldsymbol{C}_{\boldsymbol{yy}}^{-1}\boldsymbol{C}_{\boldsymbol{yx}}\boldsymbol{C}_{\boldsymbol{xx}}^{-1}\boldsymbol{C}_{\boldsymbol{xy}}\hat{\boldsymbol{b}}_{\boldsymbol{y}} = \rho^2\hat{\boldsymbol{b}}_{\boldsymbol{y}} \end{cases}$$

Table 1
Demographic and blood biochemical parameters of children with normal weight, overweight and obesity.

Parameter	Normal weight	Overweight	Obesity
Number (n)	16	13	32
Age (years)	8.7 ± 1.74	10.0 ± 1.15	8.9 ± 2.14
Height (cm)	132.5 ± 11.32	144.2 ± 12.64	140.7 ± 12.40
Weight (kg)	29.6 ± 7.15	47.6 ± 12.31[a]	49.8 ± 13.68
BMI (kg m^{-2})	16.6 ± 1.46	22.4 ± 2.65[a]	24.6 ± 3.44[b]
SBP (mmHg)	91.5 ± 10.05	105.3 ± 12.13[a]	106.6 ± 11.07
DBP (mmHg)	59.6 ± 5.06	67.2 ± 8.16[a]	69.1 ± 7.56
Waist (cm)	56.8 ± 5.98	76.8 ± 10.19[a]	78.8 ± 10.41
Hip (cm)	72.3 ± 6.63	85.2 ± 8.36[a]	87.8 ± 9.65
Waist–hip ratio	0.8 ± 0.05	0.9 ± 0.05[a]	0.9 ± 0.05
FBS (mmol L^{-1})	4.8 ± 0.21	4.8 ± 0.44	5.0 ± 0.35
TG (mmol L^{-1})	0.6 ± 0.21	1.2 ± 0.57[a]	1.1 ± 0.72
TC (mmol L^{-1})	3.7 ± 0.58	3.7 ± 0.66	3.9 ± 0.66
HDL (mmol L^{-1})	1.6 ± 0.35	1.3 ± 0.32[a]	1.3 ± 0.29
LDL (mmol L^{-1})	1.8 ± 0.47	1.9 ± 0.43	2.1 ± 0.51
HDL/CHO	0.4 ± 0.08	0.3 ± 0.09[a]	0.3 ± 0.08

[a] Significant coefficient at 0.05 significance level of children with normal weight compared with overweight.
[b] Significant coefficient at 0.05 significance level of children with overweight compared with obesity.

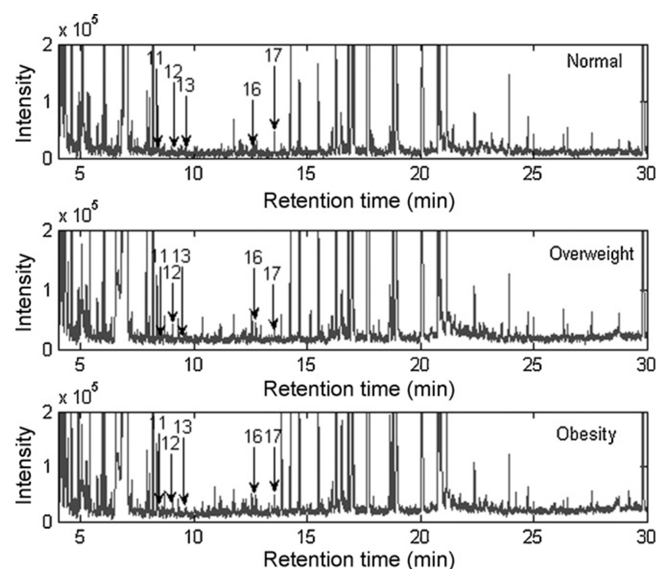

Fig. 1. Typical GC/MS total ion chromatograms (TICs) of trimethylsilylated plasma samples from children with normal weight, overweight and obesity. Labeled peaks are corresponding metabolites which were considered as potential biomarkers of childhood obesity, and the numbers coincide with the ones in Table 2.

where the eigenvalues ρ^2 are the squared canonical correlations and the eigenvectors $\hat{\boldsymbol{a}}_{\boldsymbol{x}}$ and $\hat{\boldsymbol{b}}_{\boldsymbol{y}}$ are the normalized canonical correlation basis vectors. The number of non-zero solutions to these equations are limited to the smallest dimensionality of **X** and **Y**. The so-called canonical variables **U** and **V** can be represented as **U** = **Xa** and **V** = **Yb**.

Before multivariate statistical analysis, data pretreatment was regularly used. In this study, centering was employed for PCA and ULDA, while autoscaling was utilized to preprocess the data before PLS-DA. Centering converts all the concentrations to fluctuations around zero instead of around the mean of the metabolite concentrations. Autoscaling standardizes each variable to have zero mean and unit variance [23].

3. Results and discussion

3.1. Comparison of demographic and blood chemical parameters

A child could be regarded as overweight with a BMI in the 85th–95th percentile, or as obesity with a BMI above 95th percentile for age and sex [12]. Table 1 shows the demographic and blood chemical parameters of children with normal weight, overweight and obesity. As shown in the table, several parameters such as weight, BMI, SBP (systolic blood pressure), DBP (diastolic blood pressure), waist, hip, WHR (waist–hip ratio), TG, HDL/CHO (high density lipoprotein/total cholesterol) were significantly higher in overweight group than in normal group, in contrast, HDL level was significantly lower in overweight group ($P < 0.05$). However, only the level of BMI was significantly increased in obesity group compared to overweight group ($P < 0.05$). Although these parameters may reflect the metabolic state of childhood obesity to some extent, there are various other underlying metabolic disturbances associated with childhood obesity [24]. Moreover, these metabolic perturbations may relate to serious metabolic outcomes such as type 2 diabetes mellitus [20] and cardiovascular diseases [25], and various metabolic abnormalities may exist during the progress of childhood obesity. Thus, metabolic fingerprinting may provide a useful tool for elucidating the disturbances of metabolic patterns and potential biomarkers, furthermore, shed light on the pathophysiological progress of childhood obesity.

3.2. GC/MS metabolic fingerprinting

In this study, GC/MS was employed to profile plasma samples of children with normal weight, overweight and obesity. Fig. 1 shows the typical total ion chromatograms (TICs) of plasma samples from children with normal weight, overweight and obesity. After deconvolution, various kinds of metabolites, including carbohydrates, organic acids, amino acids, fatty acids, amine, phosphate and lipids were found in the three groups. Excluded carbohydrates, 30 compounds were considered as endogenous metabolites according to the metabolomics database (www.hmdb.ca), and the keys and measurements of these compounds were listed in Table 2.

Lactate plays important roles in some biochemical processes and could be produced in the muscles during intense activity. Moreover, lactate measurement in the critically ill has been traditionally used to stratify patients with poor outcome [26]. 2-Ketoisocaproic acid is a deamination product of leucine [27]. α-Hydroxybutyric acid is an organic acid that is involved in propanoate metabolism, and it is produced in hepatic that catabolize L-threonine or synthesize glutathione [28]. β-Hydroxybutyric acid is a ketone body and the levels in blood and urine are raised in ketosis like the other ketone bodies such as acetoacetate and acetone [29]. Urea is the principal end product of protein catabolism and formed in a cyclic pathway known as the urea cycle [30]. Phosphate is an essential component of life especially ATP [31]. Glycerol is an important component of triglycerides and phospholipids, and it can also be converted to glucose in the liver and provides energy [32]. Glycerophosphoric acid is a chemical intermediate in the glycolysis metabolic pathway and can be produced from glycerol, which is the triose sugar backbone of triglycerides and glycerophospholipids [33]. Glyceric acid can be obtained from oxidation of glycerol, several phosphate derivatives of glyceric acid, including 2-phosphoglyceric acid, 3-phosphoglyceric acid, 2,3-bisphosphoglyceric acid and 1, 3-bisphosphoglyceric acid are important biochemical intermediates of lipid metabolism [34]. 2,3,4-Trihydroxybutyric acid is probably derived from glycated proteins or from degradation of ascorbic acid [35]. Alanine is one of the most important amino acids released by muscle, functioning as a major energy source [36]. Leucine and isoleucine are branched chain amino acids, which are critical to human life and are particularly involved in stress, energy

Table 2
Metabolites tentatively identified in plasma samples from healthy control, overweight and obesity groups.

ID	t_R[a] (min)	Metabolites	m/z[b]	MW[c]	Normal[f]	Overweight[g]	Obesity[h]
1	4.33	Lactate	73, 117, 147	234	130 ± 79.2	134 ± 52.6	153 ± 69.1
2	4.91	2-Ketoisocaproic acid	73, 57, 145	202	3.06 ± 1.30	2.63 ± 0.949	3.15 ± 0.911
3	5.03	Alanine	73, 116	233	0.935 ± 0.706	0.834 ± 0.596	1.11 ± 0.715
4	5.42	α-Hydroxybutyric acid	73, 131, 147	248	3.80 ± 1.89	4.00 ± 1.92	4.48 ± 1.96
5	5.96	β-Hydroxybutyric acid	73, 117, 147	248	5.08 ± 4.63	3.16 ± 2.47	3.90 ± 3.87
6	6.55[d], 7.04[e]	Urea	73[d,e], 147[d,e], 261[d], 189[e]	276[d],204[e]	346 ± 119	316 ± 73.8	312 ± 99.5
7	7.92	Phosphate	73, 299	314	4.33 ± 1.53	4.32 ± 0.858	4.40 ± 1.47
8	8.03	Leucine	73, 158	275	1.87 ± 1.13	1.38 ± 0.689	1.78 ± 1.03
9	8.20	Glycerol	73, 103, 147, 205	308	14.5 ± 7.48	12.8 ± 6.27	14.9 ± 7.74
10	8.38	Proline	73, 142	259	2.79 ± 1.83	2.94 ± 1.24	3.16 ± 1.31
11	8.43	Isoleucine	73, 158	275	0.607 ± 0.409	0.496 ± 0.266	0.663 ± 0.457
12	9.07	Glyceric acid	73, 147, 189, 292	322	0.461 ± 0.288	0.780 ± 0.473*	0.551 ± 0.314
13	9.61	Serine	73, 204, 218	321	0.332 ± 0.152	0.234 ± 0.133	0.293 ± 0.155
14	10.11	Threonine	73, 117, 219, 291	335	0.343 ± 0.279	0.309 ± 0.260	0.341 ± 0.224
15	11.77	Pyroglutamic acid	73, 147, 156, 258	273	1.22 ± 0.856	1.34 ± 1.01	1.51 ± 0.751
16	12.74	2,3,4-Trihydroxybutyric acid	73, 147, 292	424	0.279 ± 0.137	0.308 ± 0.194	0.299 ± 0.174
17	13.56	Phenylalanine	73, 192, 218	309	0.932 ± 0.673	0.637 ± 0.455	0.789 ± 0.507
18	13.87	Lauric acid	73, 117, 257	272	0.606 ± 0.393	0.580 ± 0.468	1.28 ± 3.37
19	14.27	Dodecyl acrylate	55, 83, 127	282	5.09 ± 2.75	3.74 ± 1.84	4.41 ± 2.15
20	15.68	Glycerophosphoric acid	73, 299, 357	460	0.971 ± 1.26	1.70 ± 1.49	1.28 ± 1.23
21	16.53	Myristic acid	73, 117, 285	300	1.08 ± 0.559	0.958 ± 0.411	1.12 ± 1.23
22	17.84	Tyrosine	73, 218, 280	397	0.460 ± 0.312	0.487 ± 0.335	0.519 ± 0.351
23	18.96	Palmitic acid	73, 117, 313	328	35.0 ± 9.04	37.9 ± 8.93	38.1 ± 16.0
24	20.76	Linoleic acid	73, 262, 337	352	18.9 ± 5.11	20.1 ± 7.69	20.4 ± 6.44
25	20.85	Oleic acid	73, 264, 339	354	26.7 ± 12.5	27.4 ± 13.6	29.3 ± 14.6
26	21.17	Stearic acid	73, 117, 341	356	11.3 ± 2.84	11.8 ± 2.51	12.0 ± 4.58
27	22.39	Arachidonic acid	73, 117, 175	376	1.95 ± 0.751	1.82 ± 0.647	1.98 ± 0.963
28	24.74	Monopalmitin	73, 147, 239, 371	474	1.45 ± 0.887	1.80 ± 1.02	1.91 ± 1.08
29	26.49	Monostearin	73, 147, 267, 399	502	1.11 ± 0.654	1.33 ± 0.829	1.36 ± 0.936
30	29.85	Cholesterol	73, 129, 329, 368, 458	458	26.0 ± 8.28	25.7 ± 9.93	28.7 ± 14.7

* Significant changes of levels of the corresponding metabolites from overweight children group compared to normal weight children group by the t-test ($P < 0.05$).

[a] Retention time.

[b] Masses shown are those of the ions selected for tentatively identification of individual derivatised metabolites.

[c] Molecular weight of the derivatised metabolites.

[d] The peak was tentatively identified as tri-TMS derivative of urea.

[e] The peak was tentatively identified as bis-TMS derivative of urea.

[f] One hundred times of the ratio of peak area to the internal standard on the same chromatogram from normal weight children group, the data were represented as mean ± SD.

[g] One hundred times of the ratio of peak area to the internal standard on the same chromatogram from overweight children group, the data were represented as mean ± SD.

[h] One hundred times of the ratio of peak area to the internal standard on the same chromatogram from obesity children group, the data were represented as mean ± SD.

and muscle metabolism [37]. Proline is synthesized from glutamic acid. It is an essential component of collagen and is important for proper functioning of joints and tendons [38]. Serine is important in metabolism because it participates in the biosynthesis of purines and pyrimidines [39]. Threonine could be converted to pyruvate via threonine dehydrogenase, and an intermediate in this pathway can undergo thiolysis with CoA to produce acetyl-CoA and glycine [40]. Pyroglutamic acid is a cyclized derivative of glutamic acid, and it is an uncommon amino acid derivative in which the free amino group of glutamic acid cyclizes to form a lactam [41]. Phenylalanine is an essential amino acid and the precursor for the amino acid tyrosine and catecholamines in the body [42,43]. Tyrosine is an essential amino acid that readily passes the blood–brain barrier. Once in the brain, it is a precursor for the neurotransmitters dopamine, norepinephrine and epinephrine, better known as adrenalin [44]. Dodecyl acrylate is usually used as separation media, and it may need further investigation [45]. Several free fatty acids, including lauric acid, myristic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, arachidonic acid are all important intermediates of lipid metabolism [46]. The two monoglycerides, say monopalmitin and monostearin, could be formed biochemically via release of a fatty acid from diacylglycerol [47]. Cholesterol is a lipidic and waxy steroid found in the cell membranes and transported in the blood plasma [48].

As visualized in Fig. 1, the peak (12) of glyceric acid was significantly higher in overweight than in normal weight children, which coincides with the measurement results in Table 2. Obviously, peak (17) of phenylalanine was significantly lower in overweight than in normal weight children as shown in Fig. 1, but it is not significant by the univariate *t*-test as shown in Table 2. However, the results were not controversial. The standard deviations were large although the mean values of phenylalanine from the two groups were significantly different. Since the metabolic profiles of the three groups were similar, multivariate statistical analyses were subsequently employed to explore the disturbances of metabolic patterns and biomarker candidates of childhood obesity.

3.3. *Metabolic disturbances of childhood obesity*

As shown in Section 2.5, the metabolic pattern of each sample was represented by a 30-dimensional vector, in which each element corresponding to the metabolite as shown in Table 2. First of all, PCA was employed to examine the clustering of samples for the differences of metabolic patterns of the three groups. In the score plot, four samples were found to be far away from the clustering and the distributions of the remaining samples were almost the same before and after deletion of these four samples (data were not shown). So these four samples were considered as outliers and removed in all of the subsequent analyses. Fig. 2 shows the score plot (PC1 versus PC2) of the three groups without outliers by PCA, as shown in the figure, although the first two PCs can explain up to 71% of the total variance of the data, the samples from these three groups scattered into each other. It indicates that the unsupervised method of PCA cannot separate the three groups satisfactorily. So the supervised method PLS-DA was performed to examine whether the patterns of these three groups were different. The samples from normal and obesity groups were first utilized to generate a PLS-DA model, and then the samples from the overweight group were projected into the model. A 10-fold cross validated PLS-DA model was first generated, however, the minimum error rate for prediction is 40% and Q2 for prediction is −0.78. The results showed that PLS-DA may be not suitable for exploring the differences of normal, overweight and obesity children at least in this study.

Thus ULDA was subsequently employed to explore the disturbances of metabolic patterns from normal to overweight and then obesity. In fact, the procedure of ULDA is a process of reducing

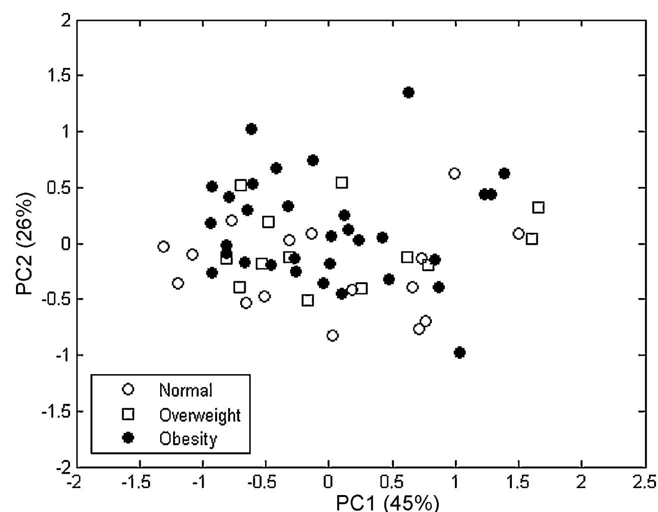

Fig. 2. PCA score plot (PC1 versus PC2) of plasma samples from normal (○), overweight (□) and obesity (●) groups.

dimensionality of the original data. Plot of the UDV1 (first uncorrelated discriminant vector) versus the UDV2 (second uncorrelated discriminant vector) was shown in Fig. 3. As illustrated in the figure, the samples from the obesity group lie in the top, while the samples from normal and overweight group locate in the left bottom and right bottom, respectively. The results indicate that the proposed metabolic patterns of plasma samples from normal to overweight and then to obesity group were different. However, the samples from overweight group were not in the middle of the samples from normal and obesity groups. This result suggests that the metabolic pattern of overweight may not simply be the middle status between normal and obesity as BMI.

For further understanding the metabolic status of the three groups, metabolic patterns of samples from overweight group were projected to the plan determined by ULDA of samples from normal and obesity groups as shown in Fig. 4. From the figure we can see that normal and obesity groups were clearly separated in the direction of UDV1, however, the samples from overweight group were not just in the middle. The samples of 7, 8 and 13 of the overweight group scattered to the left part of the obesity samples. For further

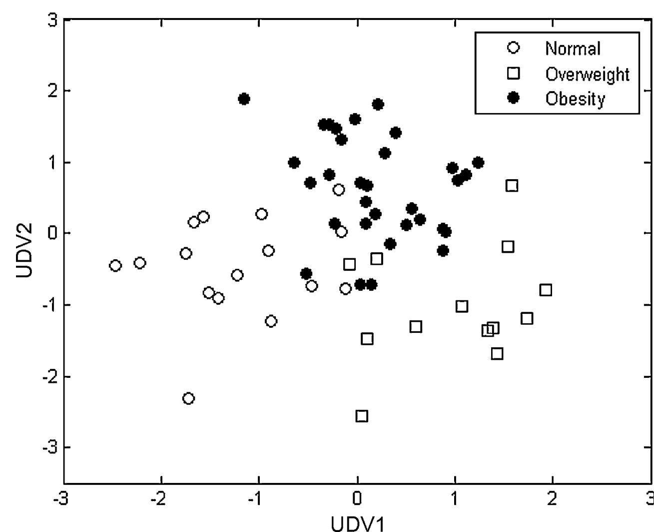

Fig. 3. Score plot of ULDA by the first uncorrelated discriminant vector (UDV1) versus the second uncorrelated discriminant vector (UDV2) of samples from normal (○), overweight (□) and obesity (●) groups.

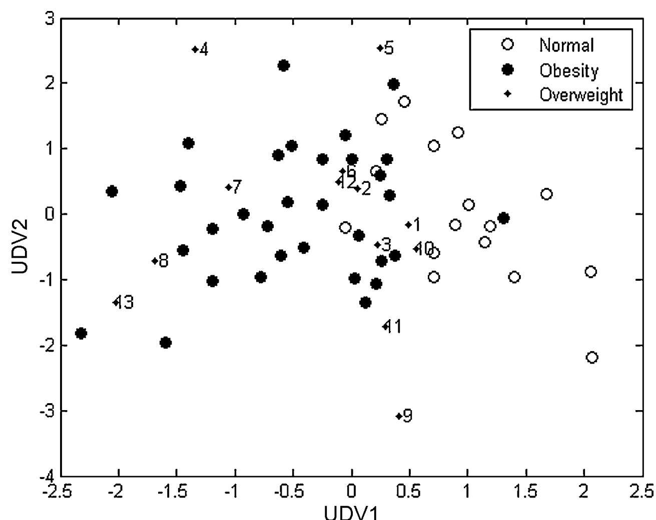

Fig. 4. Projection of the metabolic patterns of samples from overweight group (·) to the plan determined by ULDA of samples from normal (○) and obesity (●) groups.

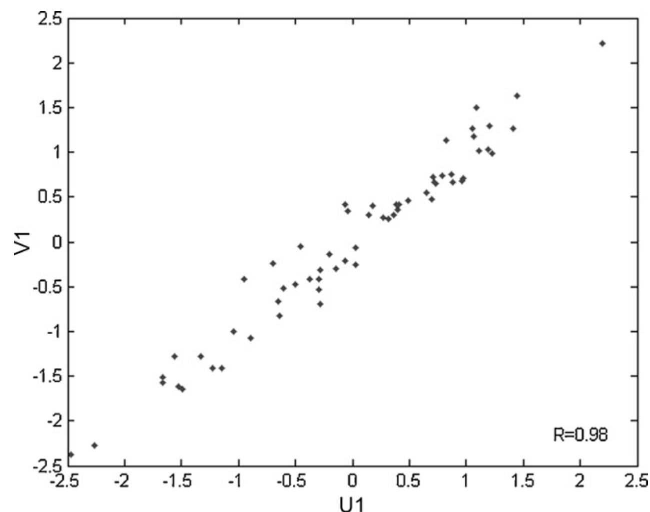

Fig. 6. Plot of the first canonical variables for both the metabolites (U1) and clinical parameters (V1).

examination of these samples, clinical parameters of samples from overweight group were projected to the plane determined by ULDA of samples from normal and obesity groups as shown in Fig. 5. In the figure, the samples of 7, 8 and 13 were also located in the right, which is the direction of obesity, although not in the very right. The results of clinical parameters in Fig. 5 coincide with the results of metabolic patterns in Fig. 4.

3.4. Canonical correlation analysis of metabolic patterns and clinical parameters

In clinical practice, not only BMI was regularly measured. However, childhood obesity was diagnosed only by BMI, and the metabolic disturbances of the overweight group were not just the middle status as the BMI as discussed above. So the correlation of metabolic patterns and clinical parameters may be important. For this purpose, CCA was conducted. Fig. 6 shows the plot of the first canonical variables of both the metabolites (U1) and clinical parameters (V1). Since only the first canonical correlation coefficient was significant ($P = 0.008$) by Bartlett's approximate chi-squared statistic, it was used in the subsequent analysis. As shown in the figure, the linearity was satisfactory with the canonical correlation coefficient as 0.98. The results indicate that the proposed metabolic patterns were closely correlated with the clinical parameters.

Since the metabolic patterns can represent the underlying metabolic disturbances of childhood obesity, and the metabolic patterns were closely correlated with the clinical parameters, we intended to exploit the important clinical parameters for the correlation, so as to be considered for monitoring metabolic disturbances of childhood obesity in clinical practice. For this purpose, the coefficients of the first canonical variable for the clinical parameters may be employed for representing the importance of the parameters. The plot of absolute values of each variable in the first canonical variable for clinical parameters was shown in Fig. 7, as shown in the figure, the absolute value of WHR together with TG, TC, HDL and LDL were significantly higher than the BMI. It indicates that these parameters may be more correlated with metabolic disturbances of childhood obesity, and more attention should be paid to these parameters in clinical practice.

As for metabolic patterns, the absolute values of each variable in the first canonical variable for metabolic profiles may represent the importance of the metabolites which closely associated with the clinical parameters. Thus, these metabolites may be considered

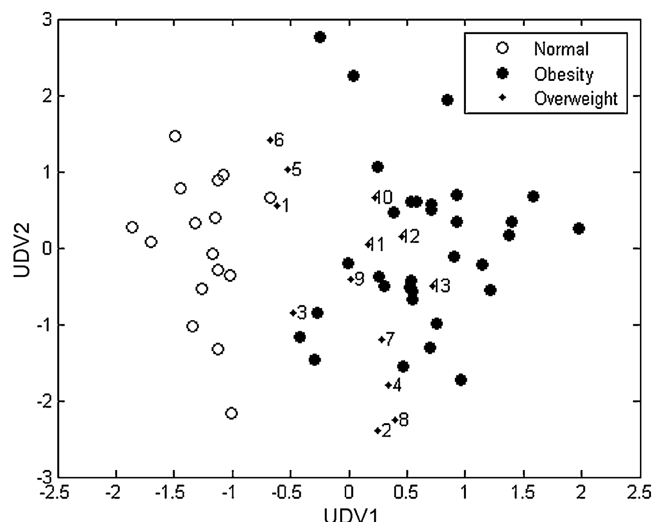

Fig. 5. Projection of the clinical parameters of samples from overweight group (·) to the plan determined by ULDA of samples from normal (○) and obesity (●) groups.

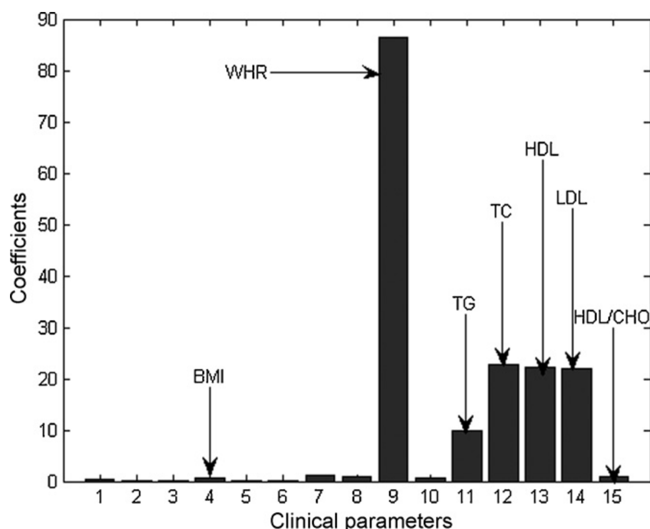

Fig. 7. Plot of the absolute coefficients of canonical variables for clinical parameters.

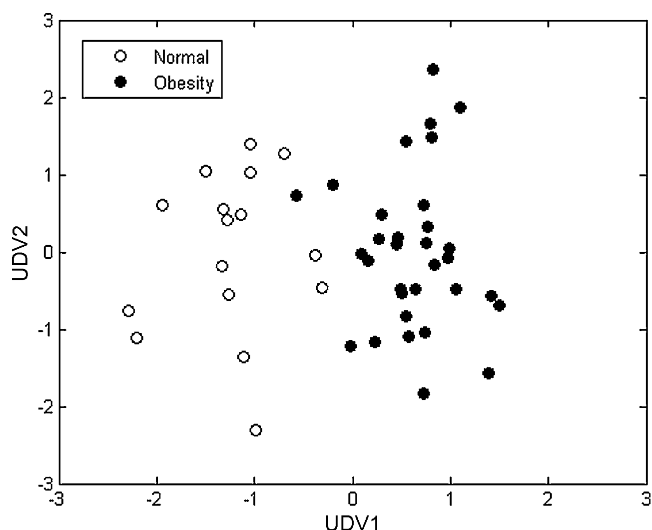

Fig. 8. Score plot of ULDA by UDV1 versus UDV2 of samples from normal (○) and obesity (●) groups. The samples from the two groups were distinguishable in the direction of UDV1.

as biomarker candidates of childhood obesity. In the other hand, the absolute values of the transformation matrix **G** can also represent the importance of the metabolites. So the potential biomarkers screened by the two approaches were compared. ULDA was performed for samples from normal and obesity groups. As shown in Fig. 8, the samples of the two groups were separated clearly. So the absolute values of the transformation matrix **G** may represent the contributions of metabolites to the separation. The corresponding absolute values were illustrated in Fig. 9(a). For CCA, Fig. 9(b) shows the absolute values of the coefficients of the first canonical variable of metabolic patterns. As shown in the figure, the importance of metabolites screened by the two approaches were similar, especially the variables from 11 to 20, and the status of variables of 11, 12, 13, 16, 17 in both Fig. 9(a) and (b) were alike, although not identical. So the corresponding metabolites, say isoleucine, glyceric acid, serine, 2,3,4-trihydroxybutyric acid and phenylalanine may be considered as potential biomarkers of childhood obesity.

Metabolic interpretation of the results is very important, although the interpretation of metabolomic research is difficult for the complexity of metabolic pathways. Isoleucine, one of the branched chain amino acids, is particularly involved in stress, energy and muscle metabolism. It plays important roles in increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis [49]. Glyceric acid can be obtained from oxidation of glycerol, several phosphate derivatives of glyceric acid, including 2-phosphoglyceric acid, 3-phosphoglyceric acid, 2,3-bisphosphoglyceric acid and 1,3-bisphosphoglyceric acid are important biochemical intermediates of lipid metabolism, which has been reported to be correlated with obesity [50]. Serine is important in metabolism that it participates in the biosynthesis of purines, whose catabolism has been found to be associated with obesity [51]. 2,3,4-Trihydroxybutyric acid may probably derived from glycated proteins, which are usually found in diseases such as obesity [52]. Phenylalanine, an essential amino acid and the precursor for tyrosine and the neurotransmitters called catecholamines in the body, has been discovered to be closely related to obesity [53].

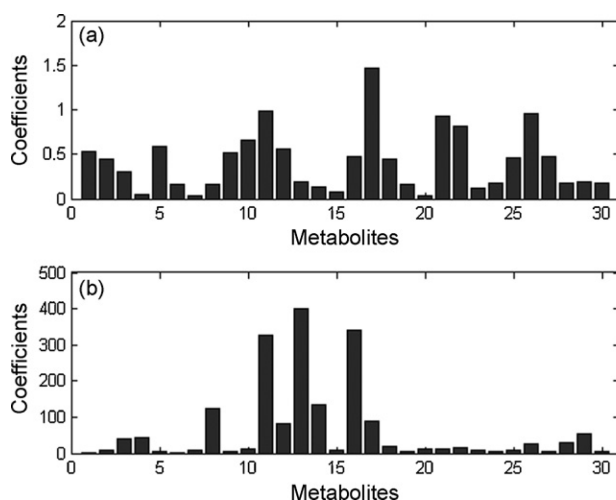

Fig. 9. Plot of the absolute values of transformation matrix G (a) and absolute coefficients of canonical variables for metabolites (b).

There are metabolomic studies about obesity, and the results coincide well with the results in this work, although they are all about obesity but not childhood obesity. A branched chain amino acid (BCAA)-related metabolite signature that is suggestive of increased catabolism of BCAA and correlated with insulin resistance was revealed by metabolomics profiling of obese versus lean humans [7]. In this work, one of the branched chain amino acids, say isoleucine, was suggested to be important and considered as potential biomarker of childhood obesity. Obesity has also been found to be related to the changes in lipidomic profile, particularly increases in lysophosphatidylcholines and decreases in ether phospholipids [8], which quite agree with our results. In this study, glyceric acid was screened and considered as biomarker candidate of childhood obesity, and its phosphate derivatives are all involved in lipid metabolism.

4. Conclusions

In this study, a metabolic fingerprinting approach by GC/MS combined with ULDA and CCA was used to explore disturbances of metabolic patterns and potential biomarkers of childhood obesity. The results indicate that the proposed metabolic patterns can reflect the metabolic states of the progress for childhood obesity, moreover, isoleucine, serine, 2,3,4-trihydroxybutyric acid and phenylalanine were screened as biomarker candidates for childhood obesity by both of ULDA and CCA. Furthermore, WHR together with TG, TC, HDL and LDL may be the most important parameters which correlated with the metabolic disturbances of childhood obesity, thus should be paid more attention to in clinical practice for monitoring metabolic disturbances of childhood obesity. The results have demonstrated that the proposed metabolic fingerprinting approach may be effective for exploring metabolic perturbations and possible biomarkers for diseases, and may be able to provide useful information for clinical practice.

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