

Original Article

Untargeted metabolomics approach (UPLC-Q-TOF-MS) explores the biomarkers of serum and urine in overweight/obese young men

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Background and Objectives: Obesity is linked to metabolic diseases characterized by insulin resistance, such as diabetes and cardiovascular disease. In this study, we investigated the metabolic disorders of uncomplicated obesity to identify early alterations in biological systems. **Methods and Study Design:** Metabolic differences between overweight/obese (n=36) and normal-weight (n=35) young Chinese men without known metabolic disorders were assessed. Metabolic profiling of the serum and urine was performed using ultra-performance liquid-chromatography quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS). Partial least squares discriminant analysis (PLS-DA) was undertaken to reveal and classify the differences between the two groups. **Results:** Compared to normal-weight men, obese men had higher levels of the serum metabolites phenylalanine, Phe-Phe, and L-tryptophan, whereas those of p-cresol sulfate and p-cresol were less in obesity. Urinary metabolites phenylacetamide, L-glutamine, phenylacetylglutamine, indoxyl sulfate, p-cresol, and p-cresol sulfate were greater in obese men. **Conclusions:** These findings indicate that disorders involving aromatic amino acids and the tricarboxylic acid cycle (TCA) have microbiomic involvement in the uncomplicated phase of obesity.

Key Words: overweight/obese, metabolomics, serum, urine

INTRODUCTION

Obesity has reached epidemic proportions worldwide.¹ It is associated with diabetes, hypertension, cardiovascular disease, and various cancers.^{2,3} Multiple factors can contribute to the development of obesity, including those that are genetic,⁴ environmental,⁵ and psychological.⁶ The metabolic disorders seen in obesity may be etiologic or consequential. From the perspective of systems biology, endogenous metabolic changes should be explored by integrative and comprehensive methods. Therefore, comprehensive metabolic profiling to identify metabolites that characterize the body's internal environment is feasible by a metabolomics approach. Specific metabolites or metabolic pathways can be identified as potential biomarkers of obesity.

Endogenous metabolites have been identified in animal and human obesity and metabolic disease using metabolomic methods.⁷⁻⁹ With targeted metabolomic methods, a cluster of obesity-associated metabolites such as amino acids,¹⁰ acylcarnitines,¹¹ organic acids,¹² and fatty acids have been detected in patients and animals with the metabolic syndrome.¹³ However, targeted metabolomic methods can only quantify the metabolites that are definitely related to obesity-associated metabolic perturbation and can only reflect metabolic alterations, not the primary metabolic disorder. Untargeted metabolomic methods can comprehensively detect low-molecular-weight metabolites in biological fluids. Therefore, we used ultra-performance liquid chromatography (UPLC) and time-of-

flight (TOF) mass spectrometry (UPLC-TOF-MS) to analyze serum and urinary biomarkers in overweight/obese young men.

Over the past several decades, metabolomics approaches coupled with chemometrics data analysis techniques have been used to select putative biomarkers. Metabolites have been identified in serum and urine owing to the abundance of intermediate and final products of metabolism in biofluids.^{7-9,14} However, studies analyzing the interaction of metabolites in serum and urine have been rare. In addition, researchers previously focused on adolescent and middle-aged obese individuals with metabolic syndrome but scarcely on young men at early stages of obesity and free of other metabolic diseases.¹⁴⁻¹⁶ Therefore, overweight/obese young men without any other metabolic diseases were selected as our research subjects to explore the primary endogenous metabolic alterations in the early phase of obesity.

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METHODS

Subjects and study design

Seventy-one healthy young men in the age group of 19–28 years were enrolled in this study. Thirty-six overweight/obese men with body mass index (BMI) ≥ 25 kg/m² and 35 normal-weight men as controls (18.5 kg/m² \leq BMI ≤ 22.9 kg/m²). Subjects were excluded if they had cardiopulmonary, renal, hepatic, or endocrine diseases, or had dieted to lose weight. All participants completed a 3-day food-frequency questionnaire, in which one of the days had to be a Saturday or Sunday. All the eligible subjects (both overweight/obese and normal-weight) confirmed that they would complete the same 4-week standard diet recipes; they were interviewed after every 3 days regarding dietary compliance. The same 4-week standard diet recipes could reduce the effect of food on metabolites in blood and urine. Both before and after the 4-week standard diet washout, clinical characteristics were recorded, and blood and urine samples were collected. We detected serum and urine metabolites by untargeted metabolomic methods before and after dietary intervention to identify the biomarkers that were consistently present in the biofluids. The dietary intervention was performed at the Food Center of the Harbin University of Science and Technology. Five participants dropped out of the study owing to personal and non-diet-related reasons (two obese men and three normal-weight men). The protocol of the study was approved by the Ethics Committee of the Jilin Medical College (No. 2014126-2) and conformed to the Declaration of Helsinki. Informed consent was provided by all the participants.

Clinical characteristics and sample collection

Participants underwent two physical examinations and provided blood and urine collections before and after the 4-week standard diet washout. Body weights, heights, and waist and hip circumferences were measured (with the participants unclothed) for the calculation of BMI and waist/hip ratio (WHR). The body fat ratio was measured using the TBF-300 Body Fat Analyzer (TANITA, Japan). Venous blood samples were collected in non-anticoagulant tubes and centrifuged at $1,002 \times g$ for 15 min at 4°C to obtain serum. The serum was stored in aliquot bottles at -80°C until analysis. The middle section 'urina sanguinis' was collected by the participants themselves, centrifuged at $1,002 \times g$ for 15 min at 4°C and stored in aliquot bottles at -80°C until analysis.

Diet surveys and standard diet recipes

The energy requirements of the subjects were assessed by standard equations from the basal metabolic rate (BMR) multiplied by 1.55, based on height, weight, and age as the activity factors. Both groups consumed the same standard diet that was designed according to energy requirements and diet surveys (breakfast accounted for 25–30% of the energy intake; lunch, 40% of the energy intake; and supper, 30–35% of the energy intake), and seven different dinners were designed to balance the dietary influence on metabolism. Participants could choose any combination of breakfast, lunch, and supper every day for 4 weeks, and all food, except fruit and milk, was obtained

from the Food Center of the Harbin University of Science and Technology.

Serum glucose and lipid

Fasting serum glucose (GLU), serum total cholesterol (TC), triglycerides (TG), high-density-lipoprotein cholesterol (HDL-C), and low-density-lipoprotein cholesterol (LDL-C) were measured using commercially available kits of Hitachi 7100 Autoanalyzer (Hitachi Ltd., Tokyo, Japan). The kits for GLU and serum lipids were purchased from Roche (Roche, Switzerland).

Chemicals and reagents

HPLC-grade acetonitrile, methanol, and formic acid were obtained from Dikma Science and Technology Co. Ltd. (Dikma, Beijing, China); deionized water was produced by the Milli-Q pure water system (Millipore, Billerica, MA, USA); and leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Untargeted metabolic profiling by UPLC-Q-TOF MS

Sample preparation

Before the analysis, serum samples were thawed at 4°C and vortexed for 30 s, followed by the addition of 900 μ L methanol to 300 μ L serum for deproteinization, and vortexing for 2 min. The mixture was centrifuged at $13,362 \times g$ for 12 min at 4°C. The supernatant was transferred to a clean tube and dried using the Bath Nitrogen Blow instrument (TTL-DCI, Beijing, China), followed by reconstitution in 300 μ L acetonitrile and deionized water (2:1, v/v), vortexing for 2 min, centrifugation at $13,362 \times g$ for 12 min, and injection of the supernatant into the UPLC-Q-TOF-MS instrument for analysis. Urine samples were thawed at 4°C, vortexed for 30 s, and centrifuged at $13,362 \times g$ for 12 min. Three hundred microliters supernatant was collected and diluted with deionized water at a ratio of 1:1 (v:v) and vortexed before UPLC/Q-TOF-MS/MS analysis.

UPLC-Q-TOF-MS analysis

A Waters ACQUITY UPLC system (Waters Corp., Milford, CT, USA) was used to separate the molecules from serum and urine samples. The UPLC separation was performed with a UPLC BEH C18 column (100 mm \times 2.1 mm, i.d. 1.7 μ m; Waters Corp., Milford, USA.) for serum analysis and a UPLC HSS T3 column (100 mm \times 2.1 mm, i.d. 1.8 μ m; Waters Corp., Milford, USA) for urine analysis. The metabolites were separated by UPLC and analyzed and assigned by Q-TOF-MS/MS. MS was performed on a Waters Micromass Q-TOF Micro Mass Spectrometer (Waters Corp., Manchester, United Kingdom) with an electrospray ionization (ESI) interface, with the ESI source operated in negative ion (ESI-) and positive ion (ESI+) modes. Mass data were collected in full scan mode from m/z 50 to 1,000 in both serum and urine analyses.

The MS data files, including retention time (RT), m/z , and ion intensities, were processed using the Masslynx4.1 software (Waters, Milford, USA). The parameters were set as follows: (peak width at 5% height, 1 s; peak-to-peak baseline noise were calculated automatically by the software) intensity threshold, 70 counts; mass window,

0.05 Da; retention time window, 0.2 min; minimum intensity, 80; noise elimination level, 6.0; and deisotope data, "Yes." The analysis was started when the initial and final retention times were 0.4 and 10.5 min, respectively.

After pattern recognition and alignment, the peak intensity of each metabolite was normalized and summed to obtain the total ion intensity of each chromatogram and to account for the variation in serum and urine samples. The three-dimensional data matrix, including RT-m/z pair, sample numbers, and normalized peak areas, were exported to the EZinfo software (Waters) for partial least squares discriminant analysis (PLS-DA). After an initial overview of the UPLC-Q-TOF-MS data, PLS-DA was applied to visualize the differences associated with obesity between the overweight/obese group and normal-weight group at the two time points.

Statistical analysis

Fasting serum glucose and serum lipids and metabolite peak intensities between the two groups were analyzed using the dependent t-test and the Mann-Whitney U-test of the SPSS software (version 16.0, Beijing Stats Data Mining Co. Ltd., China). A two-tailed value of $p < 0.05$ was considered statistically significant. Pearson's correlation was used to determine the relationships among variables.

Quality control

The reproducibility and reliability of the metabolomics data platform were assessed using a quality control (QC) sample.^{17,18} The QC sample was obtained by mixing equal volumes of five samples each from the obese/overweight and normal-weight groups. The QC sample was analyzed after the injection of every 15th sample throughout the analytical workflow. The reproducibility of the data platform was assessed using the principal component analysis (PCA) score plots of all samples, which revealed that all the QC samples clustered in the middle of the score plots (Supplemental figure 3). Five ions from the QC samples were analyzed to certify the reliability of the method (Supplementary table 1). The ranges of the relative standard deviations (RSDs) of retention times and peak intensity were 0.04–0.97% and

0.6–5.5%, respectively. These results reflected the reproducibility and reliability of the method.

Metabolite identification

In this study, the PLS-DA model was used to classify the two groups and to identify possible biomarkers in overweight/obese young men. The variable importance in the project (VIP) value was used to reflect the importance of metabolites and select potential biomarkers. The metabolites with VIP values > 1.0 and significantly different peak intensities between the two groups were selected as potential biomarkers.¹⁷ The MassFragment™ application manager (MassLynx v4.1; Waters Corp.) was used to facilitate the MS/MS fragment ion-analysis process using chemically intelligent peak-matching algorithms. The MS/MS analysis of ions using TOF-MS and tandem MS in both ion modes was performed to identify the MS structure of the potential biomarkers. The relative metabolic pathways of biomarkers were interpreted using the Human Metabolome Database (HMDB) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

RESULTS

Physiological measures

Physiological measures of overweight/obese and normal-weight young men are shown in Table 1. There was no significant difference between the two groups for age or smoking or alcohol consumption habits ($p > 0.05$). The overweight/obese group had higher BMIs, WHRs, and body fat ratios than those of normal-weight young men ($p < 0.001$), as expected. In addition, the systolic blood pressure (SBP) was significantly higher in the overweight/obese group ($p < 0.05$). Overweight/obese subjects had higher serum TC, TG, and LDL-C, but lower HDL-C than that of normal-weight subjects ($p < 0.05$).

Serum and urine metabolic profiling

Serum and urine samples were analyzed using UPLC-Q-TOF MS. After data reduction, 1,312 serum metabolites (624 in ESI- and 688 in ESI+) and 1,691 urine metabolites (676 in ESI- and 1,015 in ESI+) were identified and used for multivariate statistical analysis. According to the VIP values obtained from PLS-DA, metabolites that

Table 1. Physiological measures and clinical characteristics in normal-weight and overweight/obese young men

	Normal-weight	Overweight	<i>p</i> value
	N ₁ (n=35)	O ₁ (n=36)	
Age (years)	22.7±2.50	22.7±2.25	>0.05
Cigarette smoker, n (%) [†]	6 (17.1)	5 (13.9)	>0.05
Alcohol drinker, n (%) [†]	17 (48.6)	16 (44.4)	>0.05
Body mass index (kg/m ²)	21.2±1.57	27.8±1.10	<0.01
Waist/hip ratio	0.78±0.04	0.85±0.03	<0.01
Body fat (%)	20.1±2.81	30.2±2.69	<0.01
Systolic blood pressure (mmHg)	120±8.79	126±7.30	<0.01
Diastolic blood pressure (mmHg)	77±9.58	79±6.59	>0.05
Glucose (mmol/l)	5.04±0.38	4.88±0.34	>0.05
Total cholesterol (mmol/l)	4.29±0.85	4.81±0.89	<0.05
Triglyceride (mmol/l)	0.91±0.37	1.41±1.05	<0.01
LDL cholesterol (mmol/l)	2.15±0.60	2.68±0.66	<0.01
HDL cholesterol (mmol/l)	1.38±0.28	1.21±0.26	<0.01

Data are presented as mean±SD.

[†]Cigarette smokers and alcohol drinkers were compared by the chi-square test.

played an important role in the separation of the two groups were regarded as potential biomarkers. As shown in the PLS-DA score plots of serum (Supplementary figure 1) and urine (Supplementary figure 2), overweight/obese subjects and normal-weight subjects were clearly separated into two clusters in both negative and positive ion modes.

Identification of distinctive metabolites

Distinctive metabolites in serum and urine samples

As shown in Table 2, 12 metabolites were identified in both ion modes, and Phe-Phe was scanned in both modes. Among the 11 metabolites, p-cresol sulfate, p-cresol, phenylacetylglutamine, and L-glutamine levels in the overweight/obese group were significantly lower than those in the normal-weight group, and Phe-Phe, the phenylalanine fragment (Phe-fragment, with the same MS/MS construction as that of the fragment of phenylalanine),^{19,20} L-phenylalanine, L-tryptophan, phenylalanine (the same MS/MS spectra as that of L-phenylalanine), and sphingosine-1-phosphate levels in the overweight/obese group were significantly higher than those of the normal-weight group.

Table 3 shows the 17 metabolites identified in the urine samples in both ion modes. Therein, L-glutamine and phenylacetylglutamine were scanned in both ion modes; the identity of a metabolite with a retention time of 5.1243 min and m/z 347.1278 could not be confirmed, but it possessed similar secondary mass spectra as those of corticosteroids. Thus, among the 14 metabolites, the levels of L-glutamine, phenylacetylglutamine, indoxyl

sulfate, p-cresol, p-cresol sulfate, phenylacetamide, hydroxytestosterone, and tetrahydrodeoxycorticosterone (THDOC) in the overweight/obese group were significantly lower than those in the normal-weight group, and the uric acid, D-glucuronic acid, deoxycortisol, and tetrahydrocortisone in the overweight/obese group were significantly higher than those in the normal-weight group.

Potential biomarkers and special metabolic pathway

Correlation analysis of the metabolites in both serum and urine samples of overweight/obese subjects was performed to understand the potential relationship among the metabolites, which is shown in the heat map of Figure 1. Upon combining statistical analyses with correlation analysis, some metabolites and pathways such as those involved in aromatic amino acid metabolism and corticosteroid and L-glutamine metabolism were selected as the special metabolic pathways operational in early stages of obesity. The aromatic amino acid and L-glutamine metabolism pathways in obese/overweight subjects were constructed according to the KEGG map and are shown in Figure 2.

DISCUSSION

In this study, 11 types of serum metabolites, including amino acids and amino acid metabolites, and 14 types of urine metabolites were identified as biomarkers of obesity. Amino acid metabolism is an important physiological phenomenon, and studies indicate that disorders in amino acid metabolism are related to the occurrence of insulin resistance and other metabolic diseases. Newgand et al^{7,10}

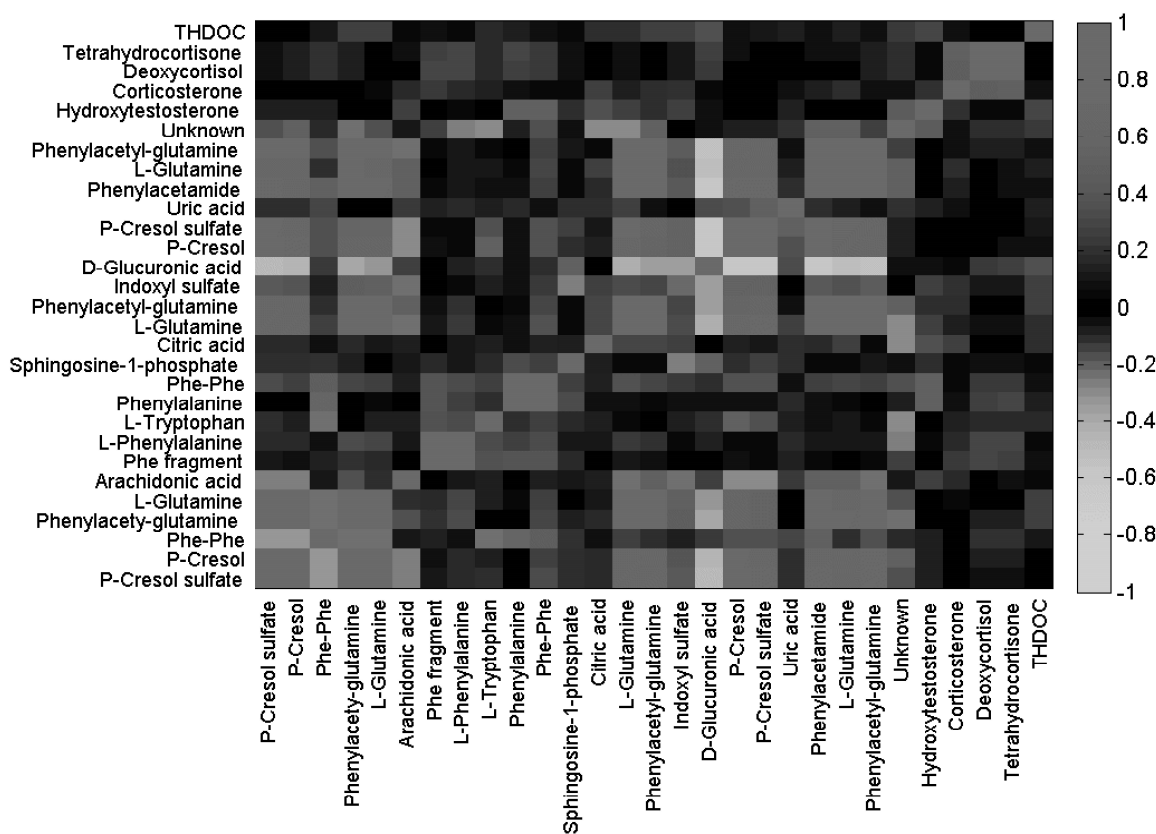


Figure 1. Correlation analysis of the 29 metabolites in the serum and urine of overweight/obese subjects. The correlation coefficients are shown in different colors, red: positive correlation, green: negative correlation.

Table 2. Identification of major metabolites in normal-weight and overweight/obese men serum samples in both ion modes

RT (min)	Postulated identity	Formula	Measured mass (Da)	Actual mass (Da)	Week 0			Week 4		
					p_1	VIP ₁	Trend ₁	p_2	VIP ₂	Trend ₂
ESI(-)										
2.8566	p-Cresol sulfate	C ₇ H ₈ O ₄ S	187.0054	187.0070	<0.05	2.4	↓	<0.05	2.0	↓
2.8580	p-Cresol	C ₇ H ₈ O	107.0482	107.0504	<0.05	2.4	↓	<0.05	2.0	↓
2.8565	Phe-Phe	C ₁₈ H ₂₀ N ₂ O ₃	311.1414	314.1401	<0.01	3.1	↑	<0.01	2.7	↑
2.5748	Phenylacetylglutamine	C ₉ H ₁₆ N ₂ O ₅ S	263.1053	263.1037	<0.05	2.4	↓	<0.05	1.9	↓
2.5785	l-Glutamine	C ₅ H ₁₀ N ₂ O ₃	145.0607	145.0619	<0.01	2.4	↓	>0.05	1.4	↓
4.5066	Arachidonic acid	C ₂₀ H ₃₂ O ₂	303.2358	303.2330	>0.05	1.3	-	>0.05	1.0	-
ESI(+)										
2.0581	Phe fragment	—	120.0748	—	<0.01	3.0	↑	<0.01	2.5	↑
2.0624	l-Phenylalanine	C ₉ H ₁₁ NO ₂	166.0883	166.0863	<0.01	3.0	↑	<0.01	2.3	↑
2.2884	l-Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	205.0996	205.0972	<0.01	1.8	↑	>0.05	1.9	-
2.9520	Phenylalanine	C ₉ H ₁₁ NO ₂	166.0887	166.0863	<0.01	3.1	↑	<0.01	2.3	↑
2.9508	Phe-Phe	C ₁₈ H ₂₀ N ₂ O ₃	313.1576	313.1547	<0.01	2.8	↑	<0.01	2.3	↑
6.2178	Sphingosine-1-phosphate	C ₁₈ H ₃₈ NO ₅ P	380.2598	380.2560	<0.01	1.8	↓	<0.05	1.3	↓

Data are presented as mean±SD. VIP: variable importance in the project; VIP >1 indicates high relevance for explaining the differences between groups.

Table 3. Identification of major metabolites in normal-weight and overweight/obese men urine samples in both ion modes

RT (min)	Postulated identity	Formula	Measured mass (Da)	Actual mass (Da)	Week 0			Week 4		
					p_1	VIP ₁	Trend ₁	p_2	VIP ₂	Trend ₂
ESI(-)										
1.2218	Citric acid	C ₆ H ₈ O ₇	191.0194	191.0197	>0.05	1.2	-	>0.05	1.0	-
5.3455	l-Glutamine	C ₅ H ₁₀ N ₂ O ₃	145.0594	145.0619	<0.015	2.9	↓	<0.05	2.1	↓
5.3375	Phenylacetylglutamine	C ₉ H ₁₆ N ₂ O ₅ S	263.0943	263.1037	<0.05	2.3	↓	<0.05	1.9	↓
5.6829	Indoxyl sulfate	C ₈ H ₇ NO ₄ S	212.0034	212.0023	<0.01	2.3	↓	<0.05	1.8	↓
6.2526	d-Glucuronic acid	C ₆ H ₁₀ O ₇	193.0364	193.0354	<0.01	2.6	↑	0.05	1.9	↑
6.2738	p-Cresol	C ₇ H ₈ O	107.0484	107.0504	<0.01	2.9	↓	<0.05	1.9	↓
6.2901	p-Cresol sulfate	C ₇ H ₈ O ₄ S	187.0032	187.0070	<0.01	2.8	↓	<0.05	1.7	↓
ESI(+)										
2.9406	Uric acid	C ₅ H ₄ N ₄ O ₃	169.0403	169.0356	<0.05	2.1	↑	<0.05	2.1	↑
4.8998	Phenylacetamide	C ₈ H ₉ NO	136.0757	136.0757	<0.01	3.5	↓	<0.01	3.0	↓
4.8995	l-Glutamine	C ₅ H ₁₀ N ₂ O ₃	147.0767	147.0764	<0.01	3.4	↓	<0.01	3.1	↓
4.8979	Phenylacetylglutamine	C ₉ H ₁₆ N ₂ O ₅ S	265.1119	265.1183	<0.01	3.6	↓	<0.01	2.6	↓
5.1243	Unknown	—	347.1278	—	<0.01	2.4	↓	<0.01	1.7	↓
7.5509	19-Hydroxytestosterone	C ₁₉ H ₂₈ O ₃	305.2085	305.2111	<0.01	2.7	↓	<0.01	3.8	↓
7.6728	Corticosterone	C ₂₁ H ₃₀ O ₄	347.2262	347.2217	>0.05	1.2	-	>0.05	1.5	-
7.9195	Deoxycortisol	C ₂₁ H ₃₀ O ₄	347.2247	347.2217	0.06	1.9	↑	0.07	1.7	↑
7.9204	Tetrahydrocortisone	C ₂₁ H ₃₂ O ₅	365.2339	365.2323	0.08	2.0	↑	0.06	1.6	↑
8.8988	THDOC	C ₂₁ H ₃₄ O ₃	335.2526	335.2581	<0.05	2.6	↓	<0.05	1.9	↓

Data are presented as mean±SD. VIP: variable importance in the project; VIP >1 indicates high relevance for explaining the differences between groups.

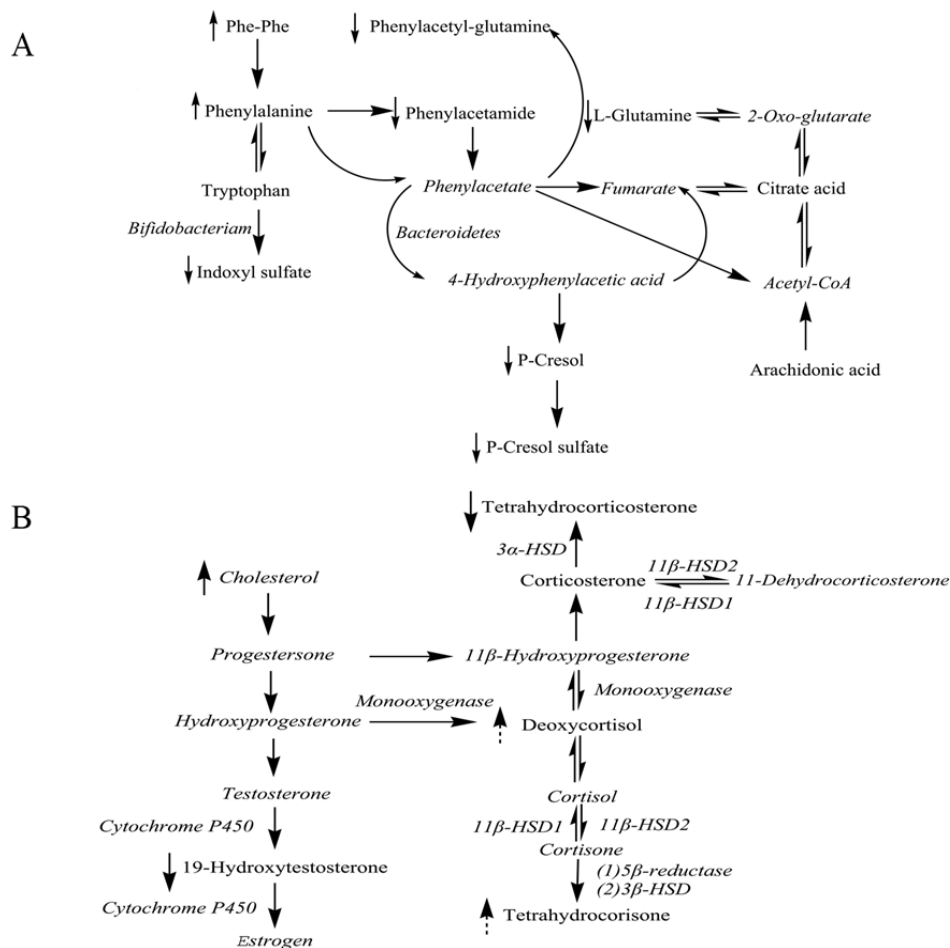


Figure 2. Schematic diagram of the metabolic pathways in overweight/obese men. The diagram was constructed according to the KEGG map. The metabolites detected by UPLC-Q-TOF-MS are shown in upright font, and undetected metabolites are shown in italicized font. The increased metabolites are marked by upward arrows (↑) and decreased metabolites, by downward arrows (↓).

found that both branched-chain amino acids (BCAA) and aromatic amino acids, including tyrosine, tryptophan, and phenylalanine, were significantly higher in obese or insulin-resistant patients. Similarly, we also observed that the serum phenylalanine levels were significantly elevated in the overweight/obese individuals. The tryptophan tended to be high in overweight/obese men; however, this was not consistently observed. The molecule with a m/z of 120.0748 had the same daughter ion as that of the L-phenylalanine fragment, which was detected and identified to be L-phenylalanine in other studies. We found that the Phe-fragment was higher in the overweight/obese group. Meanwhile, Phe-Phe, a peptide consisting of two phenylalanine molecules, was significantly increased. In addition, correlation analysis in the heat map (Figure 1) showed strongly positive correlations between L-phenylalanine and the Phe-fragment ($r=0.802$, $p<0.05$), L-phenylalanine and Phe-Phe ($r=0.316$, $p<0.05$), L-phenylalanine and L-tryptophan ($r=0.327$, $p<0.05$), and the Phe-fragment and Phe-Phe ($r=0.377$, $p<0.05$). Therefore, we concluded that aromatic amino acid metabolic disorder occurs in the early phase of obesity.

P-cresol is the degradation product of the aromatic amino acid tyrosine, and to some extent, it is also a by-product of phenylalanine metabolism. P-cresol sulfate is generated by the secondary metabolism of p-cresol. Significant differences were observed in the peak intensity of p-cresol and p-cresol sulfate in both the serum and urine

of the two groups, which can be related to the type of gastrointestinal flora. Other studies indicate that the occurrence of obesity is related to the profile of gastrointestinal flora.^{21,22} Human body fluids contain various metabolites generated from the co-metabolism of gastrointestinal flora. The Bacteroidetes and Firmicutes are the dominant beneficial bacteria in the human gastrointestinal tract. Compared to lean people, the relative proportion of Bacteroidetes is less in overweight/obese people, and the proportion increases with weight loss.²¹ P-cresol and phenol are derived from the catabolism of tyrosine catalyzed by the intestinal flora, and they are synthesized from 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid, respectively. Bacteroides tends to produce p-cresol from tyrosine.²³ Owing probably to the reduction in the number of Bacteroides in vivo in overweight/obese individuals, both serum and urine p-cresol and p-cresol sulfate in overweight/obese young men were significantly lower than those in normal-weight group individuals (Figure 2). In addition, urine indoxyl sulfate was found to be low in the overweight/obese participants. Indoxyl sulfate is a tryptophan metabolite that is catalyzed by tryptophanase produced by the flora of the large intestine. Indole is metabolized to form indoxyl sulfate by *Bifidobacterium* or indole-3-propionic acid by *Clostridium sporogenes*.^{24,25} Studies indicate that *Bifidobacterium* numbers are markedly low in obese individuals,²⁶ which could explain the low levels of indoxyl sulfate in overweight/obese subjects.

Phenylacetamide is an intermediate in phenylalanine metabolism, and it is the third last step in the synthesis of phenylacetylglutamine, which is catalyzed by phenylalanine-2-monooxygenase. It is converted to phenylacetate via the enzyme amidase. Therefore, positive correlations were found for phenylacetamide with p-cresol, p-cresol sulfate, phenylacetylglutamine, glutamine, and indoxyl sulfate (Figure 1). In this study, the urine phenylacetamide decreased significantly in overweight/obese subjects, while the phenylalanine increased significantly. This difference could be attributed to the abnormal activity of phenylalanine-2-monooxygenase.

L-Glutamic acid is not only the precursor of L-glutamine but also an important tricarboxylic acid (TCA) cycle intermediate. It can cooperate with phenylacetate in the liver and kidney to form phenylacetylglutamine and is excreted stably in the urine. Consistent with this, a strong positive correlation ($r_{\text{serum}}=0.949$, $r_{\text{urine}}=0.938$) was found between L-glutamine and phenylacetylglutamine in this study (Figure 1). Furthermore, several metabolites were found to be positively correlated with L-glutamine (Figure 1), including p-cresol ($r_{\text{serum}}=0.763$, $r_{\text{urine}}=0.716$) and p-cresol sulfate ($r_{\text{serum}}=0.763$, $r_{\text{urine}}=0.732$) in serum and urine, as well as L-phenylalanine, indoxyl sulfate, and phenylacetamide. Glutamine can be synthesized via the phenylacetamide and 4-hydroxyphenylacetic acid pathway derived from phenylalanine catabolism (Figure 2), and this pathway is influenced by the presence of Bacteroidetes. The mechanism by which an imbalance in the intestinal flora profile may lead to the development of obesity requires further investigation.

Corticosteroids are a class of steroid hormones produced by the adrenal cortex, and they are classified as glucocorticoids (GCs) or mineralocorticoids. GCs participate in the biosynthesis and catabolism of sugar, fat, and protein, and possess anti-inflammatory properties. Mineralocorticoids are involved in water–electrolyte metabolism and sodium retention. The main endogenous mineralocorticoid is aldosterone, but numerous other hormones such as progesterone and deoxycorticosterone also possess mineralocorticoid activity. Studies have shown that both aldosterone and GCs induce adipogenesis.²⁷ In addition, glucocorticoid metabolism has been shown to have a close relationship with the development of obesity and adipose distribution. Marin et al²⁸ found that urinary cortisol concentration was related to the body fat distribution in obese women, and a positive association was shown between urinary cortisol and WHR. This can be explained by the higher density of GC receptors and GC overactivity in abdominal adipose tissue, which alter the GC metabolism and cause further accumulation of vascular adipose tissue.^{29,30} Therefore, GCs may promote both the differentiation and proliferation of human adipocytes through GC receptors (GRs). The renin–angiotensin–aldosterone system (RAAS) might be overexpressed in the adipose tissue of obese animals and humans.^{31,32} Furthermore, mineralocorticoid receptor (MR) expression in adipose tissue can be activated by aldosterone during adipose differentiation.^{33–35} In the present study, the possible biomarker deoxycortisol (a mineralocorticoid), an intermediate in the biotransformation of mineralocorticoid to GC, was identified at higher levels in the urina sanguinis

of overweight/obese young men than that in normal-weight participants. The levels of corticosterone, an intermediate in the biotransformation of progesterone to aldosterone, was higher in overweight/obese subjects.

We analyzed the interrelationships of serum and urine metabolites with putative pathways related to obesity, but no clear associations were found. The role of gut flora in aromatic amino acid metabolism and the activities of steroid-converting enzymes in early obesity may provide insights for future research.

Our study showed that aromatic amino acid metabolism disorder occurred in the early stages of obesity, and that the catabolism of aromatic amino acids in the serum and urine was altered. The TCA cycle intermediates L-glutamine and phenylacetylglutamine were affected by changes in aromatic amino acids. Urine corticosteroids were higher in overweight/obese subjects. Therefore, urine-specific metabolic corticosteroids were identified as potential biomarkers, and the aromatic amino acid metabolic pathway was identified as the specific metabolic pathway related to early stages of obesity.

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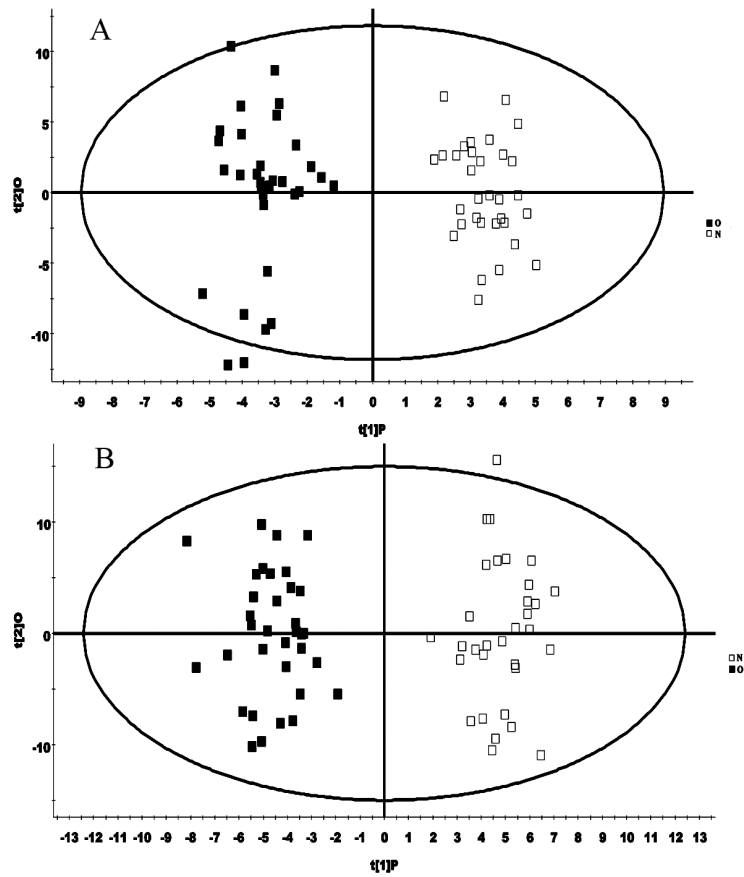
AUTHOR DISCLOSURES

We declare that we have no financial or personal relationships with people or organizations that could inappropriately influence our work. We received financial support from the National Natural Science Foundation of China (81472964), the National Natural Science Foundation of Jilin Province (No. 20150101206JC), and the Chang Bai Shan Scholars Program of Jilin Province, China.

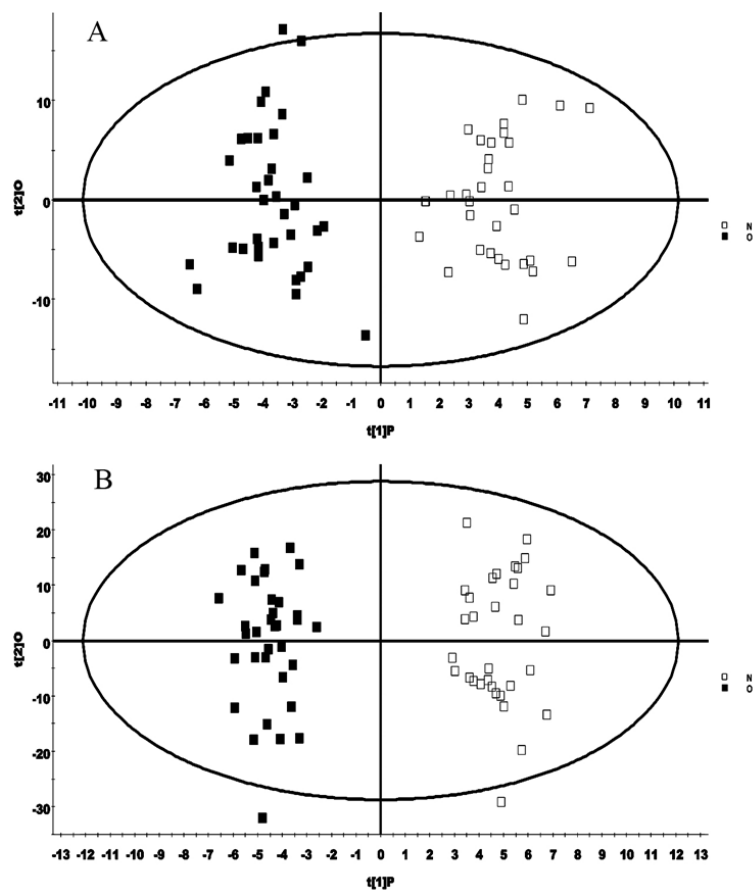
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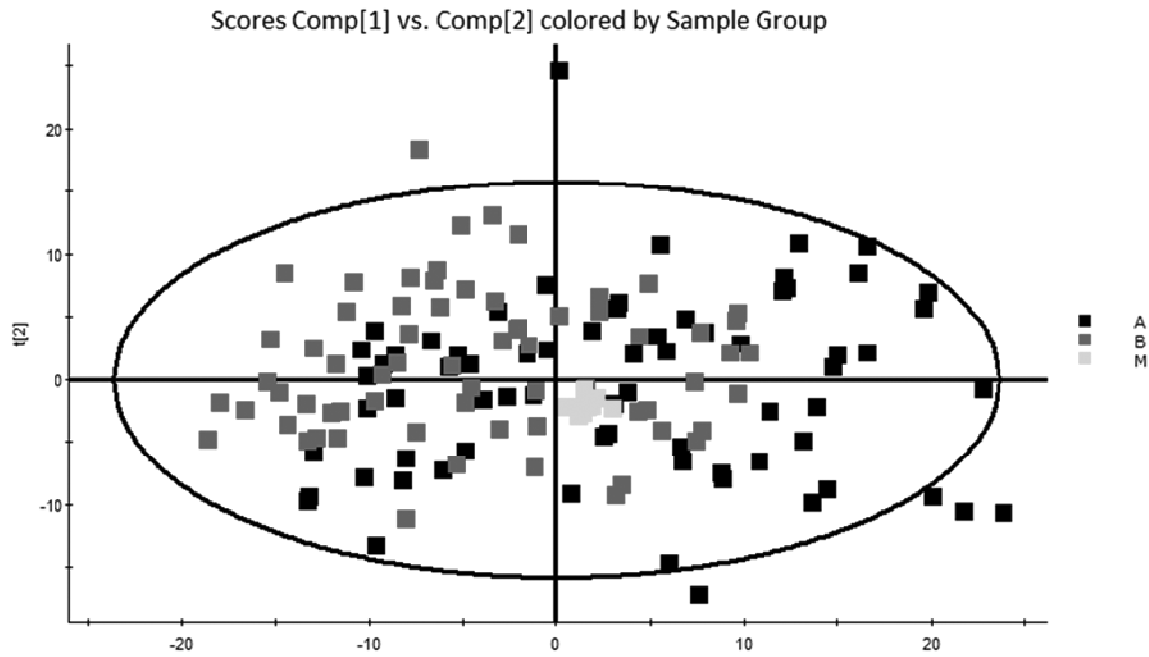
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Supplementary figure 1. PLS-DA score plots of serum samples. (A) The second time serum samples scanned in the negative ion mode. (B) The second time serum samples scanned in the positive ion mode. O: overweight, N: normal.



Supplementary figure 2. PLS-DA score plots of urine samples. (A) The second time urine samples scanned in the negative ion mode. (B) The second time urine samples scanned in the positive ion mode. O: overweight, N: normal.



Supplementary figure 3. Two-dimensional PCA scores plots of urine samples and QC samples (M) of both two time points (137 samples) in ESI negative ion mode. A: overweight/obese group; B: normal-weight group.

Supplementary table 1. Reproducibility of method from 5 ions of the quality control sample in the urine negative ESI modes (n=11)

m/z	RT			Peak Intensity		
	RT	SD	RSD	INT	SD	RSD
82.0289	0.8364	0.00809	0.0097	1.8047	0.18476	0.022
192.9819	1.7173	0.00647	0.0038	6.6230	0.36823	0.055
218.1040	3.3955	0.00522	0.0015	9.9063	0.62555	0.006
317.1280	8.0445	0.00522	0.0006	10.0792	0.44576	0.044
429.1875	8.6082	0.00405	0.0004	36.0505	1.56050	0.043