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Metabolic profiling of urine in young obese men using ultra performance liquid chromatography and Q-TOF mass spectrometry (UPLC/Q-TOF MS)

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ABSTRACT

Obesity is currently epidemic in many countries worldwide. In the young adult, obesity often accompanies hyperlipemia, which is strongly related to the occurrence and development of obesity-related chronic diseases such as diabetes mellitus, hypertension and cardiovascular disease. This study investigated the differences in metabolomic profiling between obese (with hyperlipemia, $n = 30$) and normal-weight ($n = 30$) young men. Anthropometric parameters and conventional metabolites were measured. There were no significant differences between obese and normal-weight young men in age, height and fasting plasma glucose level. Obese young men showed increased weight, body mass index, fat mass, systolic blood pressure, and triglyceride, total cholesterol and insulin levels, and lower levels of testosterone. The endogenous metabolite profile of urine was investigated by UPLC/Q-TOF MS (ultra performance liquid chromatography and Q-TOF mass spectrometry) with electrospray ionization (ESI). Partial least squares (PLS) enabled clusters to be visualized. Eight urine principal metabolites contributing to the clusters were identified; these included increased L-prolyl-L-proline, leucyl-phenylalanine, and decanoylcarnitine in positive ESI mode (m/z 213.1267, 279.1715 and 316.2459, respectively) and N-acetylornithine, 17-hydroxypregnenolone sulfate, 11β-hydroxyprogesterone, 5a-dihydrotestosterone sulfate and glucosylgalactosyl hydroxylysine in negative ESI mode (m/z 173.0931, 411.1883, 331.185, 369.1751 and 485.1875, respectively). These metabolite changes in obese men suggested early changes of metabolism in young-male obesity with hyperlipemia. The study may further aid the clinical prevention and treatment of obesity and related chronic disease.

1. Introduction

The past couple of decades have witnessed a worldwide epidemic of obesity, which poses a major threat to human health [1,2]. In young adults obesity, obesity often accompanies hyperlipemia, which is strongly related to the occurrence and development of obesity-related chronic diseases such as diabetes mellitus, hypertension and cardiovascular disease [3,4]. The fundamental cause of obesity-related chronic diseases is the metabolic disorders in organism caused by obesity [5]. Many previous comparisons of obese and lean or normal-weight subjects focused on a small group of experimental variables, whereas the organism has tens of thousands of metabolites. Therefore, obesity with hyperlipemia-induced perturbations in metabolism has not been fully characterized. Especially in obese young adults with hyperlipemia, the metabolic changes are more interesting to the occurrence and development of obesity-related chronic diseases in the future. Complex etiologies highlight the need to understand how metabolite profiles are altered at this stage.

Metabolomics is a sensitive and unbiased analytical method that assesses all metabolites in biological samples [6]. The technique can generate substantial amounts of metabolic data that can give surprisingly detailed insights into the changes in metabolic processes in whole organisms [7,8]. Metabolomics can determine the relationships between phenotype and metabolism, and can be used to identify the key metabolites associated with a particular phenotype and investigate the biological function and metabolic changes in the organism. This approach has been used to identify urinary principal metabolites involved in dietary intake [9,10], diabetes [11,12] and coronary artery disease [13] in humans and animal models. Recently, ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) has been applied widely in metabolomic studies owing to its high sensitivity and reproducibility [14,15]. In this field, ultra-performance liquid chromatography and Q-TOF mass spectrometry (UPLC/Q-TOF MS) adds a new dimension to

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metabolism studies, enabling attainment of better detection limits, better throughput, and increased chromatographic resolution, which in turn will improve data quality from the mass spectrometer.

We used metabolomics analyses based on UPLC-Q-TOF MS to gain a broader understanding of metabolic differences between obese individuals with hyperlipemia and normal-weight young men, and found the metabolic changes in young obese males with hyperlipemia that may be important for future clinical prevention and treatment of obesity and related chronic diseases.

2. Materials and methods

2.1. Subjects

Sixty young men, aged 18–26 years, were recruited at Harbin Medical University. The study cohort included both obese hyperlipemic subjects whose body mass index (BMI) was >28.0 kg/m^2 and triglyceride level (TG) ≥1.7 mmol/L (n = 30) and normal weight subjects (n = 30, 18.5 kg/m^2 < BMI < 24 kg/m^2). The inclusion criteria were: (1) stable body weight during the 6 months before the study; (2) not receiving prescribed medication and not having an oral or urinary tract infection within 1 month of commencing the study; (3) no smoking and drinking; (4) not having cardiopulmonary, renal, or liver disease. Informed consent was obtained from all subjects, and the study protocol was approved by the Ethics Committee of Harbin Medical University.

2.2. Anthropometry parameters, blood pressure, blood, and urine collection, and the control of dietary intake

Before commencing the standard diet, height and weight were measured twice, to an accuracy of ±0.1 cm and to ±0.1 kg, respectively, while fasting overnight and wearing only underwear. BMI (weight in kilograms divided by the square of height in meters) was used as a measure of overall adiposity. Fat mass (FM) was measured using the electric impedance method and a body fat mass analyzer (TANITA TBF-300, Tanita Corporation, Tokyo, Japan). Blood pressure was measured using a standard mercury sphygmomanometer on the right arm after at least 10 min of rest. Mean values were determined from two independent measurements (by the same researcher) at 2-min intervals.

Volunteers were asked to record their dietary intake over a7-day period, and a preliminary test was performed to determine the amounts and types of foods that comprised their normal daily diet. Finally, the volunteers were provided with a full range and prescribed amounts of foods to form a standard diet [16] and were asked to avoid any vigorous activity. This was intended to attenuate the inter-individual variation in metabolite profiles associated with consuming different amounts and types of foods. Before commencing the standard diet, antecubital venous blood samples were collected after overnight fasting of 12 h. After following the standard diet for 3 days, the urina sanguinis in the morning of the 4th day was collected and centrifuged at 14,000 rpm (17,968 × g) for 10 min to remove particle contaminants and stored at −80 °C until analysis.

2.3. Chemicals and reagents

Acetonitrile (chromatographic grade) was purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Deionized water was purified using an ultra-clear system (SG Water conditioning and Regeneration, Barsbüttel, Germany). Leucine enkephalin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Plasma glucose was measured using a Kyoto blood sugar test meter and test strip (Arkray, Inc. Kyoto, Japan). Serum total cholesterol (TC) and TG were assayed using standard enzymatic colorimetric techniques and commercial kits (Biosino Biotechnology Ltd, Beijing, China) with an auto-analyzer (AUTOLAB PM 4000, AMS Corporation, Rome, Italy). Serum insulin and testosterone levels were measured using commercial kits (Tosoh Corporation, Tokyo, Japan) with an auto-immunoassay analyzer (AIA-2000 ST, Tosoh Corporation, Tokyo, Japan).

2.4. UPLC/Q-TOF MS analysis

UPLC/Q-TOF MS analysis was performed using an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) coupled to a Micromass Q-tof (Quatropde-Time of Flight) microTM Mass Spectrometer (Waters Corporation, Manchester, UK) with electrospray ionization (ESI) in positive and negative modes. Urine samples were centrifuged at 14,000 rpm for 10 min and the supernatant was transferred into an autosampler vial. A 2-μL aliquot of supernatant was injected into an ACQUITY UPLC HSS-T3C_{18} column (50 mm × 4.6 mm i.d., 1.8 μm; Waters Corporation, Milford, MA, USA). The flow rate of the mobile phase was 300 μL/min. Analytes were eluted from the column with a gradient, where A was water and B was acetonitrile. The initial composition of B was 2% and increased to 20% in 5 min, 35% in 2 min, 70% in 2 min, and to 100% in a further 1.5 min, followed by re-equilibration to the initial conditions in 10 min. Each run time was 20.5 min.

For MS analysis, the source temperature was set at 100 °C with a cone gas flow of 50 L/h. A desolvation gas temperature of 300 °C and a desolvation gas flow of 600 L/h were used. The capillary voltage was set at 3.0 kV in positive ESI mode and 2.6 kV in negative ESI mode, and the cone voltage to 35 V. All analyses were performed using the lock spray to ensure accuracy and reproducibility. A lock-mass of leucine enkephalin for positive ESI mode ($[M+H]^+$ = 556.2771) and negative ESI mode ($[M+H]^-$ = 554.2615) was used via a lock spray interface. The MS data were collected in centroid mode from m/z 80 to 1000 with a lockspray frequency of 0.40 s, and data averaging over 10 scans. Order effects in the statistical analysis were avoided due to the randomized crossover design used. In addition, the repeatability of the present method was evaluated using a mixture of 10 urine samples (quality control, QC) injected interval of 12 samples. The overlapped performance of each spectral peak was evaluated, and then six single ions with different m/z were randomly selected in accordance with the six equal portions in the retention time. The reproducibility of the QC sample was examined by analyzing the differences in the retention time and peak intensity of the six ions.

2.5. Data analysis

Statistical analysis was performed using SPSS (version 13.01S; Beijing Stats Data Mining Co. Ltd., Beijing, China). Data were presented as mean ± SD. Differences between groups were analyzed using the independent samples t-test. All P values were 2-tailed and a P value < 0.05 was considered significant.

The UPLC/Q-TOF MS data were analyzed using the MarkerLynx Application Manager 4.1 SCN 714 (Waters Corporation, Milford, MA, USA). Mass window was set at 0.02 Da, noise elimination level at 10.00, RT tolerance at 0.01 min and RT window at 0.2 min. The resulting 3D matrix that contained arbitrarily assigned peak indexes (retention time-m/z pairs), sample names (observations), and normalized ion intensities for each peak area, was exported to EZINFO 2.0 (an component of MarkerLynx) for multivariate statistical analysis using partial least-squares (PLS), which were used to visualize the score plot and obtain the principal metabolites. The goodness of the fit was quantified by R2Y, while the predictive ability was indicated by Q2Y. Models with Q2Y greater than or equal to 0.5 were considered to have good predictive

Table 1
Mean levels (±SD) of study variables in normal-weight and obese young men with hyperlipemia.

	Normal weight (n = 30)	Obesity (n = 30)
Age (years)	21.4 ± 2.0	20.8 ± 1.8
Height (cm)	173.5 ± 6.0	177.7 ± 5.3
Weight (kg)	62.2 ± 6.8	101.1 ± 12.1**
BMI (kg/m^2)	20.6 ± 1.5	32.0 ± 3.8**
Fat mass (kg)	10.6 ± 3.1	30.9 ± 5.4**
SBP (mmHg)	116.0 ± 11.0	127.8 ± 15.7*
DBP (mmHg)	72.6 ± 9.3	80.2 ± 17.1
TG (mmol/L)	0.9 ± 0.3	2.2 ± 0.7**
TC (mmol/L)	3.6 ± 0.5	4.6 ± 0.9*
FPG (mmol/L)	5.37 ± 0.54	5.67 ± 0.49
Insulin (uU/mL)	5.4 ± 1.9	13.8 ± 5.5**
Testosterone (mml/L)	22.6 ± 5.0	18.9 ± 6.7*

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglycerides.
* P < 0.05: different from that in normal weight group.
** P < 0.01: different from that in normal weight group.

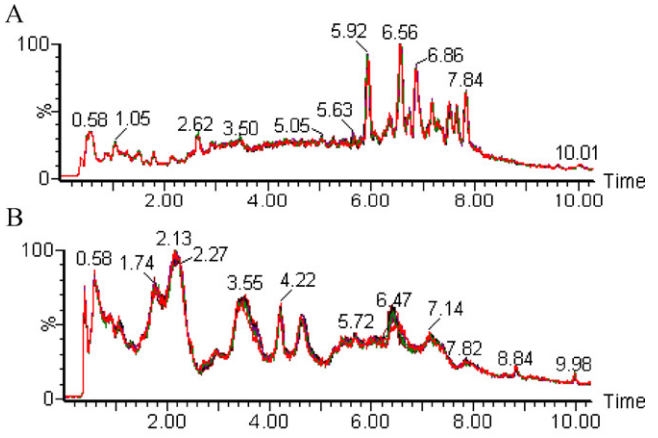

Fig. 1. The overlaps of the spectral peak of quality control samples injected 6 times in positive (A) and negative (B) ESI mode.

capability [17]. The metabolites with the greatest variable importance in projection (VIP) values [18] (VIP > 1.0) in the model were regarded as potential principal components. The principal metabolites were identified based on accurate mass, isotopic pattern, MS/MS information and comparison with the structure message of metabolites (with the same m/z as our principal metabolites) obtained from the Human Metabolome (www.hmdb.ca) or Chemspider (www.chemspider.com) databases, and the UPLC/MS/MS product ion spectrum of metabolites was matched with the structure message of metabolites using Mass Fragment software (Waters Corporation, Milford, USA) [19].

3. Results and discussion

3.1. Characteristics of subjects in the two groups

There were no significant differences between the two groups in age, height and FPG. Obese young men showed higher weight, body mass index, fat mass, systolic blood pressure, and triglyeride, total cholesterol and serum insulin levels, and lower testosterone levels than normal-weight adults (Table 1).

3.2. Quality control of the sample analysis

Quality control of the sample analysis was conducted by evaluating the repeatability of the present method using a mixture of 10 urine samples (quality control, QC) injected interval of 12 samples (n = 6). The overlapped performance of the spectral peak was evaluated (Fig. 1). The relative standard deviation (RSD) of retention time ranged from 0.00 to 0.97 for the positive ESI mode, and from 0.14 to 0.52 for the negative ESI mode. The RSD of peak intensity ranged from 2.89 to 4.91 for the positive ESI mode, and from 2.82 to 7.34 for the negative ESI mode (Table 2). The results indicated that method had excellent repeatability.

3.3. UPLC/Q-TOF MS fingerprinting and multivariate analysis

All the urine samples collected in this study were analyzed by UPLC/MS with positive and negative ESI. Pattern recognition via PLS was performed on positive and negative ESI data. Furthermore, the parameters of PLS model including the values of R2Y and Q2Y (0.990, 0.665 in positive ESI mode and 0.976, 0.597 in negative ESI mode) were all more than 0.5, which indicates that these models were suitable for these recognition analysis. The PLS score plots revealed the clustering between the two groups in positive and negative ESI modes (Fig. 2).

3.4. Principal differences in metabolites between obese and normal weight men

Components with important roles in the separation were picked out according to the parameter variable importance in the projection. Analysis of these data using PLS revealed that obesity with hyperlipemia was associated with increases in several compounds in positive and negative ESI modes. The principal ions that were seen to increase in positive ESI mode were those for m/z 213.1267, 279.1715 and 316.2459, which eluted at 5.13, 7.69 and 7.92 min and were identified as L-prolyl-L-proline, leucyl-phenylalanine and

Table 2
Reproducibility of method from six ions of the quality control sample in the positive and negative ESI modes (n = 6).

	m/z	RT (min)			INT		
		RT (min)	SD	RSD (%)	INT (10^2)	SD (10^2)	RSD (%)
ESI+	86.0717	0.57	0.005	0.97	2.05	0.06	2.89
	232.1456	1.80	0.000	0.00	70.96	2.47	3.48
	623.2509	2.70	0.007	0.26	1.11	0.05	4.81
	369.2351	5.26	0.004	0.09	9.47	0.46	4.91
	182.1075	6.56	0.000	0.00	3.77	0.15	3.99
	328.2437	8.09	0.004	0.06	21.57	0.79	3.66
ESI−	194.0461	0.86	0.004	0.52	8.66	0.24	2.82
	203.0836	2.48	0.004	0.18	9.21	0.28	3.07
	329.0734	5.41	0.018	0.34	4.86	0.19	3.88
	467.1938	6.37	0.009	0.14	3.46	0.25	7.34
	305.1889	7.26	0.019	0.27	1.71	9.34	5.45
	223.1455	8.62	0.013	0.15	0.86	0.04	4.20

RT: retention time; INT: intensity; ESI+ or ESI−: electrospray ionization in positive or negative; SD: standard deviation; RSD: relative standard deviation.

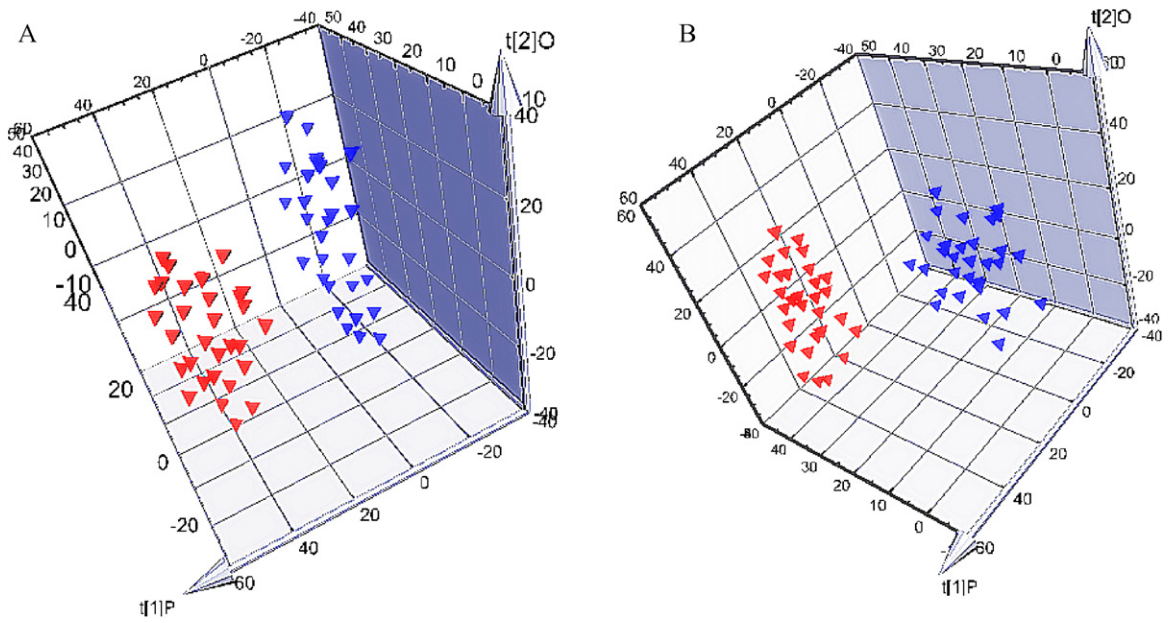

Fig. 2. Score plot with PLS of urine metabolite in obese (diamond, red) and normal-weight (diamond, blue) young men in positive (A) and negative (B) ESI mode. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

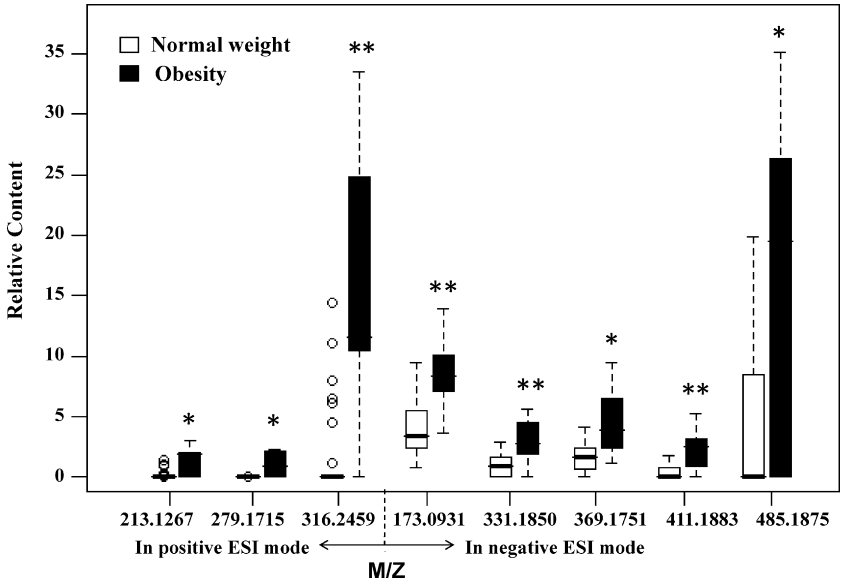

Fig. 3. Trending plot of eight markers (relative contents) in positive and negative ESI mode, and the differences seen in samples from obese young men and normal-weight men. $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 3
Principal metabolites in UPLC/Q-TOF MS positive and negative ion modes.

Retention time (min)	Measured mass (Da)	Calculated mass (Da)	Mass error (PPM)	Elemental composition	Postulated identity	VIP values	*P*-values
Positive ion mode							
5.13	213.1267	213.1239	13	$C_{10}H_{16}N_2O_3$	L-prolyl-L-proline	1.59	0.028
7.69	279.1715	279.1709	2	$C_{15}H_{22}N_2O_3$	Leucyl-phenylalanine	1.54	0.021
7.92	316.2459	316.2488	9	$C_{17}H_{33}NO_4$	Decanoylcarnitine	2.86	0.008
Negative ion mode							
0.34	173.0931	173.0926	2	$C_7H_{14}N_2O_3$	N-acetylornithine	2.72	0.003
4.44	411.1883	411.1841	10	$C_{21}H_{32}O_6S$	17-hydroxypregnenolone sulfate	3.14	0.009
5.11	331.185	331.1909	17	$C_{20}H_{28}O_4$	11β-hydroxyprogesterone	2.95	0.006
6.06	369.1751	369.1736	4	$C_{19}H_{30}O_5S$	5a-dihydrotestosterone sulfate	2.78	0.013
7.64	485.1875	485.1983	22	$C_{18}H_{34}N_2O_{13}$	Glucosylgalactosyl hydroxylysine	2.50	0.026

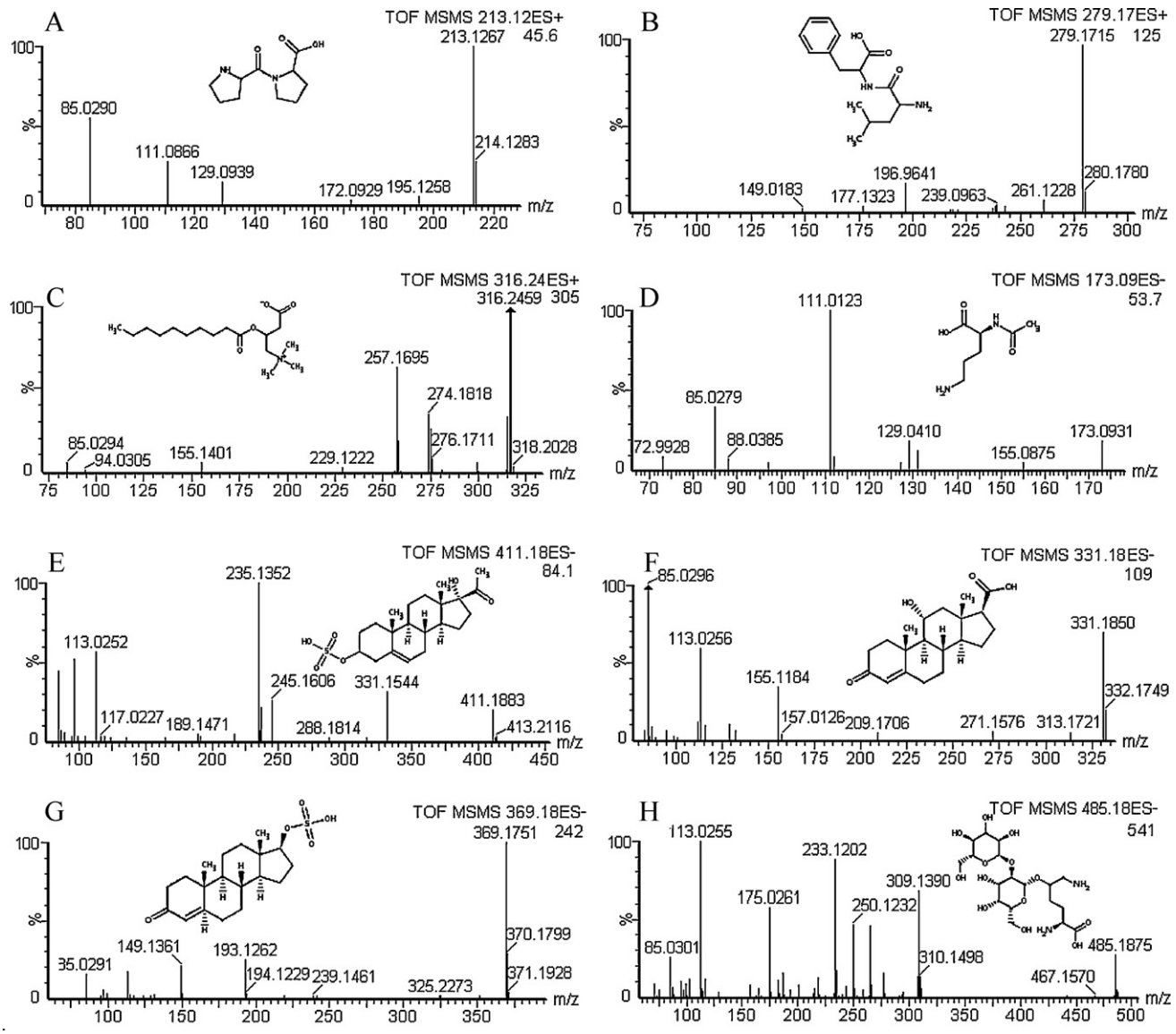

Fig. 4. Chemical structure and mass fragment information of principal metabolites with different collision energy (ev). (A) L-prolyl-L-proline (10 ev); (B) leucyl-phenylalanine (10 ev); (C) decanoylcarnitine (10 ev); (D) N-acetylornithine (15 ev); (E) 17-hydroxypregnenolone sulfate (3 ev); (F) 11β-hydroxyprogesterone (23 ev); (G) 5a-dihydrotestosterone sulfate (23 ev); and (H) glucosylgalactosyl hydroxylysine (15 ev).

decanoylcarnitine, respectively (Table 3 and Figs. 3 and 4). The principal ions that were seen to increase in negative ESI mode were those for *m*/*z* 173.0931, 411.1883, 331.185, 369.1751 and 485.1875, which eluted at 0.34, 4.44, 5.11, 6.06 and 7.64 min and were identified as N-acetylornithine, 17-hydroxypregnenolone sulfate, 11β-hydroxyprogesterone, 5a-dihydrotestosterone sulfate and glucosylgalactosyl hydroxylysine, respectively (Table 3 and Figs. 3 and 4).

Biological functions and metabolic pathways of these metabolites were investigated using databases such as HMDB and Chemspider. 17-Hydroxypregnenolone sulfate, in addition to being secreted by the adrenal glands, is also formed by peripheral sulfoconjugation of 17-hydroxypregnenolone [20], and hydroxypregnenolone can be metabolized to testosterone [21]. In addition, 5a-dihydrotestosterone sulfate, another principal metabolite, is a sulfate derivative of 5a-dihydrotestosterone produced via phase II metabolism. 5a-dihydrotestosterone (DHT) is a steroid of the androgen class, and about 70% of DHT is derived from peripheral conversion of testosterone in men [22]. Therefore, the metabolic disruptions in the levels of 17-hydroxypregnenolone sulfate (decrease the synthesis of testosterone) and 5a-dihydrotestosterone sulfate (increase the conversion of testosterone) might explain the reduced testosterone levels seen in young obese men in this study and previously in obese men [23]. 11β-Hydroxyprogesterone could stimulate Na+ absorption via its effects as a mineralocorticoid agonist [24], and also may have an important role in corticoid synthesis [25]. Both of these biochemical functions could increase blood pressure [26]. Increased levels of 11β-hydroxyprogesterone might be one reason for the higher blood pressure observed in obese young men compared with normal-weight men in the present study. The mechanism by which levels of glucosylgalactosyl hydroxylysine (GGH) are increased in obese young men is likely associated with the hydroxylation and glycosylation of collagen protein structural domain in adiponectin, which in turn could lead to the increased GGH level in urine [27]. This might also explain the reduced plasma level of adiponectin in obese versus normal-weight individuals; adiponectin present in plasma is inversely associated with blood lipid level and insulin resistance [28]. These factors might lead to the increases in insulin and blood lipid levels in the obese young men in the present study.

It is well known that obesity often accompanies the occurrence of hyperlipemia in young adults, and long-term obesity with hyperlipemia will lead to insulin resistance and, often, obesity-related chronic diseases. However, the most important metabolic changes in young obese individuals with hyperlipemia that could affect the occurrence and development of obesity-related chronic diseases remain to be determined. Metabolomics technology can achieve high-throughput and rapid detection of metabolites and analysis of large datasets, and could be used to identify, among tens of thousands of metabolites, those with greatest phenotypic contribution. Through investigating the metabolic pathways of the principal metabolites, the most important metabolic changes and the relations between these changes with obesity-related chronic diseases could be found.

To our knowledge, this is the first report to evaluate the metabolic changes in young obese men with hyperlipemia compared with normal-weight men using UPLC/Q-TOF MS. A study by Kim et al. [29] investigated the metabolic profiles, in plasma, of overweight/obese and lean men; the results demonstrated the abnormal metabolism of two branched-chain amino acids, two aromatic amino acids, and fatty acid synthesis and oxidation in overweight/obese men. Such changes have not been identified in the present study. There are several possible reasons for this difference. First, the two studies included different sets of subjects; in the present study, the subjects were young men aged 18–26 years, whereas the previous study included men aged 30–50 years. The metabolic changes reported by Kim might not occur in the young men included in the present study. Second, the biofluid used in the two studies was different; in the present study, the biofluid is urine, which has different components to plasma, which was used in Kim's study. Third, a large number of principal metabolites were detected in this study, but most remain unidentified at present; the unidentified metabolites and their metabolic pathways might include the metabolic changes found in the previous study. This is also a possible limitation of this study; although a large number of principal metabolites were detected by UPLC/Q-TOF MS, most remain unidentified at present. Unlike GC–MS or NMR, for which large databases exist, the use of LC–MS-based techniques for metabolomics research is still in the initial stage and databases of endogenous biomolecules have not yet been constructed; this limits identification and analysis of metabolic pathway to the principal metabolites.

4. Conclusions

The present study used a UPLC/Q-TOF MS-based metabolomics strategy and multivariate data analysis to show that young obese men with hyperlipidemia-associated changes in metabolites, including changes in levels of L-prolyl-L-proline, leucyl-phenylalanine, decanoylcarnitine, N-acetylornithine, 17-hydroxypregnenolone sulfate, 11β-hydroxyprogesterone, 5a-dihydrotestosterone sulfate and glucosylgalactosyl hydroxylysine. The results indicate that the metabolomics technique based on UPLC/Q-TOF MS provides an effective approach for studying organic metabolic changes. These metabolites changes are indicative of early changes in metabolism, including changes in testosterone, insulin resistance, blood pressure, and hyperlipemia, in young obese men, which may aid the early treatment of obesity and related chronic diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.08.014.

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