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^{1}H NMR-based metabolite profiling of diet-induced obesity in a mouse mode

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High-fat diets (HFD) and high-carbohydrate diets (HCD)-induced obesity through different pathways, but the metabolic differences between these diets are not fully understood. Therefore, we applied proton nuclear magnetic resonance (^{1}H NMR)-based metabolomics to compare the metabolic patterns between C57BL/6 mice fed HCD and those fed HFD. Principal component analysis derived from ^{1}H NMR spectra of urine showed a clear separation between the HCD and HFD groups. Based on the changes in urinary metabolites, the slow rate of weight gain in mice fed the HCD related to activation of the tricarboxylic acid cycle (resulting in increased levels of citrate and succinate in HCD mice), while the HFD affected nicotinamide metabolism (increased levels of 1-methylnicotineamide, nicotinamide-*N*-oxide in HFD mice), which leads to systemic oxidative stress. In addition, perturbation of gut microflora metabolism was also related to different metabolic patterns of those two diets. These findings demonstrate that ^{1}H NMR-based metabolomics can identify diet-dependent perturbations in biological pathways. [BMB Reports 2012; 45(7): 419-424]

INTRODUCTION

With the improving economic situation of developing countries, there has been a significant change in diet, physical activity, and overall body composition in the past half century (1). In particular, the diets of Asian countries are shifting away from high-carbohydrate foods toward high-fat, high-energy foods (1, 2), creating a chronic energy imbalance that leads to persistent weight gain in the form of body fat and a variety of metabolic abnormalities.

Low-fat diets and high-carbohydrate diets (HCD) have been recommended to obese populations and patients with non-insulin-dependent diabetes mellitus (3, 4). HCD are less energy dense, more rapidly oxidized, and less readily stored than HFD (5-7). Therefore, HCD promote weight loss in obese individuals and improve glycemic control and plasma triglyceride and high-density lipoprotein cholesterol levels in patients with non-insulin-dependent diabetes mellitus (8). However, several studies have recently reported that HCD consisting of large amounts of simple carbohydrates, high-sugar, and corn syrup could lead to obesity and metabolic syndrome (9-11). Thus, the influence of diet on the human body is complicated and not simply understood.

Endogenous metabolites are important factors in metabolic processes that respond to environmental, pathogenic, and dietary changes (12, 13). Therefore, metabolomics, the multi-targeted analysis of endogenous metabolites from biological samples, is commonly used as a noninvasive method to supervise pathophysiological processes and diet-induced perturbations (14-16). Many studies have reported that metabolomics can easily elucidate the biological pathway responsible for high fat-induced obesity and its complications, such as diabetes, atherosclerosis, dyslipidemia, and liver inflammation (17-20). However, no metabolomics studies regarding HCD and the difference between HCD and HFD have been reported.

In present study, we applied a proton nuclear magnetic resonance (^{1}H NMR) spectroscopy-based metabolite profiling approach to evaluate the metabolic perturbations related to diet-induced obesity and investigate the effects of HCD and HFD on metabolism. We also demonstrate how the metabolic effects of HCD and HFD relate to the mechanism of diet-induced obesity and its associated complications.

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RESULTS

Body and fat weight

Mice fed the HFD had significantly increased weight following three weeks of feeding, and an increased ratio of body weight (weight gain to weight at week 0) approximately 50% higher than mice fed the HCD after twelve weeks (Fig. 1A and B). However, there was no difference in food intake between the two groups (data not shown). In addition, the weight of epididymal white adipose tissue (EWAT) and the EWAT to body weight ratio (relative EWAT weight) was higher in mice fed the HFD compared to mice fed the HCD (Fig. 1C and D), indicating that the HFD induced obesity more rapidly than did the HCD.

^{1}H NMR spectroscopy and pattern recognition analysis

Fig. 2 shows representative 600 MHz 1D ^{1}H NMR spectra for urine samples from mice fed HCD and HFD (Fig. 2A and B). These spectra were used to identify numerous endogenous metabolites, including 1-methylnicotinamide (MNA), niacinamide, trigonelline, nicotinamide *N*-oxide (*N*-OX), formate, adenosine, phenylacetylglycine, pantothenate, hippurate, benzoate, 3-indoxylsulate, tyrosine, 4-hydroxyphenylacetate, 4-hydroxybenzoate, trans-aconitate, urea, tartrate, sucrose, fructose, glucarate, glycolate, glucose, fucose, glycerol, creatine, trimethylamine *N*-oxide (TMAO), *N*-carbamoyl-β-alanine, taurine, trimethylamine (TMA), N,N-dimethyl glycine (DMG), dimethylamine (DMA), citrate, carnitine, succinate, *N*-acetylglycine, acetate, *N*-isovaleroylglycine, alanine, lactate, isopropanol, methylsuccinate, isobutyrate, valine, and 2-hydroxyvalerate.

The spectral data from the urine of mice fed an HCD or HFD indicate that diet significantly influences metabolite alteration. The dominant metabolites in urine from the HCD mice included tyrosine, 4-hydroxyphenylacetate, 4-hydroxybenzoate, trans-aconitate, tartrate, sucrose, fructose, citrate, succinate, alanine, lactate, and isopropanol. In the mice fed an HFD, 1-methylnicotinamide, trigonelline, nicotinamide *N*-oxide, adenosine, benzoate, glucarate, glycolate, glucose, fucose, creatine, *N*-carbamoyl-β-alanine, and methylsuccinate were dominant. These metabolites include surrogate biomarkers for differentiating between HCD- and HFD-fed mice.

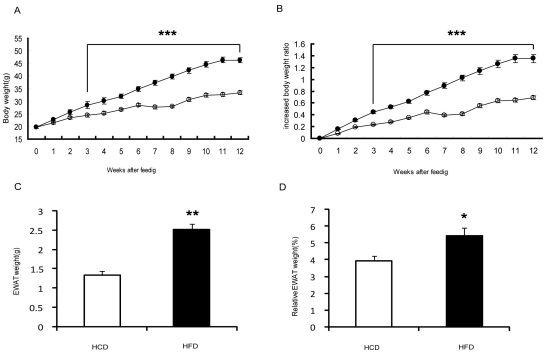

Fig. 1. Body weight (A) and increased body weight ratio (B) of mice fed a high-carbohydrate diet (HCD; open circles) or a high-fat diet (HFD; closed circles). Weight of epididymal white adipose tissue (EWAT, C) and the weight of EWAT to body weight ratio (relative EWAT weight, D). The data are presented as means ± SE (*P < 0.05; **P < 0.01; ***P < 0.001).

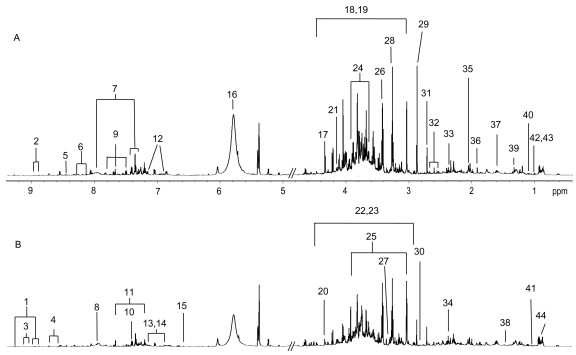

Fig. 2. Representative 600 MHz proton nuclear magnetic resonance spectra obtained from urine of (A) high-carbohydrate diet (HCD)- and (B) high-fat diet (HFD)-fed mice. Key for spectra: 1, 1-methylnicotinamide (MNA); 2, niacinamide; 3, trigonelline; 4, nicotinamide *N*-oxide (N-OX); 5, formate; 6, adenosine; 7, phenylacetylglycine; 8, pantothenate; 9, hippurate; 10, benzoate; 11, 3-indoxylsulate; 12, tyrosine; 13, 4-hydroxyphenylacetate; 14, 4-hydroxybenzoate; 15, trans-aconitate; 16, urea; 17, tartrate; 18, sucrose; 19, fructose; 20, glucarate; 21, glycolate; 22, glucose; 23, fucose; 24, glycerol; 25, creatine; 26, trimethylamine *N*-oxide (TMAO); 27, *N*-carbamoyl-β-alanine; 28, taurine; 29, trimethylamine (TMA); 30, *N,N*-dimethyl glycine (DMG); 31, dimethylamine (DMA); 32, citrate; 33, carnitine; 34, succinate; 35, N-acetylglycine; 36, acetate; 37, N-isovaleroylglycine; 38, alanine; 39, lactate; 40, isopropanol; 41, methylsuccinate; 42, isobutyrate; 43, valine; 44, 2-hydroxyvalerate.

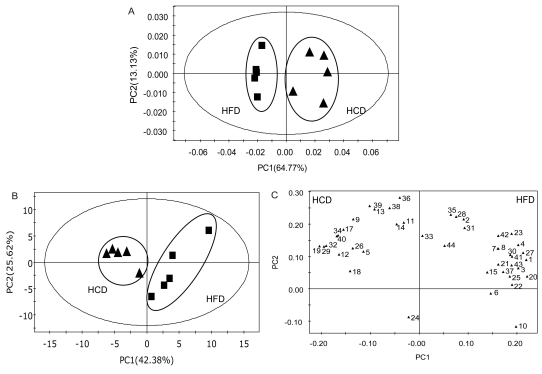

Fig. 3. Principal component analysis (PCA) score plot of urinary proton nuclear magnetic resonance spectra (A) obtained from mice fed an HCD (triangles) or an HFD (squares), and PCA scores (B) and loading (C) plots of urinary metabolite concentrations obtained through targeted profiling of mice fed an HCD (triangles) or an HFD (squares). See Fig. 2 for the metabolite key for the loading plot.

Principal component analysis (PCA) was used to investigate the differences in metabolite levels of mice fed HCD and HFD (Fig. 3A). PCA score plots derived from the NMR spectra show clear separation between the HCD and HFD groups along PC1 (R^2X = 0.779, Q^2 = 0.569).

Targeted metabolic profiling

Endogenous metabolites were identified from the ^1H NMR spectra using the Chenomx Library (Chenomx Inc., Edmonton, Alberta) and then quantified. A total of 44 metabolites that varied between the HCD- and HFD-fed mice are listed in Table 1 with their urinary concentrations. PCA score plots of the urinary metabolite concentrations obtained from the mice fed an HCD or an HFD showed diet-dependent separation along PC1 (R^2X = 0.68, Q^2 = 0.387; Fig. 3B).

A loading plot was generated to identify the metabolites re-

Table 1. Proton chemical shift and the concentration of urinary metabolites observed from mice fed a high-fat diet (HFD) -and a high-carbohydrate diet (HCD)

Metabolite	Chemical shift (ppm)	HFD (n = 5)	HCD (n = 5)	P value
		mean ± SD (μmol/L)	mean ± SD (μmol/L)	
N-Isovaleroylglycine	0.73, 1.99, 2.16, 3.75	434.94 ± 103.99	348.02 ± 49.45	0.222
Pantothenate	0.88, 0.92, 2.46, 3.43, 3.51, 3.98, 8.00	387.54 ± 69.96	321.44 ± 34.48	0.151
2-Hydroxyvalerate	0.90, 1.33, 1.37, 1.60, 1.67, 4.03	328.14 ± 154.58	373.30 ± 81.91	0.841
Valine	1.00, 1.04, 2.26, 3.60	64.76 ± 19.66	39.82 ± 6.73	0.016
Isobutyrate	1.05, 2.37	28.56 ± 11.26	21.54 ± 6.25	0.222
Methylsuccinate	1.09, 2.12, 2.51, 2.61	190.00 ± 42.05	130.00 ± 28.12	0.016
Isopropanol	1.16, 4.01	75.4 ± 10.50	119.6 ± 25.24	0.008
Fucose	1.20, 1.24, 3.44, 3.63, 3.74, 3.85, 4.18	670.92 ± 164.51	549.86 ± 29.73	0.222
Lactate	1.32, 4.11	435.2 ± 93.59	544.82 ± 76.82	0.032
Alanine	1.47, 3.78	143.16 ± 39.15	176.92 ± 38.04	0.310
Acetate	1.91	285.32 ± 105.91	373.04 ± 45.30	0.095
N-Acetylglycine	2.02, 3.74, 7.98	643.18 ± 156.26	645.86 ± 95.14	1.000
N-Carbamoyl-β-alanine	2.37, 3.30	1,931.02 ± 927.94	761.24 ± 114.23	0.008
Succinate	2.40	185.72 ± 46.69	496.46 ± 176.53	0.032
Carnitine	2.43, 3.21	231.28 ± 46.24	229.78 ± 69.30	1.000
Citrate	2.54, 2.69	292.44 ± 128.01	3,667.54 ± 1,783.15	0.008
DMA	2.71	773.74 ± 183.52	801.62 ± 117.86	0.548
TMA	2.87	747.98 ± 303.38	5,641.12 ± 1,436.91	0.008
DMG	2.91, 3.71	97.22 ± 26.74	65.56 ± 14.49	0.032
Creatine	3.02, 3.92	2,126.16 ± 1,233.52	499.32 ± 403.66	0.016
Tyrosine	3.03, 3.19, 3.94, 6.89, 7.15	231.30 ± 53.02	323.68 ± 100.63	0.222
Glucose	3.24, 3.41 3.46, 3.49, 3.53, 3.71, 3.76, 3.84, 3.89, 4.64, 5.23	3,320.78 ± 836.56	1,910.16 ± 388.21	0.016
TMAO	3.25	324.56 ± 138.92	682.98 ± 105.56	0.008
Tartrate	3.26, 3.42	10,027.02 ± 3,503.98	15,848.70 ± 3,057.53	0.032
Taurine	3.26, 3.42	16,334.24 ± 9,136.64	15,933.58 ± 5,959.59	0.841
Trans-aconitate	3.43, 6.58	134.46 ± 57.39	102.70 ± 34.93	0.421
4-Hydroxyphenylacetate	3.44, 6.85, 7.15	276.94 ± 142.70	470.58 ± 104.05	0.056
Sucrose	3.47, 3.55, 3.68, 3.80, 3.82, 3.88, 4.04, 4.21	1,911.94 ± 870.06	4,365.12 ± 2,047.24	0.032
Fructose	3.55 3.59, 3.67, 3.70 3.79, 3.82, 3.89, 3.99, 4.01	958.34 ± 286.58	14,499.26 ± 4,434.03	0.008
Glycerol	3.56, 3.64, 3.78	1,721.76 ± 119.07	1,734.62 ± 393.36	0.691
Phenylacetylglycine	3.66, 3.75, 7.34, 7.35, 7.41, 7.97	2,884.02 ± 735.97	2,390.78 ± 93.64	0.421
Adenosine	3.83, 3.91, 4.29, 4.42, 4.79, 6.06, 8.24, 8.33	165.70 ± 65.55	93.64 ± 27.94	0.056
Glycolate	3.94	891.48 ± 271.03	668.88 ± 262.83	0.095
Glucarate	3.94, 4.08, 4.12, 4.15	1,466.34 ± 410.11	853.98 ± 276.08	0.032
Hippurate	3.95, 7.54, 7.63, 7.82, 8.52	251.08 ± 120.87	474.36 ± 82.16	0.016
Trigonelline	4.43, 8 .07, 8.82, 9.15	29.72 ± 13.47	12.72 ± 5.72	0.032
MNA	4.47, 8.17, 8.88, 8.96, 9.27	645.74 ± 271.08	249.68 ± 31.28	0.008
4-Hydroxybenzoate	6.89, 7.75	86.30 ± 30.86	118.34 ± 22.04	0.151
3-Indoxylsulfate	7.20, 7.26, 7.35, 7.50, 7.69	1,464.66 ± 438.81	1,894.78 ± 408.08	0.151
Niacinamide	7.43, 7.58, 8.23, 8.25, 8.70, 8.93	116.54 ± 50.75	108.14 ± 3.55	1.000
Benzoate	7.46, 7.86	202.66 ± 28.61	131.28 ± 36.42	0.016
N-OX	7.62, 7.73, 8.11, 8.36, 8.48, 8.73	517.78 ± 311.13	229.20 ± 67.61	0.032
Formate	8.45	329.5 ± 41.78	436.98 ± 172.85	0.421

sponsible for the score plot differentiation (Fig. 3C). This plot showed increased levels of 1-methylnicotinamide (MNA), nicotinamide *N*-oxide (*N*-OX), creatine, glucose, benzoate, valine, trigonelline, *N*-carbamoyl-β-alanine, glucarate, methylsuccinate, adenosine, and *N,N*-dimethyl glycine (DMG) in the urine of mice fed HFD compared to HCD. In contrast, it showed increased levels of citrate, fructose, sucrose, succinate, isopropanol, formate, hippurate, tartrate, trimethylamine (TMA), and trimethylamine *N*-oxide (TMAO) in the urine of mice in the HCD group compared with those in the HFD group.

Although our PCA models clearly differentiated between mice fed an HCD and those fed an HFD, the models exhibited poor predictability ($Q^2 < 0.5$) to explain the differences between the groups (21). Therefore, we investigated the statistical significance of the variations in metabolite levels. Variations in the concentration of endogenous urinary metabolites according to diet type are shown in Table 1. Based on multivariate and univariate analyses, in mice fed an HFD, MNA, *N*-OX, creatine, glucose, benzoate, valine, trigonelline, *N*-carbamoyl-β-alanine, glucarate, methylsuccinate, and DMG showed statistically significant changes ($P < 0.05$). In mice fed an HCD, citrate, fructose, sucrose, succinate, isopropanol, hippurate, tartrate, TMA, and TMAO showed statistically significant changes ($P < 0.05$). Importantly, metabolites associated with the tricarboxylic acid (TCA) cycle, nicotinamide, and gut microflora pathways were significantly changed (Table 1).

DISCUSSION

To investigate the mechanisms underlying the diet-dependent changes in metabolic patterns in HCD- and HFD-fed mice, we compared the urinary metabolic profiles of mice fed isocaloric diets high in carbohydrates (70% of the total calories) and fat (60% of the total calories) using ^{1}H NMR-based metabolomics.

Carbohydrates are rapidly oxidized to maintain energy balance (22). However, fat is not used rapidly through fatty oxidation on an HFD, resulting in a positive fat balance (22-24). Thus, mice fed an HFD gained body weight and EWAT weight more rapidly than mice fed an HCD. In addition, the TCA cycle was activated in response to carbohydrates in mice fed an HCD, resulting in higher levels of TCA cycle intermediates, including citrate and succinate, compared to mice fed an HFD. Carnitine, which transports fatty acids from the cytosol to mitochondria during the breakdown of lipids (fats), was not significantly increased in mice fed an HFD compared to those fed an HCD (25).

Fatty oxidation, however, slowly increased in mice fed an HFD and required nicotinamide-adenine dinucleotide (NAD+) for normal mitochondrial fatty oxidation (26). Therefore, higher levels of *N*-OX, a precursor of NAD+ in animals that is catalyzed into NAD+ by xanthine oxidase in the liver (27), was observed in HFD-fed mice. In addition, the increased amount of adipose tissue in the HFD-fed mice became a significant source of nicotinamide N-methyltransferase (NNMT), resulting in increased levels of MNA through catalysis of the conversion of nicotinamide to MNA (28).

These two nicotinamide-associated metabolites are closely related to reactive oxygen species (ROS) production, causing complications in the obesity pathways (29). Erdei *et al*. (30) reported that HFD-induced obesity impaired the endothelium-dependent dilation of arterioles because of enhanced xanthine oxidase-derived superoxide production, which may lead to hypertension by disturbing blood flow in the tissues and increasing the development of peripheral resistance. In addition, increasing evidence suggests that systemic oxidative stress, characterized by the elevation of plasma ROS, is an important cause of insulin resistance in obesity (31). Zhou et al. (32) found that MNA elevated plasma H_2O_2 levels *in vivo* and directly stimulated H_2O_2 generation by human erythrocytes *in vitro* at physiological concentrations, which indicates that MNA is a potent trigger of diabetic oxidative stress. Moreover, chronic niacin overload, which leads to MNA-induced high levels of H_2O_2, is associated with the increased prevalence of childhood obesity (33).

Many metabolic studies have recently shown that gut microflora are closely associated with diet-induced obesity and that consumption of an HFD results in a decrease in total gut bacterial levels, leading to alterations in metabolites such as TMA and TMAO (34, 35). We also observed differences in TMA and TMAO levels between the two diet groups.

Fructose, a major cause of metabolic syndrome in HCD, was excreted at high levels in the urine of HCD-fed mice (9-11). Kanarek and Orthen-Gambill (36) evaluated the effects of sucrose, fructose, and glucose on carbohydrate-induced obesity and found that fructose and sucrose led to a decreased oral glucose load tolerance. Compared to sucrose or glucose, fructose significantly increased serum triglyceride levels.

This study demonstrated that ^{1}H NMR-based metabolomics can be used to identify metabolic differences between HCD- and HFD-fed mice and investigated diet-dependent changes in metabolic patterns. It also revealed that the weight gain in HCD-fed mice was related to enhancement of the TCA cycle and that HFD alters nicotinamide, which leads to systemic oxidative stress, and gut microflora metabolism. Therefore, global and targeted metabolic profiling via ^{1}H NMR-based metabolomics provides insight into the biological pathways involved in diet-dependent metabolic changes.

MATERIALS AND METHODS

Experimental animals

Twenty six-week-old male C57BL/6N mice purchased from Orient Bio (Seoul, South Korea) were fed a commercial chow diet for acclimation for one week. At seven weeks of age, the animals were divided into two groups of five mice each: (1) mice fed an HCD (70% carbohydrate, 10% fat, and 20% protein; D12450B: Research Diets Inc., New Brunswick, NJ, USA), and (2) mice fed an HFD (20% carbohydrate, 60% fat,

and 20% protein; D12492: Research Diets Inc.) for twelve weeks. The animals were maintained under standard light (12 h of light/12 h of darkness), temperature (24 ± 2°C), and humidity (60%) conditions and had free access to food and water. The body weight and diet uptake in each group were measured weekly at 09:00.

The procedures used to handle and care for the animals adhered to current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication 1985, revised 1996). This protocol was approved by the Institutional Animal Care and Use Committee (IACUS) of Seoul National University. All experiments were designed to minimize the number of animals used and the suffering caused.

^{1}H NMR spectroscopy of urine samples

The urine samples, which had been stored at −80°C, were thawed at room temperature and centrifuged at 13,000 rpm for 10 min prior to the NMR analysis. For the NMR analysis, 400 μl of urine supernatant was mixed with 200 μl of phosphate-buffered saline (0.2 M, 0.018% NaN_3) and adjusted to pH 7.0 ± 0.1. Aliquots of 540 μl of the supernatant and 60 μl of D_2O (containing 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate [DSS] as an internal chemical standard) were transferred to 5-mm NMR tubes.

^{1}H NMR spectra were collected on a VNMRS-600 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA) using a triple-resonance 5-mm HCN salt-tolerant cold probe. Water was suppressed by the standard one-dimensional (1D) Noesy-presat pulse sequence (recycle decay-90°-t1-90°-tm-90°-free induction decay acquisition). For each urine sample, the spectrum was collected using 64 transients into 67,568 data points using a spectral width of 8445.9 Hz, a relaxation delay of 2.0 s, and an acquisition time of 4.0 s.

NMR spectral data reduction and preprocessing

All NMR spectra were phased and baseline-corrected using Chenomx NMR Suite version 6.0 (Chenomx Inc., Edmonton, AB, Canada). The regions corresponding to residual water, urea, and DSS (4.7-5.05, 5.5-6, and 0.0-0.7 ppm, respectively) were excluded and the remaining spectral regions were divided into 0.005 ppm bins. The spectra were then normalized to the total spectral area and converted to ASCII format. The ASCII formatted files were imported into MATLAB (R2008a; Mathworks, Inc., 2008), and the spectra were aligned using the correlation optimized warping method (37). NMR spectral data analysis was accomplished using targeted profiling with Chenomx NMR Suite 6.0, and the concentrations were determined using the 600 MHz library from Chenomx NMR Suite 6.0, which compares the integral of a known reference signal (DSS-d6) with signals derived from a library of compounds containing chemical shifts and peak multiplicities.

Multivariate and univariate statistical analyses

The resulting data sets were imported into SIMCA-P version 12.0(Umetrics AB, Umeå, Sweden) for multivariate analysis. All imported data were mean-centered. PCA, an unsupervised pattern recognition method, was performed to examine the intrinsic variation and to explain the "clustering" and trends within the multivariate data set. The quality of each model was determined using the goodness of fit parameter (R^2) and the goodness of prediction parameter (Q^2) (21).

The Mann-Whitney test was applied to the concentrations of urinary metabolites using Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

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