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Correlation analysis of human urinary metabolites related to gender and obesity using NMR-based metabolic profiling

Jahan Kim[1], Jungdae Park[1], Sung-Soo Park[2], Geum-Sook Hwang[1, 3*]

[1] *Integrated Metabolomics Research Group, Korea basic science institute,,Seoul 136-701, Korea*
[2] *Department of Surgery, Korea University College of Medicine, Seoul 136-705, Korea*
[3] *Graduated School of Analytical Science and Technology, Chungnam University, Daejeon, 305-764, Korea*
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Abstract : Metabolomic studies using human urine have shown that human metabolism is altered by a variety of environmental, cultural, and physiological factors. Comprehensive information about normal human metabolite profiles is necessary for accurate clinical diagnosis of disease and for disease prevention and treatment. In this study, metabolite correlation analyses, using ^{1}H nuclear magnetic resonance (NMR) spectroscopy coupled with multivariate statistics, were performed on human urine to compare metabolic differences based on gender and/or obesity in healthy human subjects. First, we applied partial least squares discriminant analysis to the NMR spectral data set to verify the data's ability to discriminate by gender and obesity. Then, the differences in metabolite–metabolite correlation between male and female, and between normal and high body mass index (obese) subjects were investigated through pairwise correlations. Creatine and several metabolites, including isoleucine, trans-aconitate, and trimethylamine N-oxide (TMAO), exhibited different quantitative relationships depending on gender. Dimethylamine had a different correlation with glycine and TMAO, based on gender. The correlation of TMAO with amino acids was considerably lower in obese, compared to normal, subjects. We expect that the results will shed light on the metabolic pathways of healthy humans and will assist in the accurate diagnosis of human disease.

Keywords: Metabolomics; ^{1}H NMR; Multivariate analysis; Human urine

Principal Researcher Integrated Metabolomics Research Group Korea Basic Science Institute of Korea University, Seoul 136-713, Korea
Tel: +82.2.920.0737 Fax: +82.2.920.0709 email: gshwang@kbsi.re.kr
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INTRODUCTION

Metabolomics provide system-wide information about clinical and biomedical subjects, such as toxicity assessment, identification of biomarkers, and biochemical pathways associated with specific metabolites. [1–3] A quantitative description of endogenous, low molecular mass metabolites present in biological samples such as urine and plasma can be investigated through metabolic profiling.[4] Advances in systems biology will allow metabolites to be linked to their respective metabolic pathways.[5]

Two major techniques, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy have been used for the analysis of metabolomic data. Among the analytical techniques used, ^{1}H-NMR spectroscopy has been one of the major metabolite profiling tools, as it enables many endogenous metabolites to be quantified rapidly and reproducibly.[1–3,6,7,8,9] NMR-based metabolic fingerprinting provides a uniform detection system for identifying valuable metabolites directly from biofluids.[10]

The human body contains a complex array of interacting metabolic pathways. In a healthy individual, these complex chemical reactions are maintained in balance, or otherwise, the slightest metabolic deviation can generate devastating results.[11-13] Therefore, understanding the normal physiology of humans is essential for accurate diagnosis of disease.[5] Most human metabolomic studies have found that spectral results are strongly affected by inter- and intraindividual variations.[14]

Many studies investigating the influence of physiological variation in the metabolic pattern of healthy human subjects have been reported.[4,5,11,12] Some metabolomic studies have attempted to reveal the effect of cultural characteristics on metabolite profiles of urine and plasma from healthy humans in different countries.[4,15] Metabolites related to gender, age, and body mass index (BMI) have been identified by NMR spectroscopy in studies of normal humans.[5,16] As shown in these studies, the endogenous urinary metabolite profiles demonstrate considerable intersubject variability.[17] In humans, cultural and dietary diversities, as well as geographical characteristics, influence the urinary metabolite profile.[4, 14, 16–18]

The metabolites creatinine, lactate, alanine, citrate, dimethylamine (DMA), glycine, hippurate, and trimethylamine-N-oxide (TMAO) are commonly detected in 1H NMR profiles of urine from healthy humans.[19,20] One problem faced in clinical investigations is the greater variability of human biofluid samples compared with experimental animal samples.[17] Many studies on metabolite profiles in normal human subjects have been published, but no reports exist on the correlations between metabolites detected in healthy human subjects. Correlation analysis of metabolite data derived from NMR can provide meaningful information about the relationship between biomarkers and their corresponding metabolic pathway(s).

In this report, we demonstrate the interindividual variation in the metabolic urinary profile of a healthy population in Korea using NMR spectroscopy coupled with chemometric analysis, and investigate the correlations among metabolites associated with gender and obesity.

EXPERIMENTAL

Subjects

We recruited 78 healthy volunteers from the Korea University Hospital in Korea. Urine samples were collected from the subjects and immediately stored at –80°C until analysis. The study protocol was approved by the institutional review board of Korea University Hospital. The participants were required to complete a form disclosing personal information, including age, height, weight, disease history, smoking habits, and liquid consumption.

Preparation of urine samples

All frozen urine samples were thawed at 21 ± 2°C and centrifuged at 12,000 × g for 10 min at 4°C to remove any precipitates. Prior to NMR analysis, 400 μl aliquots of the urine samples were mixed with 230 μl of phosphate buffer (0.2 M NaH_2PO_4/Na_2HPO_4, pH 6.8) to minimize variations in pH, and then were added to 70 μl of an internal standard solution consisting of 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and 0.2% sodium azide (to prevent bacterial growth) in 99% D_2O. The final pH adjustment was carried out with a HCl or KOH solution and a pH meter. A 600 μl aliquot of the prepared urine sample was then transferred to a 5-mm NMR tube (Norell,

Landisville, NJ, USA). D_2O provided a field frequency lock and DSS provided a chemical shift reference (^1H, δ 0.00).

^1H NMR spectroscopy

^1H NMR spectra of the prepared urine samples were acquired at 298 K using a VNMRS-600MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a triple-resonance 5-mm HCN salt-tolerant cold probe. A NOESY-presat pulse sequence was applied to suppress the residual water signal. For each sample, 64 transients were collected into 67 K data points using a spectral width of 8445.9 Hz and a relaxation delay of 2 s, an acquisition time of 4 s, and a mixing time of 100 ms. All spectra were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz.

NMR data processing

All NMR spectra were manually baseline-corrected and phased. The total area from 0.7 to 9.4 ppm, excluding the DSS (0.0–0.7 ppm), water (4.684–5.161 ppm), urea (5.495–6.500 ppm), and imidazole regions (8.220–8.490, 7.294–7.393ppm), was reduced into 6948 variables with spectra binning of 0.001 ppm. Individual integral regions were normalized to the total area. The spectra binning data were imported into MATLAB 2008a (ver 7.6; Mathworks Inc.). NMR peak alignments

were performed by a MATLAB script using correlation-optimized warping (COW) algorithms. Analysis of the NMR spectral data was accomplished with targeted profiling through the use of Chenomx NMRSuite 5.1 (Chenomx, Edmonton, AB, Canada), and metabolite concentrations were determined using the 600-MHz library from Chenomx NMR Suite 4.6, 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 statistical analysis of urine metabolites

For multivariate analysis of multidimensional data, partial least squares discriminant analysis (PLS-DA) was applied to the NMR spectral binning data using SIMCA-P+12 (ver. 11.0; Umetrics, Kinnelon, NJ, USA). The binning data were transformed using Pareto scaling as a preprocessing step prior to the multivariate analysis. A supervised approach was used to maximize the separation of samples based on group and to suggest a predictive model for class membership. This was done by applying PLS-DA to the NMR binning data. PLS-DA was used to derive components (latent variables) to maximize the covariance between X (measured NMR data) and Y (response variable). Variable importance in projection (VIP) was calculated to measure the importance of X (variable in NMR data) for X and Y (its correlation to the response) in the model as a variable selection method. A value of VIP larger than 1 indicates a statistically more plausible variable for explaining the correlation Y.[21]

To compare differences in metabolite concentrations between groups, we examined the normality of metabolites by applying the Shapiro–Wilk test to log-transformed (base 10) variables from the raw concentration data. An unpaired t-test was performed under the assumption of equal variance and normality. Variables that exhibited no equal variance and non-normality were analyzed by the nonparametric two-tailed Mann–Whitney test.

Correlation analysis of pairwise metabolites

Pearson and Spearman correlation analyses were performed to investigate the correlations among metabolites detected in human urine. For the correlation analysis, the metabolites were quantified and the raw concentration data were then transformed to their logarithms (base 10). The correlation analysis between urinary metabolites according to gender and obesity (BMI) was analyzed using Graph Pad (Prism 5). The two correlation coefficients, Pearson (PR) and Spearman (SR), were determined to investigate the difference in metabolite correlations for male versus female and for normal weight versus obese subjects, respectively. A p-value of less than 0.05 was considered to be statistically significant. The critical values corresponding to a two-tailed test at the 0.05 levels for Pearson and Spearman coefficients were 0.316 and 0.317, respectively.

RESULTS AND DISCUSSION

Urinary metabolite analysis

Healthy male and female subjects were selected from the Korean population. Table 1 summarizes the demographic characteristics of the subjects. The mean age was approximately 42 years for both sexes, and no significant difference was observed between male and female ages ($P > 0.05$).

Table 1. Demographic information for healthy human subjects selected in Korea

Groups	Number of each group	Age (years)	Weight (kg)	BMI (kg/m^2)
Males	38	42.48 ± 8.46	71.51 ± 7.23[a]	23.86 ± 2.42[b] (0:14:26)
Females	38	42.00 ± 10.18	55.69 ± 7.75[a]	22.22 ± 3.05[b] (5:21:14)

All values of age (<40, ≥50), weight, and BMI represent the mean ± SD. [a], $P < 0.0001$; [b], $P < 0.01$. ( ) indicates thin (<18.5), normal (18.5–22.9), and obesity (overweight 23–25 and >25).

Figure 1 shows the ^{1}H NMR spectra obtained from healthy male and female urine. The typical metabolites, such as amines, amino acids, carbohydrate derivatives, and organic acids, were identified in the human urine samples. Major metabolites have already been assigned in previous studies.[4, 5, 16, 17, 22, 24–27] PLS-DA was applied to the NMR spectral data to investigate the differences in metabolic profiles between the groups. As shown in Figure 2, the PLS-DA score plots for the two genders showed significant separation between male and female. The model generated by the PLS-DA was

able to explain 76.6% of the response variance between males and females by the first two components. In addition, the model based on the first two principal components was able to predict gender with 54.9% accuracy. Metabolites that differ between the genders included hippurate, glycine, creatine, creatinine, citrate, succinate, glycolate, and TMAO. The urinary concentrations of hippurate, creatine, glycine, TMAO, citrate, succinate, acetate, glycolate and histidine were greater for females than for males, whereas the concentrations of creatinine, 2-hydroxyisobutyrate, 3 hydroxyisovalerate, and taurine were lower for females than for males. Metabolites that closely overlap in the NMR spectra were excluded from the comparison of metabolite concentrations. The VIP statistic indicates how much each metabolite contributes to the separation of the groups (Table 2). Creatinine, creatine, glycine, citrate, and succinate had large contributions to the separation between males and females (VIP = 20.9, 4.3, 6.8, 7.2, and 3.2, respectively). In addition, an unpaired t-test and two-tailed Mann–Whitney test indicated that creatinine, creatine, glycine, citrate, and succinate had significantly different concentrations in male versus female urine. In particular, succinate exhibited the most significantly different concentration between the genders. The concentration of creatinine was significantly greater in male than in female urine because this metabolite is associated with muscle mass.[5]

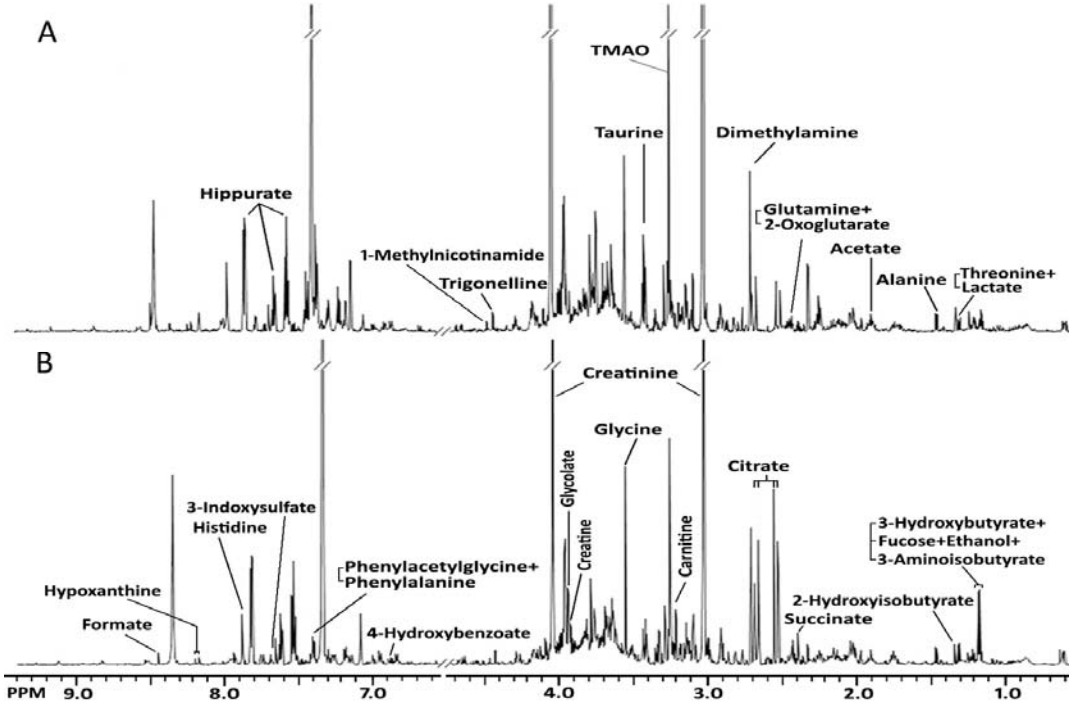

Figure 1. ^{1}H NMR spectra, using NOESYpresat, of urine samples from healthy human subjects. (A) Male. (B) Female. (+) represents metabolites that ovelap in a peak. TMAO, trimethylamine-*N*-oxide.

Table 2. A comparison of relative levels by gender for healthy subjects in urinary metabolites

The superscirpt number represents overlapped metabolites in identical PPM. ↑, increased concentration of metabolites; [a], variable importance by component 2.

Metabolites	**^{1}H chemical shift region**	**Gender**		
		Variable importance[a]	**Male (n = 39)**	**Female (n = 39)**
3-Aminoisobutyrate[1]	1.172–1.191	1.2	↑	-
3-Hydroxyisovalerate	1.256–1.261	<1.0	↑	-
Lactate[2], Threonine[2]	1.310–1.329	1.8	-	↑
2-Hydroxyisobutyrate	1.345–1.350	1.4	↑	-
Acetate	1.906–1.912	1.0	-	↑
Succinate	2.394–2.401	3.2	-	↑
Citrate	2.666–2.701	7.2	-	↑
	2.515–2.548			
Creatinine	3.029–3.036	20.9	↑	-
	4.039–4.050			
Creatine	3.021–3.028	4.3	-	↑
	3.918–3.926			
TMAO[3]	3.253–3.260	2.9	-	↑
Taurine[3]	3.408–3.437	1.6	↑	-
Glycine	3.555–3.562	6.8	-	↑
Glycolate	3.931–3.937	<1.0	-	↑
Histidine	7.079–7.088	1.0	-	↑
	7.862–7.873			
Phenylacetylglycine[4]	7.395–7.427	3.6	-	↑
Phenylalanine[4]				
Hippurate	7.810–7.832	3.6	-	↑
	7.523–7.554			
	3.952–3.967			
Unknown	1.211–1.226	2.2	↑	-
Unknown	2.239–2.260	1.0	-	↑

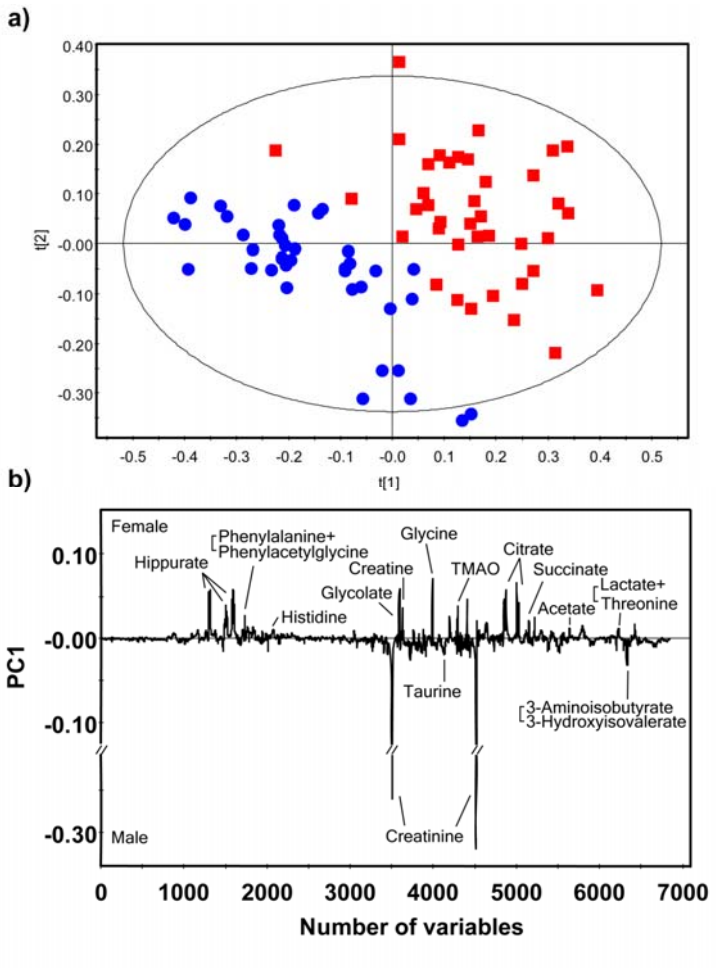

Figure 2. PLS-DA score and loading plot based on gender from the spectral binning data. Red squares, male group; blue circles, female group; +, metabolites that overlap in an identical interval (R^2X: 0.188, R^2Y: 0.766, Q^2: 0.549).

We compared the metabolite profiles between subjects with normal BMI versus high BMI (obese) from the same population sample (Fig. 3). The PLS-DA was performed for 56 subjects

selected from the sample. The score plot showed a clear separation between normal and obesity, indicating significant metabolic differences in the urine of normal weight versus obese people. The urine from obese subjects was characterized by higher levels of histidine, creatinine, lactate, and threonine, and lower levels of hippurate, phenylalanine, phenylacetylglycine, glycine, TMAO, and 3-aminoisobutyrate, compared to the normal group.

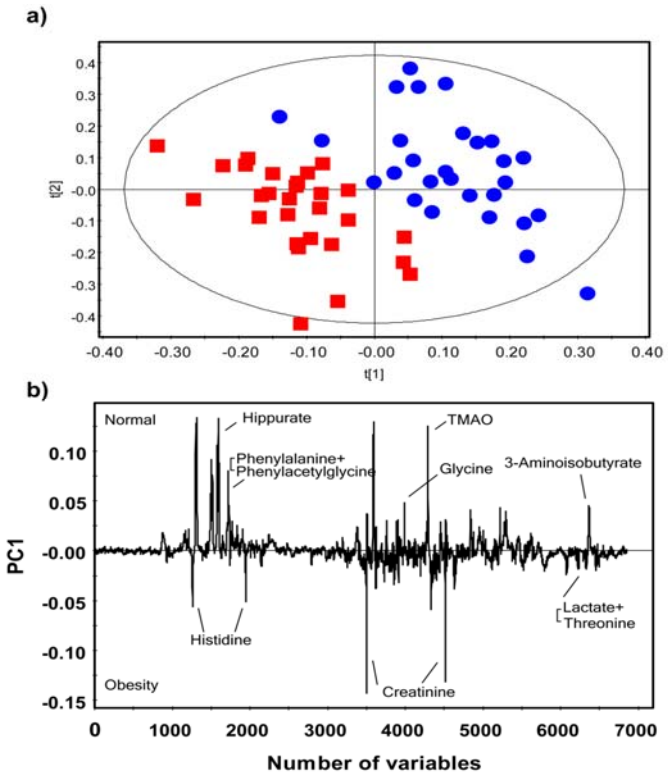

Figure 3. PLS-DA score and loading plot based on BMI from the spectral binning data. Red squares, obese group; blue circles, normal group; +, metabolites that overlap in an identical interval (R^2X: 0.18, R^2Y: 0.781, Q^2: 0.477).

Correlation analysis of urinary metabolites

A correlation analysis was performed to investigate the differences in metabolite–metabolite correlations between male and female (Fig. 4) and normal BMI and obesity (Fig. 5). The correlation analysis showed a positive correlation for most of the metabolites in all the groups, and the Pearson (PR) and Spearman (SR) coefficients gave comparable results in each of the groups.

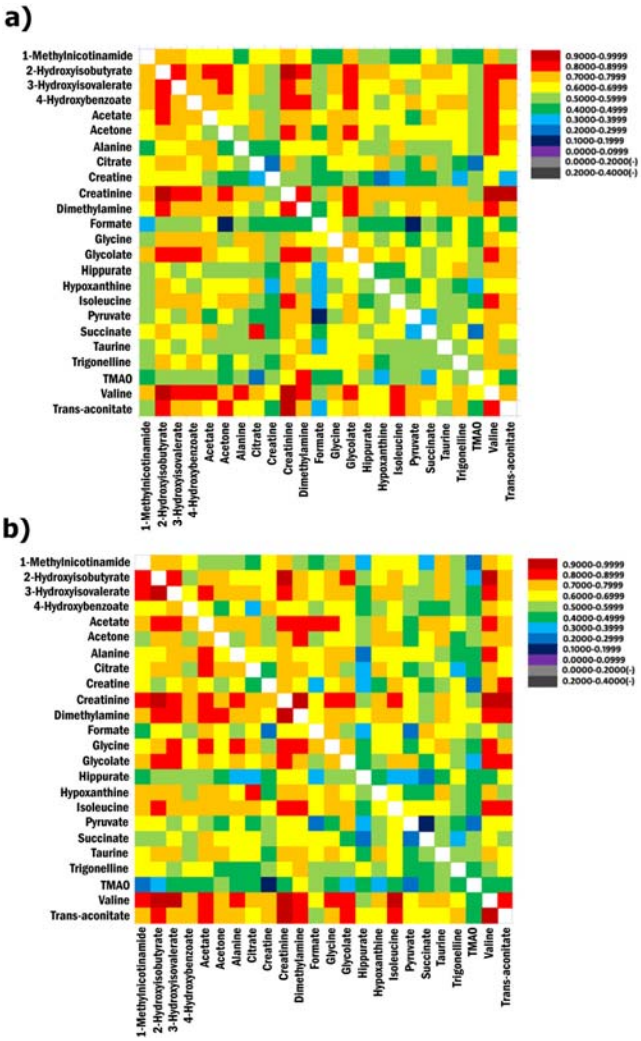

Figure 4. Correlation analysis of metabolite pairs by gender. (a) Female (n = 39); (b) male (n = 39). The cells in the lower triangular region indicate the Pearson correlation coefficent. The cells in the upper triangular region indicate the Spearman correlation coefficent. The scale in the upper right corner of the figure shows the range of the correlation coefficient corresponding with each color.

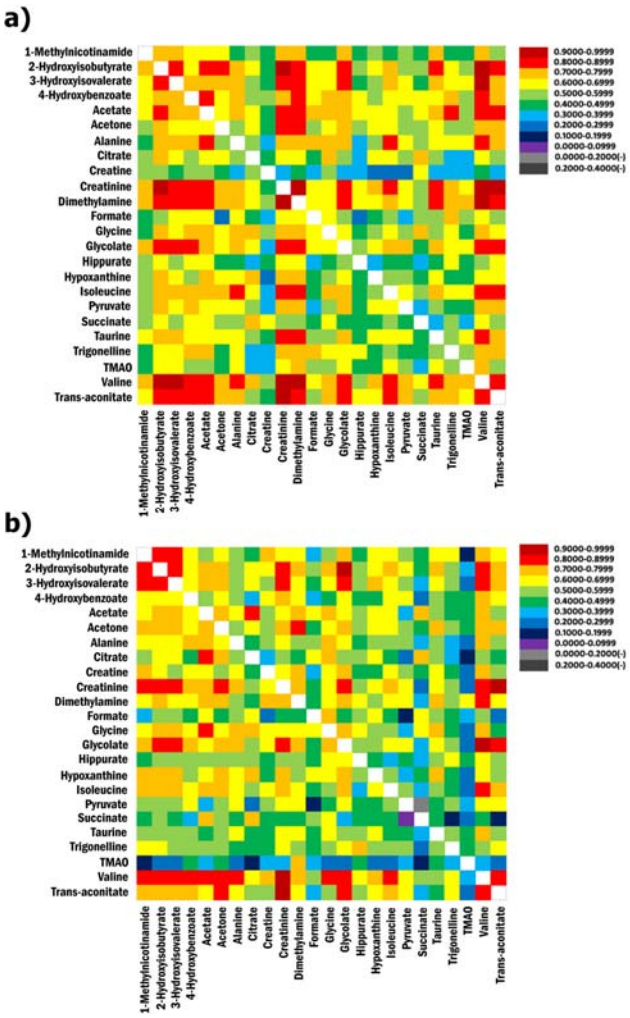

Figure 5. Correlation analysis of metabolite pairs for obesity versus normal BMI. (a) Normal BMI (n = 28); (b) obese (n = 28). The cells in the lower triangular region indicate the Pearson correlation coefficent. The cells in the upper triangular region indicate the Spearman correlation coefficent. The scale in the upper right corner of the figure shows the range of the correlation coefficient corresponding to each color.

The correlation coefficient for trans-aconitate and creatine showed a large difference between males and females. The correlation between the two metabolites was smaller for females (PR = 0.4461, SR = 0.3427) than for males (PR = 0.7741, SR = 0.8109). Similarly, the correlation between creatine and isoleucine was smaller for females (PR = 0.4460, SR = 0.3674) than for males (PR = 0.6820, SR = 0.7326). The correlations between formate and isoleucine (female: PR = 0.3446, SR = 0.4294; male: PR = 0.6377, SR = 0.6363), amine derivatives such as DMA (female: PR = 0.4090, SR = 0.4464; male: PR = 0.6653, SR = 0.6854), and hypoxanthine (female: PR = 0.3667, SR = 0.4034; male: PR = 0.6477, SR = 0.6719) were also smaller in females than in males. On the other hand, the correlations between TMAO and creatine (female: PR = 0.5153, SR = 0.5168; male: PR = 0.1824, SR = 0.2603), glycolate (female: PR = 0.6426, SR = 0.5879; male: PR = 0.3162, SR = 0.3332), and pyruvate (female: PR = 0.5941, SR = 0.6105; male: PR = 0.2461, SR = 0.4336) were greater in females than in males. The number of correlations involving creatine that were significantly different between the sexes implies that the role of this metabolite varies with gender. Creatine is a naturally occurring compound found in abundance in skeletal muscle and is associated with exercise performance. The difference in exercise capacity between genders may affect the abundance and mechanism of creatine. Slupsky et al. reported that the level of metabolites related to the TCA cycle, such as creatine and trans-aconitate, was significantly different between male and female.[5]

Similarly, the concentrations of creatine and isoleucine are greater in plasma from males than in plasma from females.[16]

The correlation between DMA and glycine was relatively greater in males (PR = 0.8145, SR = 0.7731) than in females (PR = 0.6441, SR = 0.6271), whereas the correlation between TMAO and DMA was greater in females (PR = 0.8013, SR = 0.7783) than in males (PR = 0.6481, SR = 0.6879). DMA biosynthesis may occur from intestinal bacterial demethylation of trimethylamine and partially by methylation of methylamine formed from glycine.[26] DMA is also formed from TMAO and trimethylamine.[27] The correlation between DMA and the pathway related to glycine was greater than between DMA and the pathway related to TMAO in males. Therefore, we identified different correlations for the two metabolic pathways according to gender. In our study, the concentration of glycine in urine was significantly different between males and females. This is in agreement with another study on normal subjects, which found that DMA and glycine concentrations were significantly different between genders.[15]

In a comparison of normal versus obese subjects, the correlation of metabolites paired with creatine was greater in the obese group, whereas the correlation of metabolites paired with TMAO, hippurate, and DMA was relatively smaller in the obese group (Fig. 5). In particular, the correlations of TMAO with 3-hydroxyisovalerate, alanine, isoleucine, and valine were less in the obese group than in the normal group. Obesity (high BMI) is associated with a fatty liver,[28, 29] and TMAO, derived from choline, is a product in fatty livers.[30–32] TMAO paired with isoleucine and valine showed a

considerably lower degree of correlation in the obese than in the normal weight group. Isoleucine and valine are associated with the β-oxidation of fatty acids. Propionyl-CoA is the end product of β-oxidation of odd-numbered carbon-chain fatty acids and is a common intermediate in the degradation of the amino acids isoleucine and valine.[33] In a previous study, the concentrations of several metabolites related to fatty acid oxidation were higher in males.[5] If fatty acid oxidation is reduced as a consequence of obesity, then the concentrations of isoleucine and valine, both of which are associated with β-oxidation, may also decrease; thus, changes in the concentrations of metabolites paired with isoleucine and valine may be indicative of significant changes in metabolic pathways associated with β-oxidation.

The correlations between metabolites could not be uncovered solely by PLS-DA as a classification method. Thus, the correlation analysis described here has major importance to metabolomics studies. Understanding the metabolic differences between groups combined with correlation analysis of their metabolites will help further define normal urinary metabolic.

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