

# Association between metabolic profiles in urine and bone mineral density of pre- and postmenopausal Chinese women

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## Abstract

**Objective:** In the present study, we aimed to characterize the pathological development of menopausal osteoporosis, as well as to explore potential biomarkers and metabolic pathways involved in osteoporosis.

**Methods:** Urine samples from 322 female participants categorized by menopause status and different bone conditions were collected and analyzed based on a gas chromatography–mass spectrometry (GC–MS) approach. Multivariate and univariate statistical analyses were carried out for urinary metabolomic profile characterization and comparison.

**Results:** Seventeen metabolites in the low bone mineral density (BMD) groups were clearly differentiated from those in normal BMD groups. Among these 17 differentiating metabolites, taurine,  $\beta$ -alanine, and 5-hydroxycaproic acid were found to be potential biomarkers of osteoporosis. The taurine metabolic pathway and the  $\beta$ -alanine metabolic pathway were found to be related to menopause and bone loss.

**Conclusions:** Based on the GC–MS metabolomic platform, four typical pathological phases during the progression of postmenopausal osteoporosis were described. Several differentiating metabolites and metabolic pathways were found to be closely related to the pathology of postmenopausal osteoporosis. Our results provided a solid foundation for further studies on early diagnosis and pathomechanistic evaluation.

**Key Words:** Gas chromatography–Mass spectrometry – Metabolomics – Osteoporosis – Urine.

Osteoporosis is a systemic skeletal disease characterized by bone microarchitecture deterioration and low bone mass. Osteoporosis is a classic age-related disease and is often considered a “silent disease” due to its lack of symptoms for years before bone fractures. To prevent osteoporosis, early diagnosis and intervention are essential. In China, the older population, aged 60 and above, continues to significantly increase. In 2013, a total of 202 million

people aged 60 and above lived in China,<sup>1</sup> which, when compared with the year 1999, is 1.59-fold higher.<sup>2</sup> Accordingly, the incidence of osteoporosis in China rapidly increased, which subsequently leads to an increased risk of osteoporotic fractures. Osteoporosis is known to be more prevalent in women compared with men. This is particularly true for women after menopause. The link between menopause and osteoporosis has been well documented.<sup>3,4</sup> A woman is considered to undergo menopause when she has not experienced menstrual flow for a year.<sup>5</sup> Premenopause refers to the years of ovarian recession stage before the final menstrual period, whereas postmenopause refers to the time of not having had a menstrual period for at least 12 months.<sup>6</sup> There is a direct relationship between the lack of estrogen after menopause and the development of osteoporosis. Estrogen deficiency resulting from loss of ovarian follicles can accelerate bone loss, thereby increasing the risk of fractures. A significant percentage of osteoporosis in postmenopausal women is caused by the loss of estrogen. Therefore, considering the high risk of fragility fractures in this population, prevention, diagnosis, and treatment of osteoporosis among postmenopausal women are critical.

Bone mineral density (BMD), a golden criterion recommended by the WHO, is the primary diagnostic index for osteopenia and osteoporosis. Due to its lack of sensitivity to bone loss progression, BMD is not, however, a reliable marker for early diagnosis.<sup>7,8</sup> Once BMD decline occurs, reversal of bone loss is almost impossible. As a result, measuring of

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BMD alone as the standard marker of bone condition increases the risk of missing the important therapeutic window because of the time lag between the initial subtle reduction of bone density/quality and the diagnostic accuracy of BMD. Several biochemical bone turnover indicators have served for osteoporosis diagnosis, including bone formation and bone resorption markers.<sup>9,10</sup> Although these markers are useful in evaluating bone metabolism, there are disadvantages in their application, including the lack of specificity and circadian rhythmicity.<sup>11-13</sup> Due to the issues with existing methods, there is a great unmet need in identifying early and better biomarkers, which can characterize system-level bone metabolism and monitor the pathophysiological process of osteopenia and osteoporosis.

Metabolomics, together with genomics, transcriptomics, and proteomics, constitute the core of systems biology. Metabolomics provide a complete repertoire of low-molecular metabolites in biological samples to obtain an overview of the metabolome and high-throughput compound analysis of an organism.<sup>14</sup> As an emerging technology, metabolomics exhibit unique advantages in early diagnosis and pathogenesis to investigate metabolic diseases. Therefore, it is highly valuable to combine clinical research and metabolomics analysis to determine the correlation between overall metabolic changes and disease.

In previous studies, serum or plasma from pre- and postmenopausal women has been used to investigate the relation between the bone metabolome and BMD.<sup>15,16</sup> A group of differentiating metabolites were found to be closely related to low levels of BMD. To the best of our knowledge, urine, the most convenient and noninvasive body fluid available, has not yet been used for metabolomic research of osteoporosis development. Given that metabolites in urine are known to be very different from those in blood, investigating metabolic profiles in urine of female participants with different menopause statuses and bone conditions may offer unique and valuable information. In our study, urine samples from 322 female participants who were categorized by menopause status and different bone conditions were collected and analyzed. Metabolic changes were compared at different stages of osteoporosis based on a gas chromatography–mass spectrometer (GC–MS) approach, and metabolic alterations along with bone loss were comprehensively evaluated with the aim of characterizing the pathological development of postmenopausal osteoporosis as well as to provide a foundation for future early diagnosis and pathomechanistic studies.

## MATERIALS AND METHODS

### Participants and study setting

A total of 322 Chinese female volunteers aged 40 to 68 years who visited the Jiangsu Province Geriatric Hospital between July 2013 and July 2014 were selected. All procedures performed in the present study were in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Nanjing Tech University (Nanjing, China)

and Jiangsu Province Geriatric Hospital (Nanjing, China). Written informed consent was obtained from each participant before the start of the study. All participants were asked about their menopausal status. Exclusion criteria were as follows: (1) calcium or bone metabolic dysfunction; (2) cardiovascular, hepatic, or renal disease, which might affect bone metabolism; (3) history of smoking or alcohol consumption; and (4) medications or therapy that may affect BMD. BMD at the hip and spine was measured by dual-energy X-ray absorptiometry (DXA) (DPX-L; GE Lunar Health Care, Madison, WI) and calculated according to WHO criteria. Participants were divided into four groups: premenopausal women with normal BMD (group I,  $n = 82$ ), postmenopausal women with normal BMD (group II,  $n = 71$ ), postmenopausal women with osteopenia (group III,  $n = 92$ ), and postmenopausal women with osteoporosis (group IV,  $n = 77$ ). From each participant, urine samples were collected and stored at  $-80^{\circ}\text{C}$ . Data acquired from each participant, including height, weight, body mass index (BMI), and routine indexes are presented in Supplemental Table S1, <http://links.lww.com/MENO/A324>.

### Chemicals and materials

Myristic acid- $d_{27}$  (internal standard, IS), methoxyamine, pyridine, *N*-methyl-*N*-trimethylsilyltrifluoroacetamid (MSTFA), trimethylchlorosilane (TMCS), heptane, methanol, and acetonitrile were supplied by Sigma-Aldrich (St. Louis, MO). Urease was obtained from Bailingwei (J&K, Shanghai, China). All the other chemicals used were of analytical grade unless stated otherwise.

### Sample preparation and GC–MS conditions

A total of  $50\ \mu\text{L}$  of urease ( $297\ \text{U/mL}$ ) was added into  $50\ \mu\text{L}$  of urine, mixed, and incubated at  $37^{\circ}\text{C}$  for 1 hour to degrade the urea present in urine. After addition of  $400\ \mu\text{L}$  methanol containing myristic-1,2- $^{13}\text{C}_2$  (IS,  $12.5\ \mu\text{g/mL}$ ), the mixture was vortexed for 5 minutes, and centrifuged at  $12,000 \times g$ ,  $4^{\circ}\text{C}$  for 10 minutes. The supernatant ( $250\ \mu\text{L}$ ) was evaporated to dryness under vacuum in a SpeedVac concentrator (ThermoFisher Scientific, Asheville, NC). The resulting residue was then added to  $40\ \mu\text{L}$  methoxyamine in pyridine ( $30\ \text{mg/mL}$ ), incubated for 16 hours at room temperature, followed by a trimethylsilylation process for 1 hour with  $40\ \mu\text{L}$  MSTFA containing 1% TMCS at room temperature. Then,  $40\ \mu\text{L}$  of heptane with methyl myristate (ES,  $30\ \mu\text{g/mL}$ ) was added and mixed. After centrifugation at  $12,000 \times g$ ,  $4^{\circ}\text{C}$  for 10 minutes,  $1\ \mu\text{L}$  of supernatant was injected into the GC–MS equipment for analysis.

Detailed information of the GC–MS approach is presented in the Supplementary Material, <http://links.lww.com/MENO/A323>, and has been published in previous reports.<sup>16,17</sup>

### Data processing and multivariate data analysis

As previously described,<sup>16</sup> all compound peaks were processed with NIST EPA NIH Mass Spectral Library 08 software and Automated Mass Spectral Deconvolution System (AMDIS). Metabolites were identified by comparing mass

spectra, retention time, and retention index of detected compounds using authentic reference standards as well as the National Institute of Standards and Technology (NIST) 2.0 and Wiley library. Before data analysis, the intensity of each peak was normalized using IS (myristic-1,2-<sup>13</sup>C<sub>2</sub>). Multivariate data analysis was performed using SIMCA-P 11.5 for principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), whereas MetaboAnalyst 3.0 was used for the assessment of variable influence on projection (VIP) value as well as for evaluation of the fold change. Score plots of unsupervised PCA and supervised PLS-DA exhibiting similarities and differences between groups were obtained by visualizing sample clustering and segregation. To evaluate the metabolomics model in our GC-MS analysis, R<sup>2</sup>X, R<sup>2</sup>Y, and Q<sup>2</sup> (cum) parameters, ranging from 0 to 1, were calculated. R<sup>2</sup>X referred to the variation in GC-MS response, whereas R<sup>2</sup>Y referred to the model variation. Q<sup>2</sup> represented the predicted variation of the model. The closer the values of those parameters were to 1, the stronger the ability of the model to explain or predict variations. Typically, a model was valid if R<sup>2</sup> and Q<sup>2</sup> were higher than 0.3 (30%). Loading plots of PLS-DA served to distinguish between metabolites that were critical for separation of different groups. Metabolites with a larger VIP value had a greater contribution to group separation. Univariate statistical analysis was conducted by SPSS 16.0 software using two-way ANOVA analysis. *P* < 0.05 was considered statistically significant.

## RESULTS

### Metabolic changes and differentiating metabolites at different stages of osteoporosis

A typical GC-MS chromatogram of urine samples from participants is presented in Supplemental Figure S1, <http://links.lww.com/MENO/A326> (representative peaks are labeled). A total of 82 compounds (listed in Supplemental Table S2, <http://links.lww.com/MENO/A325>) were identified and authenticated according to AMDIS software, NIST, and Wiley libraries. These compounds mainly involved low-molecular endogenous metabolites, including fatty acids, amino acids, and several carbohydrates.

PCA and PLS-DA models served to visualize the clustering and separation of metabolites among different groups, as well as to identify specific metabolites that were crucial for sample segregation. As these four groups did not exhibit obvious separation in unsupervised PCA score plots,

pairwise PLS-DA models using a supervised approach were then established. Model evaluation parameters, R<sup>2</sup>X, R<sup>2</sup>Y, and Q<sup>2</sup> (cum) of the urinary metabolome, which exhibited the degree of group separation and prediction ability of PLS-DA model are listed in Table 1.

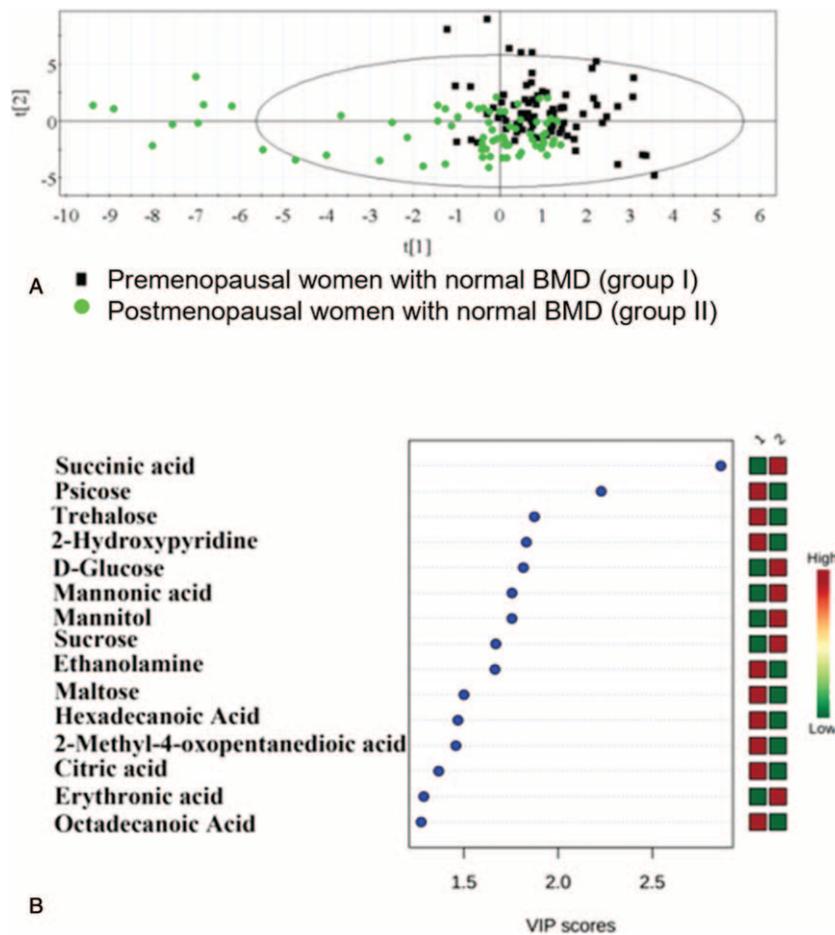
As shown in PLS-DA score plots (Fig. 1A), pre- and postmenopausal women with normal BMD (groups I and II) were only slightly separated, indicating that menopause only contributed partially to metabolic disorders in urine. Metabolites with a variable level of importance in the projection (VIP values >1 and *P* < 0.05) are shown in corresponding loading plots (Supplemental Figure S2a, <http://links.lww.com/MENO/A327>, VIP values are presented in Fig. 1B). 3D score plots achieved by a parallel metabolomics analysis platform exhibited similar results (Supplemental Figure S2b, <http://links.lww.com/MENO/A327>).

Without considering the contribution of menopause to metabolic perturbation, the metabolome of premenopausal women with normal BMD (group II) and postmenopausal women with osteopenia (group III)/osteoporosis (group IV) showed a clear segregation in PLS-DA score plots (Figs. 2A and 3A) and 3D plots (Supplemental Figure S3b, <http://links.lww.com/MENO/A328> and Supplemental Figure S4b, <http://links.lww.com/MENO/A329>). These findings suggested that, when comparing premenopausal women with normal BMD, there were significant metabolic changes in postmenopausal women with bone loss, especially in those with osteoporosis. Loading plots (Supplemental Figure S3a, <http://links.lww.com/MENO/A328> and Supplemental Figure S4a, <http://links.lww.com/MENO/A329>) highlighting metabolites that were of high importance for group differentiation, and metabolites with VIP values >1.0/1.5 are shown in Figures 2B and 3B.

Such metabolic changes resulted from combined action of menopause and bone loss, where menopause status was considered. For evaluating the role of bone loss, women with different BMD at same menopause stage (groups II, III, and IV) were compared based on PLS-DA score plots. Data from postmenopausal women with normal BMD (group II) are clearly deviated from women with osteopenia (group III, Fig. 4A) and osteoporosis (group IV, Fig. 5A), indicating the significant role of bone loss in progressive metabolic alterations. Metabolites with a VIP value >1.4, which contributed most to the differentiation of groups, are presented in Figure 4B and Figure 5B and corresponding PLS-DA loading plots (Supplemental Figure S5a, <http://links.lww.com/MENO/>

**TABLE 1.** Parameters for evaluating the partial least squares discriminant analysis (PLS-DA) quality of urine samples

Groups	Components	R <sup>2</sup> X	R <sup>2</sup> Y	Q <sup>2</sup>
Premenopausal women with normal BMD ( <i>n</i> = 82) vs				
Postmenopausal women with normal BMD ( <i>n</i> = 71)	2	0.383	0.377	0.104
Postmenopausal women with osteopenia ( <i>n</i> = 92)	2	0.352	0.524	0.363
Postmenopausal women with osteoporosis ( <i>n</i> = 77)	3	0.483	0.68	0.511
Postmenopausal women with normal BMD ( <i>n</i> = 71) vs				
Postmenopausal women with osteopenia ( <i>n</i> = 92)	4	0.232	0.448	0.314
Postmenopausal women with osteoporosis ( <i>n</i> = 77)	3	0.455	0.651	0.421



**FIG. 1.** Metabolic changes between premenopausal women with normal bone mineral density (BMD) (group I,  $n = 82$ ) and postmenopausal women with normal BMD (group II,  $n = 71$ ) and differentiating metabolites: (A) partial least squares discriminant analysis (PLS-DA) score plots; (B) significantly changed metabolites based on variable importance in the projection (VIP) values of PLS-DA.

A330 and Supplemental Figure S6a, <http://links.lww.com/MENO/A331>). Parallel PLS-DA evaluation based on MetaboAnalyst 3.0 resulted in similar results; score plots are presented in Supplemental Figure S5b, <http://links.lww.com/MENO/A330> and Supplemental Figure S6b, <http://links.lww.com/MENO/A331>.

### Global metabolic alterations in urine from pre- and postmenopausal women

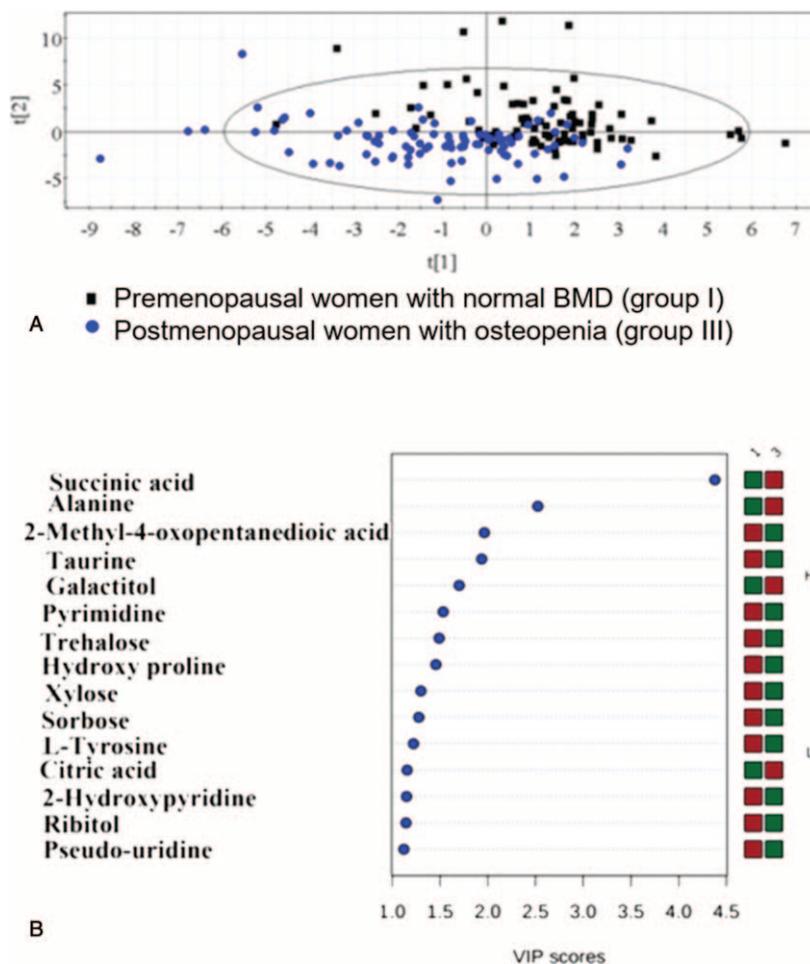
Metabolic data of premenopausal women with normal BMD (group I) and postmenopausal women with different BMDs (groups II, III, and IV) were evaluated using MetaboAnalyst 3.0 to validate and supplement the abovementioned results when comparing between groups. PLS-DA score plots and differentiating metabolites demonstrated a significant correlation and variation within groups (Fig. 6A, B). A similar comparison was performed between postmenopausal women with normal BMD (group II) and low BMD (groups III and IV) (Fig. 6C, D).

*T*-test and one-way ANOVA analysis used by SPSS 16.0 were performed to further characterize and evaluate these metabolites. Relative fold changes and *P* values of

17 urine-differentiating metabolites are presented in Table 2. With menopause and bone density decrease, urine metabolism exhibited obvious and significant disorders, including disorders involved in energy metabolism, amino acid metabolism, and carbohydrate metabolism.

Compared with the premenopausal group (group I), levels of succinic acid,  $\beta$ -alanine, and glucose in urine samples from the postmenopausal group with normal BMD (group II) increased 6.7-, 2.1-, and 1.7-fold, respectively, whereas taurine decreased 1.6-fold. These findings suggested that menopause might lead to systemic metabolic perturbation, including upregulation of galactose metabolism and  $\beta$ -alanine metabolism, as well as downregulation of taurine metabolism. As reported previously, estrogen deficiency plays an important role in the pathology of osteoporosis as estrogen affected not only bone turnover but also energy metabolism.<sup>18</sup> Therefore, estrogen deficiency could result in glucose intolerance and insulin resistance.<sup>19</sup> This may explain menopause-induced upregulation of the galactose metabolism.

Considering the impact of estrogen on body metabolism, directly comparing group I with groups III and IV without taking menopause status into account may exaggerate the



**FIG. 2.** Metabolic changes between premenopausal women with normal BMD (group I,  $n = 82$ ) and postmenopausal women with osteopenia (group III,  $n = 92$ ) and differentiating metabolites: (A) partial least squares discriminant analysis (PLS-DA) score plots; (B) significantly changed metabolites based on variable importance in the projection (VIP) values of PLS-DA.

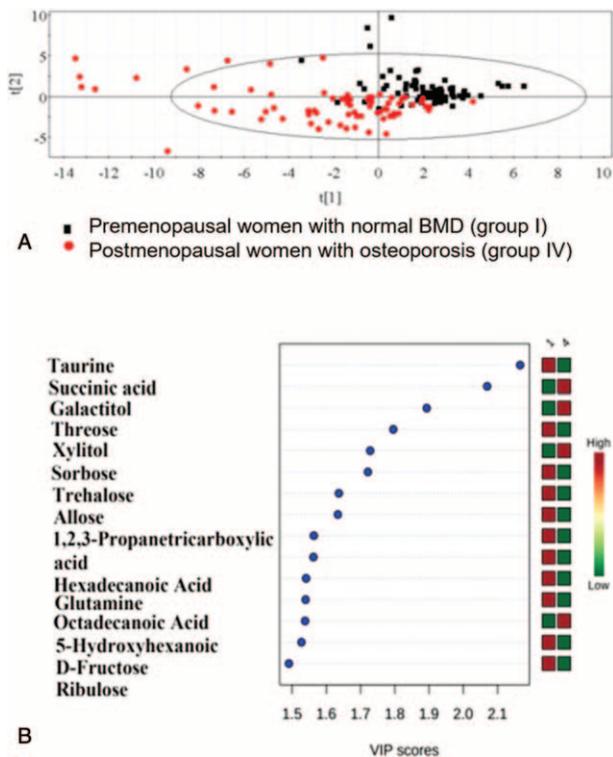
influence of bone loss on metabolic disorders. Hence, group II was further explored in our study to evaluate the role of bone loss in metabolic disorders. Succinic acid, galactitol,  $\beta$ -alanine, glucose, citramalic acid, and 5-hydroxyhexanoic acid in group III were significantly enhanced (11.2-, 2.8-, 2.3-, 1.4-, 1.4-, and 1.4-fold, respectively) compared with women in group I. In contrast, fold changes of the abovementioned metabolites in group III when compared with group II were significantly different (1.7-, 2.4-, 1.1-, 0.9-, 1.3-, and 1.2-fold, respectively). On the contrary, urine taurine was inhibited 1.9- and 1.2-fold when compared with that in groups I and II. Differentiating metabolite levels of postmenopausal women with osteoporosis compared with pre- and postmenopausal women with normal BMD exhibited distinct levels, indicating that urinary metabolic disorder was mainly subject to the combined action of menopause and bone loss.

Severe bone loss was observed in postmenopausal women with osteoporosis (group IV). Succinic acid, citramalic acid, galactitol, 5-hydroxyhexanoic acid, and  $\beta$ -alanine in group IV were upregulated more than threefold when compared with levels in the normal BMD group (groups I and II, Table 2). It

is worth mentioning that succinic acid was increased 14.7-, 2.2-fold; 5-hydroxyhexanoic acid was enhanced 7.9-, 6.9-fold; galactitol was upregulated 4.0-, 3.4-fold;  $\beta$ -alanine was improved 3.9-, 1.8-fold when compared with that in group I and group II, respectively. These changes revealed that menopause status induced metabolic disorder, including abnormal tricarboxylic acid (TCA) cycle, galactose, and amino acid metabolism, as well as fatty acid metabolism, whereas bone loss intensified such disorders.

#### Pathway analysis for metabolites that associated with postmenopausal osteoporosis

Evaluation of the most relevant pathways related with osteoporosis was achieved based on MetaboAnalyst 3.0 software using 17 differentiating metabolites that are listed in Table 2. Each correlated pathway was arranged by  $-\log$  of  $P$  value on the  $y$  axis and pathway impact value on the  $x$  axis. The node color was dependent on the  $P$  value, and the node radius was determined by the pathway impact value. As shown in Figure 7, the top 2 most important metabolic pathways identified included taurine metabolism (impact



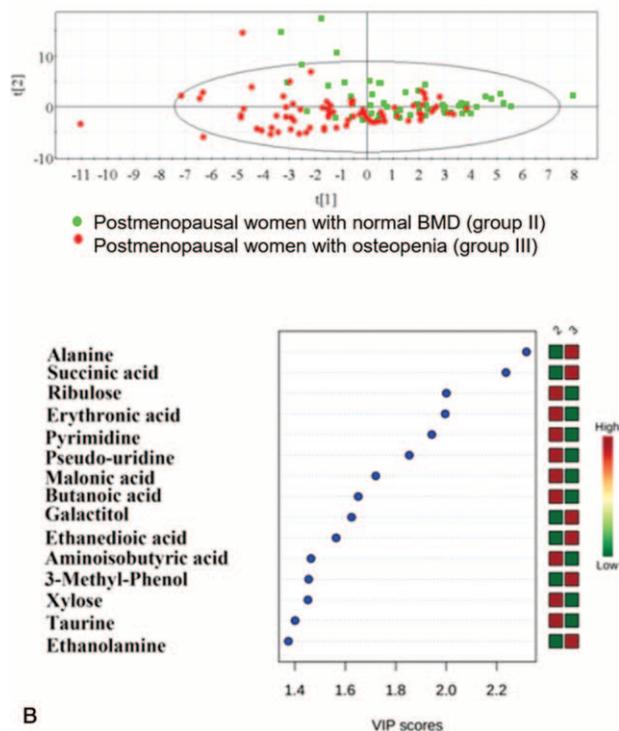
**FIG. 3.** Metabolic changes between premenopausal women with normal BMD (group I,  $n = 82$ ) and postmenopausal women with osteoporosis (group IV,  $n = 77$ ) and differentiating metabolites: (A) partial least squares discriminant analysis (PLS-DA) score plots; (B) significantly changed metabolites based on variable importance in the projection (VIP) values of PLS-DA.

value = 0.33) and  $\beta$ -alanine metabolism (impact value = 0.27), suggesting that these amino acid metabolic pathways were closely associated with menopause and bone loss. In addition, galactose metabolism (impact value = 0.09) and TCA cycle (impact value = 0.07) were, to some extent, affected by menopause and bone loss.

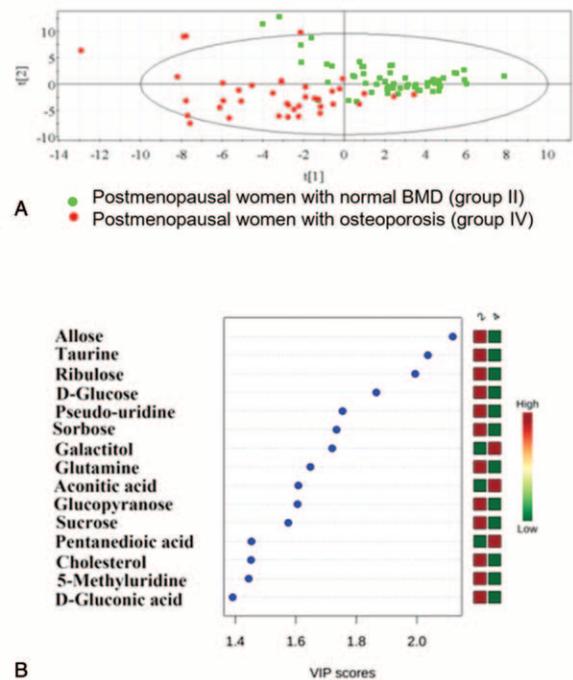
## DISCUSSION

Metabolomics characterized metabolome alterations in biology systems in response to pathophysiological changes or external stimuli. Through metabolic profiling and related pathway analysis, metabolomics represents a robust tool to elucidate dynamic disease processes and diagnose disease at an early stage. In the present study, a GC-MS approach was used to characterize for the first time the metabolomics profile of urine samples of 322 pre- and postmenopausal Chinese women with different BMD levels to monitor four typical pathological phases during postmenopausal osteoporosis progression. Using multivariate data analyses including PLS-DA,  $t$  test, and one-way ANOVA, in combination with metabolic pathway analyses of metabolites authenticated by GC-MS, an estrogen- and BMD-dependent metabolomic change was identified and typical postmenopausal osteoporosis stages were demonstrated.

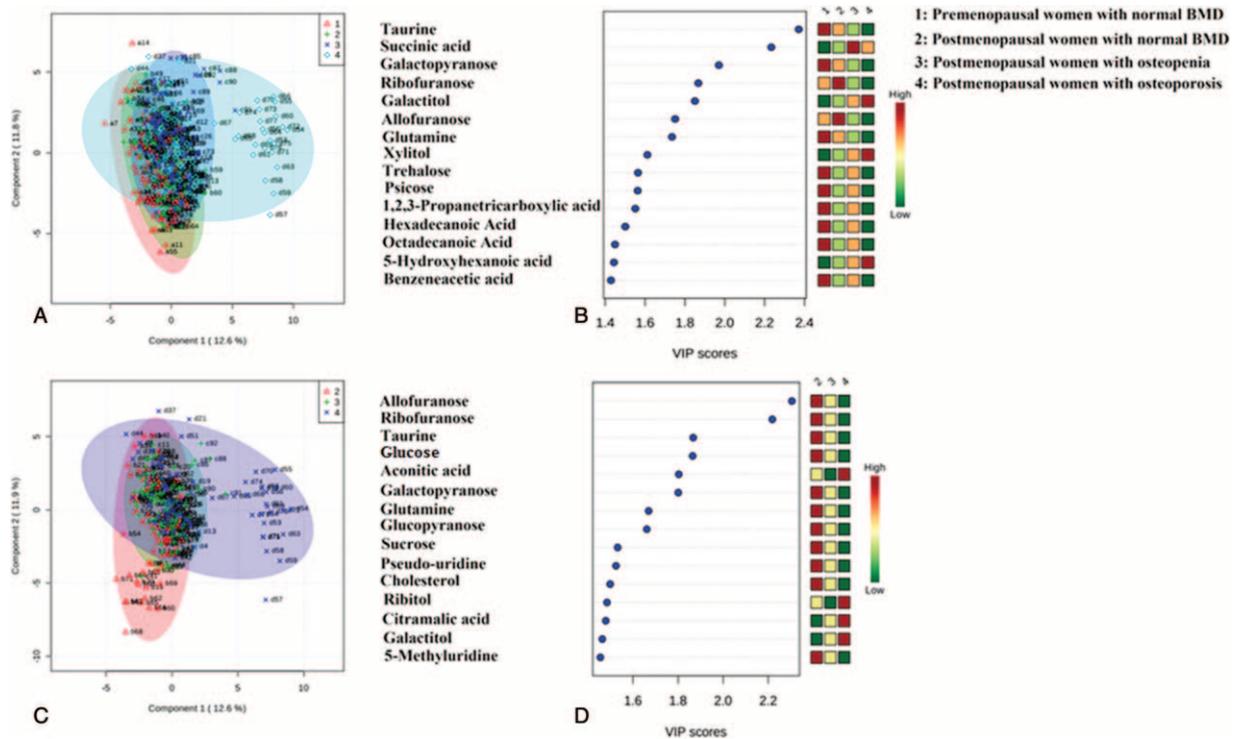
PLS-DA score plots exhibited complete discrimination of metabolomes of the normal BMD group (groups I and II) and



**FIG. 4.** Metabolic changes between postmenopausal women with normal BMD (group II,  $n = 71$ ) and postmenopausal women with osteopenia (group III,  $n = 92$ ) and differentiating metabolites: (A) partial least squares discriminant analysis (PLS-DA) score plots; (B) significantly changed metabolites based on variable importance in the projection (VIP) values of PLS-DA.



**FIG. 5.** Metabolic changes between postmenopausal women with normal BMD (group II,  $n = 71$ ) and postmenopausal women with osteoporosis (group IV,  $n = 77$ ) and differentiating metabolites: (A) partial least squares discriminant analysis (PLS-DA) score plots; (B) significantly changed metabolites based on variable importance in the projection (VIP) values of PLS-DA.



**FIG. 6.** Global metabolic changes in urine from pre- and postmenopausal women based on partial least squares discriminant analysis (PLS-DA) score plots and variable importance in the projection (VIP) values: (A) PLS-DA score plots of pre- and postmenopausal women (group I,  $n = 82$ ; group II,  $n = 71$ ; group III,  $n = 92$ ; group IV,  $n = 77$ ); (B) differentiating metabolites of four groups based on VIP values; (C) PLS-DA score plots of postmenopausal women (groups II, III, and IV); (D) metabolites contributed to segregation of groups II, III, and IV based on VIP values.

low BMD group (groups III and IV), especially the osteoporosis group (group IV). The continuous metabolic changes starting from pre- and postmenopausal women with normal BMD (groups I and II) to postmenopausal women with osteopenia (group III), and eventually to postmenopausal

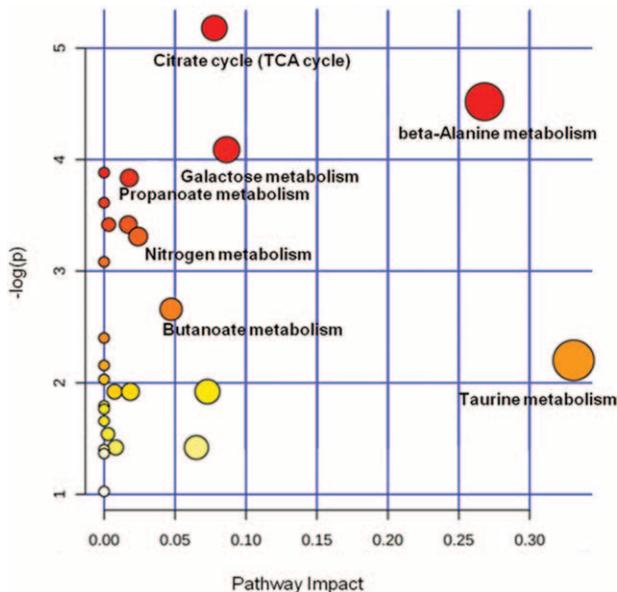
women with osteoporosis (group IV), delineated the different metabolic features in urine from an early to a late stage of osteoporosis.

One-way ANOVA analysis and  $t$  test identified 17 differentiating metabolites, including fatty acid, amino acid, and

**TABLE 2.** Relative fold changes and  $P$  values calculated by SPSS 16.0 software by comparing urine metabolites between groups I, II, III, and IV

Metabolites	Fold changes					$P$				
	Group I vs II	Group I vs III	Group I vs IV	Group II vs III	Group II vs IV	Group I vs II	Group I vs III	Group I vs IV	Group II vs III	Group II vs IV
Succinic acid	6.7	11.2	14.7	1.7	2.2	<0.001	<0.001	0.00563	0.05428	0.14339
Glucose	1.7	1.4	1.7	0.9	1.0	0.00251	0.02019	0.03401	0.32396	0.97960
Citramalic acid	1.1	1.4	3.1	1.3	2.9	0.52555	0.00127	<0.001	0.01367	<0.001
Erythronic acid	1.2	1.2	3.0	1.0	2.5	0.01811	0.01064	<0.001	0.94264	0.00152
Galactitol	1.2	2.8	4.0	2.4	3.4	0.17047	<0.001	<0.001	<0.001	<0.001
Butanoic acid	1.2	1.2	2.7	1.0	2.3	0.01165	0.04090	<0.001	0.69956	<0.001
Cis-aconitic acid	-1.0	1.2	2.9	1.3	3.0	0.75799	0.01190	<0.001	0.00356	<0.001
Pentanedioic acid	1.0	1.2	3.4	1.2	3.4	0.97956	0.30388	<0.001	0.27227	<0.001
Malonic acid	1.4	1.1	2.0	0.8	1.5	0.09841	0.42372	<0.001	0.25576	0.03430
Citric acid	1.2	1.3	1.7	1.1	1.4	0.28920	0.03055	<0.001	0.26010	0.01344
2-Methyl-4-oxopentanedioic acid	-1.1	1.0	3.0	1.1	3.3	0.22349	0.99014	0.02083	0.11494	0.02312
Ribitol	1.1	1.1	3.6	1.0	3.3	0.47109	0.43247	0.00001	0.96368	<0.001
Mannitol	1.7	1.4	2.4	0.8	1.4	0.01915	0.10145	0.00793	0.37694	0.24655
Taurine	-1.6	-1.9	-1.9	-1.2	-1.2	0.03732	0.00290	0.00958	0.31927	0.44346
5-Hydroxyhexanoic acid	1.1	1.4	7.9	1.2	6.9	0.35541	0.01457	<0.001	0.09405	0.00156
Tyrosine	-1.2	1.0	1.8	1.2	2.1	0.23483	0.97573	0.00188	0.27658	<0.001
Beta-alanine	2.1	2.3	3.9	1.1	1.8	0.02372	0.00285	0.00120	0.83749	0.09029

Group I, premenopausal women with normal BMD,  $n = 82$ ; group II, postmenopausal women with normal BMD,  $n = 71$ ; group III, postmenopausal women with osteopenia,  $n = 92$ ; and group IV, postmenopausal women with osteoporosis,  $n = 77$ .



**FIG. 7.** Overview of pathway analysis in urine from pre- and postmenopausal women with a normal bone mineral density, osteopenia, and osteoporosis.

several carbohydrates. This indicated that menopause and bone loss exerted serious influences on metabolic disorder. Among these metabolites, succinic acid, 5-hydroxycaproic acid, galactose, and  $\beta$ -alanine levels were significantly increased, whereas taurine was the only compound that continuously decreased. Pathway analysis exhibited a distinct contribution of taurine and  $\beta$ -alanine metabolism to an abnormal metabolome. In addition, galactose metabolism and the TCA cycle as well as energy metabolism pathways were also involved in metabolic perturbation.

Based on the significant changes in metabolites obtained from the metabolome analysis, our results clearly showed that menopause caused an abnormal amino acid metabolism and energy metabolism including the TCA cycle, galactose and lipid metabolism, which was further aggravated along with postmenopausal osteoporosis.

Taurine is an endogenous antioxidant with a variety of physiological effects, including inhibition of lipid peroxidation, regulation of calcium ions, and promoting glycolysis.<sup>20</sup> Taurine receptors have been reported to be extensively expressed in osteoblasts, suggesting that taurine may have direct effects on bone metabolism.<sup>21</sup> Taurine not only increases the expression of connective tissue growth factor (CTGF) in osteoblasts by activating the ERK pathway,<sup>22</sup> but also inhibits osteoclast formation and proliferation, thereby resulting in the suppression of bone resorption.<sup>23</sup> In this study, metabolomics data indicated that taurine was the only metabolite that continuously decreased, which was in parallel with the osteoporosis process. Considering the antioxidative effects and the inhibition of lipid peroxidation by taurine, together with the fact that oxidative stress and lipid disorder were enhanced with bone loss, we anticipated that taurine played a role in metabolic regulation with osteoporosis development.

$\beta$ -Alanine is known to play a key role in bone metabolism, primarily through improved production of insulin and insulin-like growth factor 1, and the synthesis of collagen and muscle protein.<sup>24</sup>  $\beta$ -alanine metabolism has been found to be related to the increase of energy consumption and therefore correlated with osteoarthritis in sclerotic processes.<sup>25</sup> In addition,  $\beta$ -alanine enhanced the working capacity and power output of both muscle and bone cells.<sup>24,26</sup> In the present study, a significant increase in  $\beta$ -alanine was observed in urine from postmenopausal women with low BMD. These findings further confirmed the close association between  $\beta$ -alanine and bone health.

Galactose has been reported to have direct adverse effects on ovarian function, which may subsequently result in estrogen insufficiency.<sup>27,28</sup> This may explain our finding that galactose metabolism correlated with the development of postmenopausal bone loss. Marked bone loss was also observed in galactose-induced aging rat models, which represented supporting evidence of the significance of galactose in BMD.<sup>29</sup>

The distinct increase of 5-hydroxycaproic acid in urine from postmenopausal women with low BMD, especially in women with osteoporosis was quite interesting. Given that 5-hydroxycaproic acid is generated by the hydroxylation of fatty acids, its level in urine could be elevated because of abnormal lipid oxidation. Our previous findings indicated that along with the bone loss process, serum omega-3 fatty acids and omega-6 fatty acids were greatly increased, which further promoted lipid oxidation.<sup>14</sup> These results may explain the elevation of 5-hydroxycaproic acid in urine, whereas in return, the increase in urine 5-hydroxycaproic acid levels further confirmed the abnormalities of lipid oxidation during bone loss. Considering its extremely limited physiological role, abnormal enhancement of 5-hydroxycaproic may play an important role in postmenopausal osteoporosis. Further investigation will be warranted to evaluate these findings.

## CONCLUSIONS

In the present study, we characterized for the first time the metabolic profiles of clinical urine samples derived from 322 Chinese women with different menopause statuses and BMD using a sensitive GC-MS approach. Data from the urinary metabolome (including 82 metabolites) showed distinct segregation of pre- and postmenopausal groups with normal BMD from postmenopausal women with low BMD, especially the osteoporosis group. A total of 17 metabolites involved in taurine and  $\beta$ -alanine metabolism, galactose metabolism, and the TCA cycle were found to be important in differentiating low BMD groups from normal BMD groups. Taurine,  $\beta$ -alanine, and 5-hydroxycaproic acid may represent potential promising biomarkers of osteoporosis. In summary, four typical pathological phases during postmenopausal osteoporosis progression were well described in this study. This may not only provide a theoretical foundation for early diagnosis and pathomechanistic research, but may also facilitate the development of medical therapies to treat postmenopausal osteoporosis.

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