Letter to the Editor

Changes in Urinary Metabolomics of Female Kashin-Beck Disease Patients in Qinghai-Tibet Plateau, China*

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Kashin-Beck disease (KBD) is a chronic, endemic, degenerative osteoarthropathic condition that is predominantly found in mainland China. KBD is a progressive disease, and the signs and symptoms become more severe in adulthood owing to the continuous development of lesions in the joint cartilage[1]. Although childhood KBD has largely been controlled, adult KBD is an important health concern in rural regions. Unfortunately, in the last century, many adult KBD patients still remain during serious epidemics.

Metabolomics, an important subdiscipline of systematic biology, is a research field of systematic biology in the class of genomics, proteomics, and transcriptomics. Metabolites are defined as the final products of cellular regulatory progression, and their levels can be regarded as the ultimate response of biological systems to genetic or ecological alterations[2]. As such, metabolomics has the potential to identify associations between metabolism and phenotype, and to further support studies on related metabolic pathways and networks in organisms.

Urine has a considerable value as a diagnostic biofluid, and previous studies have shown that the pathological changes associated with KBD are reflected in the biological changes in urine. Furthermore, urine detection is an acceptable method for KBD investigation in the Qinghai-Tibet Plateau and its neighboring regions, and has a non-invasive nature, enables easy sampling, and good operability. Thus, metabolomic detection of urine is necessary for an in-depth study of KBD and its prevention and control.

The present study was carried out in compliance with the ethical principles outlined in the world medical association Declaration of Helsinki. This study was approved by the ethics committee of the Qinghai institute for endemic disease prevention and control (Ethics Protocol number: 2017-002). All patients and controls signed an informed consent form for participation.

According to the national diagnostic criteria for KBD in China (WS/T207-2010), female patients were diagnosed with KBD based on epidemiological investigation, clinical symptoms, and radiographic findings. Controls were women with no clinical symptoms or radiographic findings. Individuals who suffered from osteoarthritis (OA), rheumatoid arthritis (RA), other osteoarticular diseases, or joint lesions or those who underwent joint operation within one year or had taken therapeutic drugs for arthritis were excluded. Women diagnosed with chronic systemic or acute inflammatory diseases were also excluded. Finally, 60 female participants (30 patients and 30 controls) were included in this study.

The morning urine samples were collected from the target population after fasting for 10 h. Ultra-performance liquid chromatography and Q-TOF mass spectrometry (UPLC/Q-TOF MS) (Beijing Mass Spectrometry Medical Research Co., Ltd) were used to analyze all the urinary samples. The chromatographic and mass spectrometry conditions were as follows: Mass spectrometry full-scan data were acquired in positive and negative electrospray ionization (ESI) modes from 50 to 1,200 D. The parameter conditions were as follows: 2.5 kV capillary voltage, 350 °C desolvation temperature, 35 V sample cone voltage, 50 L/h cone gas flow,

doi: 10.3967/bes2023.065

*This study was supported by basic research projects of Qinghai province [2017-ZJ-770]; as well as direction projects of Qinghai province health and family planning commission [2017-wjzdx-38]; and the 2020 "Kunlun Talents, High-tech Innovation and Entrepreneurial Talents" project to cultivate leading talents of Qinghai province.

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700 L/h desolvation gas flow, and 350 °C source temperature. A Waters Xevo G2 Q-TOF (Quatropde Time-of-Flight) mass spectrometer (Waters Corp.) was connected to the UPLC system *via* an ESI interface. Chromatographic separation was performed on a Waters UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm). Analytes were eluted from the column using a gradient of water (A) and acetonitrile containing 0.1% formic acid (B) as the mobile phase. The gradient conditions were: 0 min, 60% A and 40% B; 2 min, linear from 60% to 57% A and linear from 40% to 43% B; 2.1 min, linear from 57% to 50% A and linear from 43% to 50% B; 12 min, linear from 50% to 46% A and linear from 46% to 54% B; 12.1 min, linear from 46% to 30% A and linear from 54% to 70% B; 18 min, linear from 30% to 1% A and linear from 54% to 99% B; 18.1–20 min, linear from 1% to 60% A and linear from 99% to 40% B. Each run time was of 20 min, with a flow rate of 0.30 mL/min.

UPLC/Q-TOF MS data were processed using the Micromass Marker Lynx application version 4.1 (Waters Corporation, MA, USA). After denoising, deconvolution, peak extraction, alignment, and merging, the data matrix containing assigned peak numbers (retention time-m/z pairs), sample names, and normalized ion intensities was exported into SIMCA-P 11.5 software (Umetrics, Umea, Sweden) for partial least squares discriminant analysis (PLS-DA) and principal component analysis (PCA), which were used to obtain the greatest variable importance in projection (VIP) values, as well as to visualize the score plot. Variables with VIP values above 1.0 in the model were considered potential biomarkers. Goodness of fit was quantified by R^2, and predictive ability was indicated by Q^2.

Independent-samples *t*-test and nonparametric statistical test were used for statistical analysis using SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA), and a P value < 0.05 was considered statistically significant. The databases HMDB (http://www.hmdb.ca) and METLIN (http://metlin.scripps.edu/) were used to identify the potential metabolic marker candidates based on their tandem mass spectrometry (MS/MS) spectra.

Initial analysis revealed no statistically significant differences in age, height, weight, or body mass index between patients with KBD and controls (P > 0.05) (Supplementary Table S1, available in www.besjournal.com). Urinary samples were analyzed by UPLC/QTOF-MS in both the positive and negative ionization modes, and the primary data of the mass spectrograph were obtained. The total ionization graphs of patients with KBD and controls are shown in Figure 1 and Supplementary Figure S1 (available in www.besjournal.com).

The mass-to-charge ratio ranged from 85.029–835.29. A total of 1,663 metabolites with VIP values above 1.0 were selected for subsequent analysis. After *t*-test or nonparametric statistical test, a statistically significant difference in 586 metabolites was found between patients with KBD and controls (P < 0.05). PCA was performed through multivariate data analysis using Simca-p software. PLS-DA was further carried out on the module (R^2 = 0.802, Q^2 = 0.731), which showed stable and significant clusters (Supplementary Figure S2 and Supplementary Figure S3, available in www.besjournal.com).

Structural identification of the selected substances was performed by comparing the exact mass data, retention times, and corresponding MS/MS fragments with those of reference standards in the HMDB and METLIN databases. Metabolites identified in the negative ionization mode included cis-aconitic acid, but-2-enoic acid, and acetyl-N-formyl-5-methoxykynurenamine. Metabolites identified in the positive ionization mode included beta-D-glucose, uracil, 4,6-dihydroxyquinoline 2-keto-glutaramic acid, tryptophanol, N-acetylserotonin, 3-hydroxybutyric acid, pyridoxine 5'-phosphate, 5'-methylthioadenosine, and acetyl-N-formyl-5-methoxykynurenamine. Overall, 12 differential metabolites were associated with KBD (Tables 1–2, and Supplementary Figure S4, available in www.besjournal.com).

KBD is predominantly distributed in the diagonal broad belt ranging from southeastern Siberia in Russia to southwest China. The main pathological changes are chondrocyte necrosis, apoptosis, cartilage degeneration, and matrix degradation[3]. Environmental risk factors, such as selenium deficiency, grain contamination by mycotoxin-producing fungi, and high fulvic acid levels in drinking water, are closely associated with KBD[4]. As the etiology of KBD is uncertain, further analysis of the progression of OA could provide many new clues and opinions for KBD research.

Many studies have found that OA may be associated with differences in amino acid and collagen metabolism, the tricarboxylic acid cycle (TCA), and fatty acid metabolism. A prior metabolomics study examining serum samples from controls and patients with OA, RA, ankylosing spondylitis, and gout found that patients with arthritis could be differentiated from controls, and

that each form of arthritis had a distinct metabolite profile[5]. Similarly, another study found that the progression of joint space narrowing in adults with OA was associated with a different metabolite pattern[6]. Metabolomics is a promising tool for identifying biomarkers for OA diagnosis, stratification, and treatment.

Metabolomic studies have been used to probe biomarkers and the pathogenesis of KBD. NMR revealed that serum metabolite changes in patients with KBD were involved in glucose metabolism, while eight metabolites were related to the metabolism of phospholipids and amino acids in Wistar rats with KBD induced by T-2 toxin[7].

In the present study, we identified 12 metabolites involved in TCA, amino acid, fatty acid, energy, and glycolytic metabolism. Of these, cis-aconitic acid and 2-keto-glutaramic acid are related to TCA, which is involved in energy metabolism. Because the key enzyme in TCA is located in the mitochondrial matrix, changes in cis-aconitic acid and 2-keto-glutaramic acid indicate functional disorders of the mitochondria in chondrocytes. Liu et al. found that mitochondrial dysfunction may play an important role in chondrocyte apoptosis, which is involved in the pathophysiology of KBD[8]. In the present study, the relative levels of cis-aconitic acid and 2-keto-glutaramic acid in patients were lower than in controls, indicating mitochondrial dysfunction.

Tryptophanol, acetyl-N-formyl-5-methoxykynurenamine, 4,6-dihydroxyquinoline, and N-acetylserotonin were related to tryptophan metabolism, which is a key pathway in melatonin biosynthesis and degradation. The metabolites are also associated with beta-alanine metabolism, while

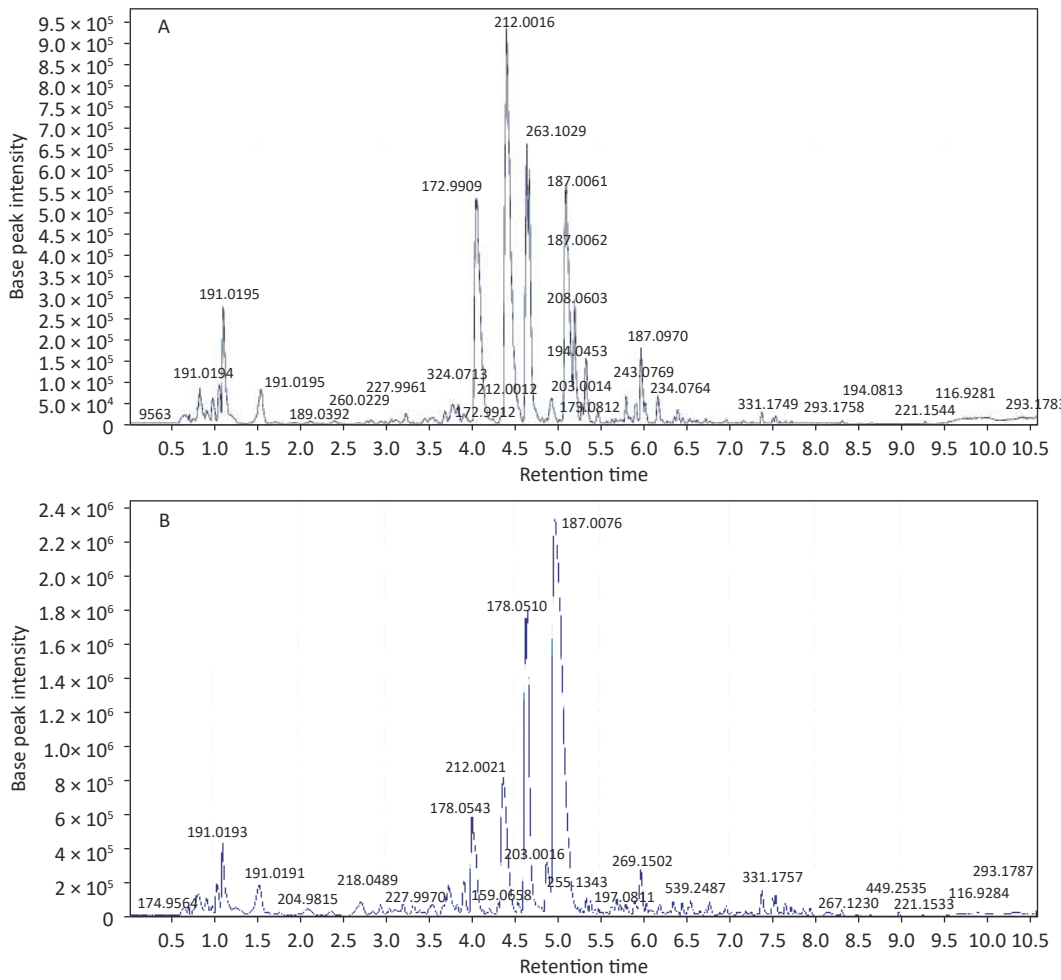

Figure 1. The total ionization graph between patients and controls in positive ESI mode. (A) KBD; (B) Control; the horizontal axis represents flight time, and the longitudinal axis represents the integral area. ESI: electrospray ionization; KBD, Kashin-Beck disease.

5'-methylthioadenosine was associated with cysteine and methionine metabolism, together indicating a disorder of amino acid metabolism.

But-2-enoic acid is associated with fatty acid metabolism. Active processing of fatty acids occurs in the cytosol, whereas further oxidation of fatty acids occurs in the mitochondria matrix. Similarly, 3-hydroxybutyric acid is associated with ketone body metabolism[9]. In the present study, the relative level of 3-hydroxybutyric acid in patients was lower than that in controls, indicating a disorder in energy metabolism.

Beta-D-glucose is a metabolite associated with glycolysis. Pathological changes in KBD include focal chondrocyte death (necrosis) and associated proteoglycan (PG) depletion, which indicates risk factor-induced disruption of PG metabolism and subsequent abnormal biomechanical strain distribution in chondrocytes[10]. In the present study, the relative level of beta-D-glucose in patients was lower than that

Table 1. Differences in urinary metabolite between patients and controls

No.	Metabolite	Formula	Retention time/min	Meraure mass	VIP	Related pathway
1	cis-aconitic acid	$C_6H_6O_6$	1.08	174.017	6.2721	Tricarboxylic acid cycle
2	But-2-enoic acid	$C_4H_6O_2$	1.12	86.037	2.1377	Fatty acid metabolism
3	Acetyl-N-formyl-5-methoxykynurenamine	$C_{13}H_{16}N_2O_4$	4.64	264.154	13.7420	Trptophan metabolism
4	2-keto-glutaramic acid	$C_5H_7NO_4$	1.07	145.020	1.6551	Tricarboxylic acid cycle
5	Uracil	$C_4H_4N_2O_2$	5.87	112.032	1.7565	Beta-alanine metabolism
6	Tryptophanol	$C_{10}H_{11}NO$	4.27	161.084	3.1972	Trptophan metabolism
7	N-acetylserotonin	$C_{12}H_{14}N_2O_2$	3.06	218.103	1.5789	Trptophan metabolism
8	4,6-dihydroxyquinoline	$C_9H_7NO_2$	4.94	161.048	2.2091	Trptophan metabolism
9	3-hydroxybutyric acid	$C_4H_8O_3$	4.03	104.050	1.5387	Ketone body metabolism
10	Pyridoxine 5'-phosphate	$C_8H_{12}NO_6P$	6.40	249.040	2.8699	Vitamin B6 metabolism
11	5'-Methylthioadenosine	$C_{11}H_{15}N_5O_3S$	3.87	297.090	2.3111	Cysteine and methionine metabolism
12	Beta-D-glucose	$C_6H_{12}O_6$	4.16	180.053	3.9685	Glycolysis

Note. VIP, variable importance in projection.

Table 2. Comparison of the peak areas of twelve differences metabolites among the two groups (x ± S)

Differences metabolites	KBD patients		Controls		*P*
	N	Mean ± SD	*N*	Mean ± SD	
Cis-aconitic acid	30	95,423 ± 37,912	30	130,128 ± 56,621	0.007
But-2-enoic acid	30	20,461 ± 10,010	30	26,779 ± 4,969	0.003
Acetyl-N-formyl-5-methoxykynurenamine	30	702,743 ± 410,729	30	1,042,346 ± 554,197	0.009
2-Keto-glutaramic acid	30	14,297 ± 8,504	30	91,952 ± 5,072	0.007
Uracil	30	5,517 ± 3,823	30	9,651 ± 6,589	0.005
Tryptophanol	30	5,897 ± 3,137	30	10,156 ± 4,402	< 0.001
N-acetylserotonin	30	1,492 ± 997	30	3,806 ± 2,739	< 0.001
4,6-dihydroxyquinoline	30	22,739 ± 7,228	30	32,261 ± 14,914	0.003
3-hydroxybutyric acid	30	8,628 ± 4,216	30	12,971 ± 6,496	0.003
Pyridoxine 5'-phosphate	30	6,798 ± 6,107	30	15,767 ± 10,993	< 0.001
5'-Methylthioadenosine	30	8,042 ± 4,482	30	14,029 ± 7,407	< 0.001
Beta-D-glucose	30	77,254 ± 50,143	30	111,332 ± 39,204	0.004

Note. KBD, Kashin-Beck disease.

in controls, indicating a glycolysis disorder.

Vitamin B6 deficiency is also involved in the pathogenesis of KBD, and in this study, the relative level of pyridoxine 5'-phosphate in KBD patients was lower than that in controls, indicating a disorder of vitamin B6 metabolism.

Overall, in the present study, we applied UPLC/Q-TOF MS technology to conduct urinary metabolomics research on women in a KBD-endemic region. This analysis revealed 12 metabolites associated with KBD. These were involved in TCA metabolism, fatty acid metabolism, energy metabolism, glycolysis metabolism, and amino acid metabolism. Further metabolomics research is required to verify these metabolites and to screen for biomarkers of KBD.

Competing Interests The authors declare that they have no competing interests.

Acknowledgements We wish to express our gratitude to ZHANG Xue Fei for their assistance with the experiments.

Author Contributions ZHAO Zhi Jun and WANG Li Hua conceived the study, LI Qiang wrote the paper and carried out all experiments. ZHOU Xin, XUE Hong Mei, WANG Jian Ling, and LI Ji Quan conducted the investigations and sample collection. ZHAO Yan Mei, XU Li Qing, CHAO Jie, and CHEN Yang Yang performed the statistical analyses.

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Received: January 5, 2023;
Accepted: April 10, 2023

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