European Review for Medical and Pharmacological Sciences 2020; 24: 9591-9600

Metabolomics and its application in the mechanism analysis on diabetic bone metabolic abnormality

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Abstract. – OBJECTIVE: This study is aimed at analysing the endogenous metabolites profiling of patients with diabetic osteoporosis, so as to provide the reference for pathogenesis research of diabetic osteoporosis.

PATIENTS AND METHODS: The 1H-NMR metabolomics technology, combined with pattern recognition analysis and SIMCA-P 12.0 statistical analysis, were employed to identify the metabolites differences between diabetic patients with disordered bone metabolism (research group) and healthy volunteers (normal group) in this study.

RESULTS: Compared with normal group, the results show that in research group, the levels of O-acetyl glycoprotein, proline, 1-methyl histidine, tricarboxylic acid cycle (TCA cycle) product (citric acid and α-ketoglutaric acid) decline, while the levels of branched chain amino acids (leucine, isoleucine, valine), glucose, choline, creatine, inositol, glutamine, aspartic acid, alanine, glycine, and citrulline increase.

CONCLUSIONS: There are disordered metabolic pathways and imbalanced bone synthetic materials and regulatory substances in diabetic patients with bone metabolic abnormality. These metabolic abnormalities could be the specific indicators in early diagnosis of diabetic osteoporosis.

Key Words:
Diabetic osteoporosis, Metabolomics, Pattern recognition, Biomarker.

Introduction

Diabetic Osteoporosis (DOP) is a type of osteoporosis secondary to diabetes, which is one of the chronic complications of diabetes mellitus (DM) as a consequence of severe metabolic disorder. Patients with DOP are prone to osteoporotic fractures, causing a high level of disability and mortality[1]. As for type 1 diabetes mellitus (T1DM), due to secretion deficiency of insulin and amylin, the anabolic effects reduce in patients with recent onset of T1DM, bringing about impaired bone formation, whereas in long-standing T1DM patients, poor nutrient supply resulting from vascular complications may be attributable to low bone mass and increased fracture risk. Despite patients with type 2 diabetes mellitus (T2DM) show a higher bone mineral densities (BMD) than T1DM patients, they are also vulnerable to osteoporotic fracture for the increased risk of falling. Therefore,

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various strategies should be taken in different cases. Optimal glycaemic control and effective prevention and treatments of vascular complications are strategies to improve BMD and prevent osteoporotic fractures in patients with T1DM. To prevent patients with T2DM from falling, visual assessment should be arranged as early as possible, additionally, aerobic exercise which is helpful to strengthen muscle and keep one's balance should be part of the treatment[2]. At present, non-invasive methods, such as dual-energy X-ray absorptiometry (DXA), quantitative bone ultrasound, trabecular bone score (TBS), and FRAX software are widely used for evaluating bone quality in patients with diabetes. However, these tools may underestimate the fracture risk in patients with DOP. For some T1DM and T2DM patients, bone quality is compromised and the risk of fracture increases even if they displayed a similar or higher BMD than individuals without diabetes[3,4]. In cases where such measurement fail to capture the actual tendency, bone turnover markers are helpful to reflect the state of bone tissue in early stages of DM.

Even though plenty of genetic researches, concerning genome, transcriptome, epigenome, and even proteome, has greatly enriched our knowledge in the etiology of osteoporosis recently, biological mechanisms underlying the development of DOP are still unclear. Moreover, there are very limited medications and prediction tools for DOP[5,6]. Therefore, for better understanding the pathogenesis and developing more comprehensive prediction/diagnosis/prognosis tools of DOP, novel biomarkers remain much needed.

As an emerging and rapidly developing field, metabolomics provides an efficient approach to recognize biomarkers or characterize perturbations of diseases through a series of processes, including detection, identification, and quantification, whose research objects are metabolites with low molecular-weight (< 1000 Da) in biological samples[7-9]. In recent years, metabolomics has been successfully applied to identify abnormal signals or biomarkers in early stage[10], characterize biological pathway[11], and diagnose diseases[10,12]. For advantages, such as simple sample preparation, high reproducibility, and fast analysis, ^{1}H nuclear magnetic resonance (NMR) spectroscopy has become one of the most widely used tools in metabolomic research. Also, ^{1}H NMR-based metabolomics is suitable for analysing metabolite fingerprinting of multiple compounds simultaneously and systemically[13].

However, there are few reports on diabetic osteoporosis concerning about its overall metabolism, adopting metabolism parameters to diagnose DOP are still needed further exploration. In this study, we investigated the differences of metabolites between diabetic patients with disordered bone metabolism and healthy volunteers by using ^{1}H-NMR spectroscopy combined with pattern recognition analysis and SIMCA-P 12.0 statistical analysis to reveal the pathogenesis of DOP and offer a potential approach in early diagnosis of DOP.

Patients and Methods

Ethics Statement

This study was approved by the Institutional Ethics Committee of Guangdong Provincial Hospital of Chinese Medicine. It was conducted in accordance with the principles of the Second Revision of the Declaration of Helsinki, and written informed consent was signed by every participant.

Participants

In this study we recruited participants from Guangdong Provincial Hospital of Chinese Medicine. Participants were divided into two groups: normal group (18 healthy volunteers, including 11 males, 35.5%, 7 females, accounting for 64.5%, mean age 45.70±4.87 years old), and research group (diabetic patients with disordered bone metabolism, including 11 males, 35.5%, 7 females, accounting for 64.5%, mean age 46.60 ± 5.03 years old). Participants with cancer, hepatic disease, kidney disease or genetic bone disease, or patients using medications (e.g., diphosphonate, glucocorticoids, oestrogen) that might influence bone metabolism were excluded. There are no statistical differences in age and gender between the two groups.

Sample Collection and Preparation

Blood samples from all participants were collected in tubes with sodium ethylene diamine tetraacetic acid (EDTA). After centrifugation, the plasma samples were frozen at -80°C. Before running, samples were thawed and centrifuged with 10,000 Hz for 10 min at 4°C. 300 μL of each sample was transferred into a 5 mm NMR tube; we added 200 μL 0.2 mol/L phosphate buffer solution and 50 μL D_2O (Qingdao Dragon Technology Co. ltd., Qingdao, China), and then, mixed

thoroughly for further analysis. All chemical and reagents used were of analytical grade and purchased from related companies.

^1H NMR Analysis

^1H NMR spectra of plasma samples were recorded on a Bruker AVANCE III 500 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with an ultra-low temperature probe. The ^1H NMR spectra were recorded with the relaxation edited Carr-Purcell-Meiboom-Gill (CPMG, RD-90°-(τ-180°-τ)n-acquisition) pulse sequence to detect low-molecular-weight metabolites over a spectral width of 1000 Hz with 4 s relaxation delay, 1 ms echo time, 64 loops, 128 transients and 64 k data points. The temperature during all experiments was kept at 298 K. The free induction decay (FID) signal is of the spectra were Fourier transformed to an NMR spectrum with FT size of 32 K.

Statistical Analysis

Automatic integration was conducted by Topspin 2.0 software package (Bruker Biospin, Rheinstetten, Germany) in ^1H-NMR spectrum. It ranged from 0.5 to 9 and the integral separation was 0.05 ppm. In order to eliminate the influence caused by the residual water peak, the integral value on a scale of δ 4.7-5.2 were set to zero. In addition, to eliminate the analysis error of sample resulting from different concentrations, the subsection integral was normalized before principal component analysis (PCA).

After the normalization, we used SIMCA-P+12.0 (UMETRICS AB, Malmo, Sweden) for PCA, partial least squares-discriminant analysis (PLS-DA), orthogonal projections to latent structures-discriminant analysis (OPLS-DA). R^2 and Q^2 are the main parameters of model validation. R^2 explains the model differences, while Q^2 predicts the model differences. If numerical value of R^2 and Q^2 were closer to 1, the model fitting accuracy was better. If numerical value of R^2 and Q^2 were higher than 0.5 (50%), we assume that the model is better. The result of pattern recognition is commonly presented in the form of score plot and loading plot. The samples in the same pathophysiological state were supposed to contain similar components. Accordingly, they should also be in a similar position in the figure. If the distance between disease group and control group in the picture was far, the biochemical and metabolic responses induced by diseases are abnormal, and vice versa. The loading plot reflects the contribution of each integral segment to the sample principal component score. Each point on the loading plot represents information about the components detected in the sample. Loading plots of OPLS-DA models were obtained from MATLAB 7.1 (Mathworks Inc., Natick, MA, USA) combined with correlation coefficients in this research. The specific metabolites between the groups were interpreted by variable importance in the projection (VIP) and correlation coefficients. The variables with a high VIP are considered to be statistically significant. In general, metabolites with VIP scores > 1 could be identified as biomarkers that might separate DOP patients from healthy controls.

All data were described as mean ± SD (standard deviation) and the statistical analyses were performed by Statistical Product and Service Solutions (SPSS) 17.0 statistical software (SPSS Inc., Chicago, IL, USA). The comparison between multiple groups was done by using One-way ANOVA test followed by post-hoc test (Least Significant Difference). A value of $p<0.05$ was considered as statistically significant.

Results

^1H NMR Spectroscopy

The CPMG ^1H NMR spectra (Figure 1) of plasma samples acquired from diabetic patients with disordered bone metabolism and healthy volunteers displayed the average signals of metabolites. In total, 19 metabolites were identified in plasma samples which included lipids, glucose, amino acids and organic acids, as shown in Figure 1.

Metabolites were detected in both groups of samples, containing branched chain amino acids (leucine, isoleucine, valine), tricarboxylic acid cycle products (α-ketoglutaric acid, citric acid), other amino acids, such as alanine, proline, glutamine, glutamic acid and citrulline, and other metabolites, such as glucose, creatine, inositol, glycerol, choline, betaine, N-acetylglycoprotein and O-acetylglycoprotein (Table I).

Compared with normal group, many metabolites in research group significantly changed: the levels of glucose, branched chain amino acids, proline, and betaine increased, while the levels of glutamine, inositol, and O-acetylglycoprotein reduced (Table I).

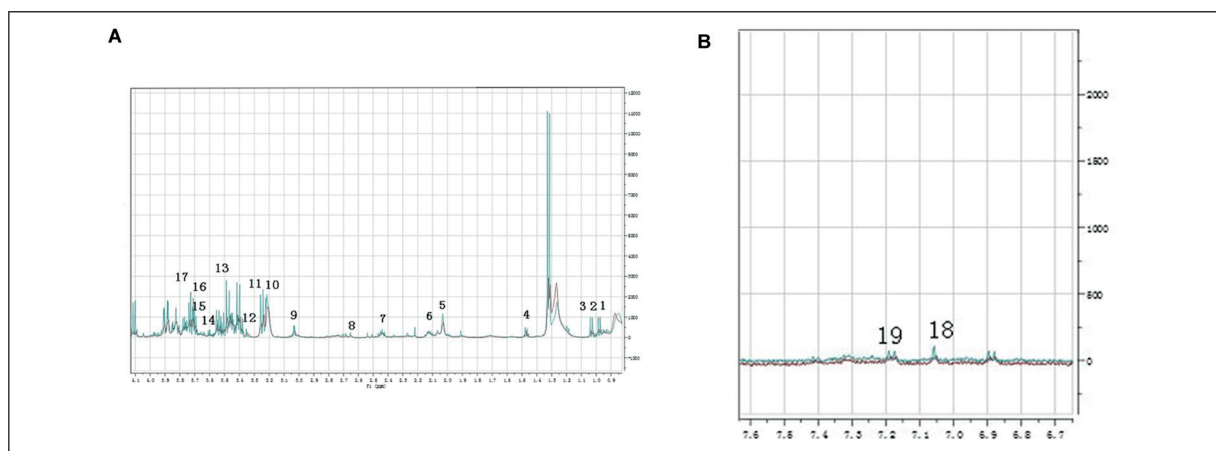

Figure 1. Representative serum ^{1}H NMR Spectra from Diabetic patients with bone metabolism disorder group (red line) and healthy volunteer group (green line). Key: 1. Leucine; 2. Isoleucine; 3. Valine; 4. Alanine; 5. N-acetylglycoprotein; 6. O-acetylglycoprotein; 7. a-ketoglutaric acid; 8. Citrate; 9. Creatine; 10. Choline; 11. Betaine; 12. Citrate; 13. Glucose; 14. Inositol; 15. Citrulline; 16. Glutamate; 17. Glutamine; 18. 1-methyl-histidine; 19. Tyrosine; The regions of δ 0.5~2.0 and δ 6.2~9.2 (**A**) in the serum spectra were vertically expanded by eight and four times respectively compared with the region of δ 2.0~4.6 (**B**).

PCA Score Plot

To illustrate the differences in the metabolic profiles, the ^{1}H NMR spectra dataset were further analysed by PCA. The score plot exhibited a distinct separation of research group from normal group (Figure 2A and 2B, R^2=79.2%, Q^2= 53.3%). As for the overlap in the score plot, we need more analysis like PLS-DA and OPLS-DA to demonstrate.

PLS-DA and OPLS-DA

To observe the clustering trends of samples obtained from diabetic patients with disordered bone metabolism and healthy volunteers, plasma metabolic profiling was conducted by PLS-DA and OPLS-DA.

As Figure 3A shown, research group could basically distinguish from normal group in the PLS-DA score plot (R^2x=65.8%, R^2Y=83.8%, Q^2(cum)=48.8%). In order to further describe the featured changes of metabolic substances between research group and normal group, the OPLS-DA model (R^2x=80.5%, R^2Y=96.2%, Q^2(cum)=67.2%) was built. In the score plot and loading plot (Figure 3B and 3C), the two groups could be completely separated from each other

Table I. Variation of the related metabolites between diabetic patients with bone metabolism disorder group and healthy volunteer group.

Chemical shift	Metabolites	|r|	Variation trend
δ 0.97	Leucine	0.579	↑
δ 1.02ϱ	Isoleucine	0.561	↑
δ 0.99, 1.04	Valine	0.643	↑
δ 1.46	Alanine	0.414	↑
δ 2.03, 2.06	N-acetylglycoprotein	0.445	↑
δ 2.14	O-acetylglycoprotein	0.404	↓
δ 2.45, 2.46	α-ketoglutaric acid	0.467	↓
δ 2.54, 2.66	Citrate	0.407	↓
δ 3.03	Creatine	0.620	↓
δ 3.63	Inositol	0.532	↑
δ 3.34, 3.35	Proline	0.511	↑
δ 3.40~3.90	Glucose	0.551	↑
δ 3.75	Glutamine	0.543	↑
δ 7.06	1-methyl-histidine	0.511	↑
δ 7.19	Tyrosine	0.427	↑

Note: |r| >0.392, $p < 0.05$.

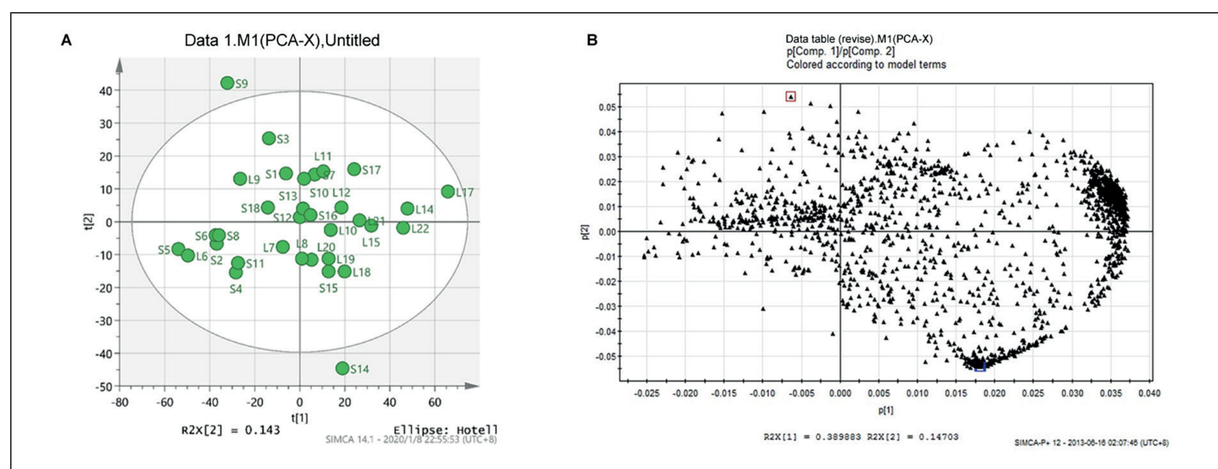

Figure 2. Principal component analysis of serum ^{1}H NMR spectra from diabetic patients with bone metabolism disorder group (S▲) and healthy volunteer group (Z▲). **A,** Scores plot. **B,** Loadings plot. PC1 *vs.* PC2, PC1 *vs.* PC2, R2=79.2%, Q2= 53.3.

and there was no cross or overlap between them. Since the numerical value of R^2 and Q^2 was close to 1 in OPLS-DA model, it was more credible than PCA and PLS-DA.

Furthermore, the correlation coefficient-loading plot of metabolites was built (Figure 4). The upward peaks in the diagram represented a decrease of the correspondent metabolites in research group, while the downward valleys indicated their increase. The redder the colour of the metabolites referred to in the figure, the greater the difference of them between the two groups will be. Whereas, the bluer the colour of the metabolites referred to, the smaller the difference between the two groups. Since the total sample size in this research was 38, the critical value of the correlation coefficient |r| was supposed to be 0.392 according to the relevant Department Boundary Value Table. Differential metabolites with |r| >0.392 between the two groups indicated that the change was remarkable statistically significance. As Figure 4 and Table I shown, there were declined levels of O-acetyl glycoprotein, proline, 1- methyl histidine, tricarboxylic acid cycle product (alpha ketone glutaric acid, citric acid), as well as increased levels of branched chain amino acids (leucine, isoleucine, valine), glucose, choline, creatine, inositol, glutamine, aspartic acid, alanine, glycine and citrulline in research group compared with normal group.

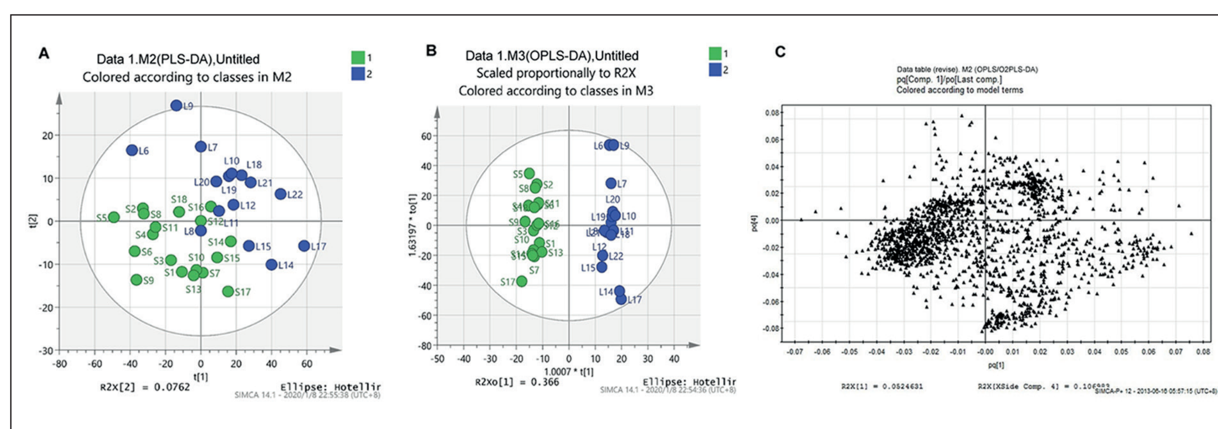

Figure 3. PLS-DA and OPLS-DA of serum ^{1}H NMR spectra from diabetic patients with bone metabolism disorder group (♦) and healthy volunteer group (■). **A,** Scores plot of PLS-DA (R^2x=80.5%, R^2Y=96.2%, Q^2 (cum)=67.2%). **B,** Scores plot of OPLS-DA (R2=92.6%, Q2= 81.4%); **C,** Loading plot of OPLS-DA.

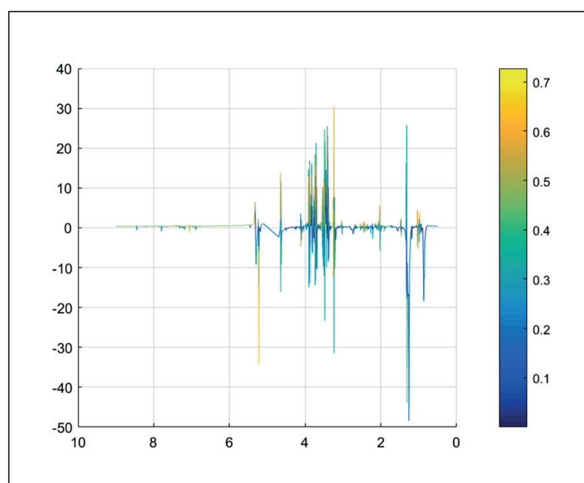

Figure 4. Correlation coefficient-loading plot of the differentiation among diabetic patients with bone metabolism disorder group and healthy volunteer group.

Discussion

The pathophysiological mechanisms of diabetic osteoporosis are not clear so far. For osteoporotic patients secondary to TIDM, bone remodeling slows down for relatively fast resorption of bone resulting from deficient insulin secretion, leading to low BMD, descendant mineralization, and impaired microarchitecture[14]. Differently, in patients with T2DM-induced osteoporosis, in spite of high BMD, decreased microarchitecture quality of bone is noticed due to factors, such as sensorimotor deficiency and neuropathy caused by disturbed metabolism. Hyperglycemia, oxidative stress and the accumulation of advanced glycation end products (AGEs) are likely to compromise collagen properties, increase marrow adiposity, release inflammatory factors, and adipokines from visceral fat. All these factors could induce functional changes of osteocytes, potentially leading to DOP. Additionally, factors like hypoglycemia caused by treatment, certain antidiabetic medications (such as thiazolidinediones) that exert direct effect on bone and mineral metabolism, may account for DOP possibly[15-23.]

In this study, the ^{1}H-NMR metabolomics technology combined with pattern recognition analysis were used to characterize the endogenous metabolites differences between diabetic patients with disordered bone metabolism and healthy volunteers, through which we could basically identify subtle changes between the two groups and offer evidence for the existence of metabolic disturbance in DOP patients. In addition, metabolomics is expected to be a novel diagnostic method of DOP in the future.

The Association Between Blood Glucose Control and DOP

It was found that the bone mass arose as the HbA_{1c} reduced in DOP patients with treatment, which showed a negative effect of hyperglycemia on osteoblasts and emphasized the significance of hypoglycemic therapy to DOP. In the cases of well glucose control, the risk of DOP declined and the BMD increased[24,25]. By osteoblasts in culture, it was proved that chronic hyperglycaemia downregulated the expression of osteocalcin gene (BGLAP) and had influence on the uptake of calcium[26,27]. Moreover, acidosis caused by hyperglycemia might also enhance the resorption of bone[28]. Hyperglycemia and oxidative stress might impact mesenchymal stem cell differentiation with adipogenesis having advantage over bone formation. Due to hyperglycemia and enhanced levels of oxidative stress, the accumulation of AGEs was increased in patients with diabetes. All these above-mentioned factors reflected a close relationship between glucose and the development of DOP.

The Association Between Energy Metabolism and DOP

In the process of bone remodeling, a large amount of energy are required, especially in the dissolution of crystalline calcium phosphate or hydroxyapatite and degradation of fibrillary collagen. Once the energy metabolism is disturbed, the bone formation would be impeded, accompanying impaired skeletal neurosensory function according to the theory of use and disuse.

It was observed that the concentration of citric acid and α-ketoglutaric acid declined in research group, which are intermediate in the TCA cycle and utilized by all aerobic organisms to produce usable chemical energy, whereas the levels of glutamine and other metabolites in the TCA cycle increased, indicating a low energy status of the osteoblasts and adverse effect on bone metabolism[29-34].

As a type of cyclitol, the structure of inositol allows the construction of a great quantity of stereo chemically unique molecules which are involved in every regard of cellular regulation[35,36] . Also, as the highest proportion of organic molecules in phosphate groups, inositol is taken for

one of the most important constituents of inositol pyrophosphates, and able to adjust a lot of biological processes by the metabolism of energy and the production adenosine triphosphate (ATP) possibly[37]. In comparison with normal group, the level of inositol elevated in research group, reflecting a declined status of energy metabolism in bone regeneration.

The Association Between Amino Acid Metabolism and DOP

In the formation of DOP, perturbations of amino acid metabolism was found, accounting for deficient bone biosynthetic material and disordered bone metabolic signal.

Compared to normal group, the content of N-acetylglycoprotein was measured higher in research group, while the concentration of O-acetylglycoprotein was detected lower in that, showing a broken homeostasis of glycosylation and modification for the participation of these metabolites. The cytoplasmic and nuclear proteins are modified after translation by O-linked β-N-acetylglucosamine (O-GlcNAc), because of glucose flux through the hexosamine biosynthetic pathway. In the plasma membrane, O-GlcNAc transferase (OGT) is assembled from the nucleus, where the OGT catalyzes the insulin signaling pathway by O-GlcNAc for its dynamic modification. The excessive expression of OGT in liver damages the expression of insulin-responsive genes and gives rise to insulin resistance. These findings underlined the contribution of nutritional cues regulating insulin signaling through O-GlcNAc to the molecular mechanism and verified the relationship between this modification and the etiology of insulin resistance and T2DM[38-49].

As the results shown, the proline level decreased in research group. As one of the main substrates in the biosynthesis of collagen biosynthesis, proline is demanding for the formation of collagen molecule[50]. In trabecular bone, the spiral structure of the polypeptide chain has a close relationship with proline and hydroxyproline in collagen. Thus, hydroxyproline can strengthen the structure of bone by producing hydrogen and oxygen bridge and the lack of proline could influence the synthesis of new collagen in bone tissues, promoting the develop of DOP to some extent.

The contents of branched chain amino acids and alanine were higher in the research group than normal group. The increase of these branched chain amino acids and alanine appeared to be a signal of disordered glucose utilization in diabetic patients, for their engagement in the glucose-alanine cycle, which imply the process of hepatic gluconeogenesis[51,52]. Besides, the content of 1-methyl-histidine declined while creatine elevated in research group, which is commonly seen in malnourished or decomposed skeletal muscle, reflecting an abnormal stability of bone. Evidently, these changes in metabolites leads to DOP somehow.

It was detected that the level of glutamate rose in the research group. With the expression of their receptors on bone cells, glutamate could lead to bone resorption[53]. Furthermore, glutamate is one of the important ingredients for osteoblasts producing osteocalcin, which is considered as marker of bone formation. The accumulation of AGEs induced by hyperglycemia might inhibit the synthesis of osteocalcin, contributing to a recession of bone formation[54,55]. The metabolism of glutamine regulates the bioenergetics of osteoblasts and osteocytes directly or indirectly as well, revealing a tight link between glutamine metabolism and degenerative diseases like osteoporosis[56].

In the research group, the level of tyrosine lifted. In the regulation of all biological processes, tyrosine phosphorylation of cellular proteins plays a vital role as signaling event. Both protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) are essential in this signaling network[57]. Yu et al[56] showed that the inhibition of PTK, particularly kinases encoded by c-Src and c-Fms proto-oncogenes, could suppress bone remodelling. Consequently, the regulation of tyrosine phosphorylation is critical in bone function. Still some studies demonstrated that in T2DM, insulin production, and beta cell growth or insulin signaling were regulated by protein tyrosine phosphatase Meg2 (PTPMeg2) by inhibiting the dephosphorylation of insulin receptor[58-61]. All these studies explained the influence of tyrosine on DOP.

Conclusions

In this NMR-based metabolomics study, significant differences between diabetic patients with bone metabolic disorder and normal human in metabolic profiles of their plasma samples were found. There were remarkable changes in TCA, glucose metabolism, energy metabolism, amino acid metabolism, and glycosylation in re-

search group. All these metabolic abnormalities might bring about insufficient of bone synthetic materials and disordered bone metabolism regulation, revealing the underlying mechanism of diabetic bone metabolic abnormalities. Potentially, these metabolic abnormalities could be applied as specific indicators to early diagnosis of diabetic osteoporosis in the near future.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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