

# Plasma metabolite profiling reveals potential biomarkers of giant cell tumor of bone by using NMR-based metabolic profiles

## A cross-sectional study

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### Abstract

Giant cell tumor (GCT) of bone is a locally aggressive bone tumor, which accounts for 4% to 5% of all primary bone tumors. At present, the early diagnosis and postoperative recurrence monitoring are still more difficult due to the lack of effective biomarkers in GCT. As an effective tool, metabolomics has played an essential role in the biomarkers research of many tumors. However, there has been no related study of the metabolomics of GCT up to now. The purpose of this study was to identify several key metabolites as potential biomarkers for GCT by using nuclear magnetic resonance (NMR)-based metabolic profiles.

Patients with GCT in our hospital were recruited in this study and their plasma was collected as the research sample, and plasma collected from healthy subjects was considered as the control. NMR was then utilized to detect all samples. Furthermore, based on correlation coefficients, variable importance for the projection values and *P* values of metabolites obtained from multidimensional statistical analysis, the most critical metabolites were selected as potential biomarkers of GCT. Finally, relevant metabolic pathways involved in these potential biomarkers were determined by database retrieval, based on which the metabolic pathways were plotted.

Finally, 28 GCT patients and 26 healthy volunteers agreed to participate in the study. In the multidimensional statistical analysis, all results showed that there was obvious difference between the GCT group and the control group. Ultimately, 18 metabolites with significant differences met the selection condition, which were identified as potential biomarkers. Through Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Metabolome Database (HMD) database searching and literature review, these metabolites were found to be mainly correlated with glucose metabolism, fat metabolism, amino acid metabolism, and intestinal microbial metabolism. These metabolic disorders might, in turn, reflect important pathological processes such as proliferation and migration of tumor cells and immune escape in GCT.

Our work showed that these potential biomarkers identified appeared to have early diagnostic and relapse monitoring values for GCT, which deserve to be further investigated. In addition, it also suggested that metabolomics profiling approach is a promising screening tool for the diagnosis and relapse monitoring of GCT patients.

**Abbreviations:** 3-HB = 3-hydroxybutyrate, ACC = acetyl-CoA carboxylase, ACLY = adenosine triphosphate citrate lyase, BCAAs = branched-chain amino acids, GCT = giant cell tumor, GPC = glycerophosphocholine, GSH = glutathione, HMD = Human Metabolome Database, KEGG = Kyoto Encyclopedia of Genes and Genomes, MS = mass spectrum, MENK = methionine enkephalin, NMR = nuclear magnetic resonance, OAG = O-acetyl-glycoprotein, OPLS-DA = orthogonal projection to latent structure with discriminant analysis, PCA = principal component analysis, PLS-DA = partial least squares discriminant analysis, ROS = reactive oxygen species, SCFAs = short-chain fatty acids, TG = triglyceride, TMSP = 3-trimethylsilyl-2,2,3,3-d4-propionate, VEGF = vascular endothelial growth factor, VIP = variable importance for the projection.

**Keywords:** biomarkers, GCT, metabolomics

Editor: Raffaele Pezzilli.

Wei Wang, Xilin Liu, and Juan Wu contributed equally to this work as co-first authors.

The study was funded by the following 4 projects: Department of Science and Technology of Sichuan Province (CN) Project, 2014YJ0009; Medical Association of Sichuan province Project, 2016GK001; National Natural Science Foundation of China, 81701625; and Youth Science and Technology Innovation Talent Project of Chengdu Military General Hospital, 41732C112.

The authors have no conflicts of interest to disclose.

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How to cite this article: Wang W, Liu X, Wu J, Kang X, Xie Q, Sheng J, Xu W, Liu D, Zheng W. Plasma metabolite profiling reveals potential biomarkers of giant cell tumor of bone by using NMR-based metabolic profiles. *Medicine* 2019;98:40(e17445).

Received: 4 April 2019 / Received in final form: 27 August 2019 / Accepted: 11 September 2019

<http://dx.doi.org/10.1097/MD.00000000000017445>

## 1. Introduction

Giant cell tumor (GCT) of bone, which accounts for approximately 6% of all primary bone tumors,<sup>[1]</sup> is characterized by extensive bone resorption,<sup>[2]</sup> leading to regional pain and the predisposition to pathologic fractures.<sup>[3]</sup> A slight predilection for females has been reported, with a female-to-male ratio ranging from 1:1.1 to 1:1.5.<sup>[4]</sup> Although GCT may affect all races, there is a strangely high prevalence (20%–30%) for Chinese and southern Indian population, which, however, has not been explained to date.<sup>[4,5]</sup> The most common GCT site is the knee (50%–65% of all cases), followed by the sacrum (approximately 9%) and proximal humerus (4%–8%).<sup>[6–8]</sup> Early diagnosis and surgical resection in the treatment of GCT is recommended, but the overall recurrence rate from 18% to 60% remains high.<sup>[9,10]</sup> Therefore, postoperative follow-up monitoring of tumor recurrence is also very important in the treatment of GCT.

Nowadays, diagnosing GCT and postoperative monitoring is a clinically driven process based on the observation of clinical symptoms and structural changes on x-rays.<sup>[11]</sup> The structural changes on x-rays are the result of the pathological process, but do not make known the pathological process itself; it provides little prognostic information regarding the risk for local recurrence.<sup>[12,13]</sup> In addition, there is currently no well-accepted biomarker available for GCT,<sup>[14]</sup> which leads to early diagnosis of GCT, and relapse monitoring remains difficult.

With the advent of microarray techniques, many pathological processes can be explored and monitored in a global view by the aid of omics-driven, high-throughput technologies.<sup>[15]</sup> In several genomics, transcriptomics, and proteomics researches, a series of genes and protein molecules (parathyroid hormone-related protein, nuclear factor Ib, zinc finger protein-14, glutathione peroxidase 1, etc) have been found to be abnormally expressed in GCT patients,<sup>[16–19]</sup> which were also expected to be potential biomarkers for GCT. The genomics, transcriptomics, and proteomics bring the concept of global systems biology by means of which integrating multivariate biological information is advocated for the better understanding of complex systems.<sup>[20]</sup> However, these profiling methods only focus on upstream genetic and protein variations.<sup>[21]</sup>

As a rapidly developing field of systems biology, metabolomics represents a new method that delineates a wide panel of metabolic parameters and thus allows a global and potentially more personalized diagnostic means to be used in combination with conventional protocols.<sup>[22]</sup> The fundamental for the application of clinical metabolomics is that perturbations in a biological.<sup>[23]</sup> Due to changes in the tumor microenvironment, many researches have previously identified tumor cells can exhibit significant alterations in metabolic pathways such as glycolysis, respiration, the tricarboxylic acid cycle, oxidative phosphorylation, lipid metabolism, and amino acid metabolism.<sup>[24]</sup> Metabolic profiling has been used to identify potential biomarkers for osteosarcoma, and glycolysis, inositol cyclic phosphate, and carnitine were found to be potential generic prognostic biomarkers in osteosarcoma patients.<sup>[25,26]</sup> But until now, there have been no reports of GCT metabolomics researches.

In this study, we performed metabolic profiling to observe metabolites in plasma from GCT patients and healthy controls using nuclear magnetic resonance (NMR) spectroscopy. Plasma is the most frequently used specimen for exploring the systematic alteration of metabolite in humans because it directly reflects the

global metabolic state of the human body, and the collection and handling of plasma is relatively easy.<sup>[27]</sup> Plasma samples were collected from patients with GCT who were scheduled for operation in our hospital, and plasma samples from volunteers without other underlying diseases were extracted as the control group. The difference of metabolites between the 2 groups was analyzed subsequently. Then, potential biomarkers, which could be used for early diagnosis of GCT and monitoring of postoperative recurrence, were screened afterwards. To the best of our knowledge, it was the first metabolomics study for GCT.

## 2. Methods

### 2.1. Patients

The case group recruited in our study comprised of those volunteers who were hospitalized and treated for GCT in the Department of Orthopedics of our hospital (General Hospital of Western Theater Command) from January, 2016 to December, 2017. Inclusion criteria were as follows: patients who were aged >18 years with independent power to sign; and patients who had definite diagnosis of GCT based on the clinical manifestations combined with the results of x-ray and magnetic resonance imaging. Exclusion criteria were as follows: patients who had other systemic diseases such as diabetes and high blood pressure; patients with distant metastases; patients who had a previous history of medication within a week; and postoperative pathological results of patients suggested to be suffering from nongiant cell tumors. In addition, volunteers with a basic match of age and sex were recruited as the control group in the healthy population of our hospital. All volunteers incorporated in this study must carefully review and sign informed consent. At the same time, the Ethics Committee in our hospital (General Hospital of Western Theater Command) has approved the clinical study.

### 2.2. Sample collection and preparation

Fasting plasma samples were collected from all volunteers in every morning. Blood samples were first loaded into a sterile anticoagulant tube containing ethylene diamine tetra-acetic acid at room temperature and then centrifuged at 4°C (5000 rpm for 10 minutes).<sup>[28]</sup> At last, plasma on the upper level was taken in the Eppendorf tube with 300  $\mu$ L each and stored at  $-80^{\circ}\text{C}$  for further analysis.

The plasma samples were prepared for NMR analysis by mixing 300  $\mu$ L of plasma with 300  $\mu$ L of Phosphate buffer saline (1.5M  $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ , pH 7.4, 10% v/v  $\text{D}_2\text{O}$ ) containing 0.6 mg 3-trimethylsilyl-2,2,3,3-d<sub>4</sub>-propionate (TMSP) as a chemical shift reference ( $\delta$  0.00 ppm). All of the samples were then centrifuged at 4°C (12,000 rpm for 10 minutes), and the supernatants were pipetted into 5-mm NMR tubes for NMR analysis.<sup>[29]</sup>

### 2.3. NMR spectroscopy

The proton NMR spectra of the plasma were recorded at 300K on a Bruker Avance II 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a  $^1\text{H}$  frequency of 600.13 MHz and equipped with a broadband-observe probe.

A water-presaturated Carr-Purell-Meiboom-Gill pulse sequence (recycle delay  $-90^{\circ}-(\tau-180^{\circ}-\tau)_n$ - acquisition) was employed to attenuate the NMR signals from macromolecules.

A spin-spin relaxation delay ( $2n\tau$ ) of 76.8 ms and a spin-echo delay  $\tau$  of 400  $\mu$ s were used. Typically, the 90° pulse was set to 13.7  $\mu$ s, and 32 transients were collected in 49,178 data points for each spectrum using a spectral width of 9590 Hz and an acquisition time of 2.56 seconds.<sup>[30]</sup> After the Fourier transformation, phase correction and baseline correction were performed using the TopSpin software package, version 3.0 (Bruker Biospin, Rheinstetten, Germany). The <sup>1</sup>H chemical shifts referred to the TMSP peak at  $\delta$  0.00.

The corrected NMR spectra, corresponding to the chemical shift range of  $\delta$  0.8 to 9.0, were imported into AMIX 3.9.5 (Bruker Biospin). All of the spectra were reduced into integral regions of equal lengths of 0.002 ppm. The region  $\delta$  4.20 to 5.20 that contained the resonance from residual water and the region of  $\delta$  5.50 to 6.00 that contained the resonance from urea were removed to avoid any contributions of H<sub>2</sub>O and urea to intergroup differentiations.

#### 2.4. Multivariate data analysis

Multivariate statistical data analysis included principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal projection to latent structure with discriminant analysis (OPLS-DA), which were performed to globally understand the metabolic changes of GCT.

Principal component analysis was performed using the software packages Simca-P version 11.5 (UmetricsAB, Umea, Sweden). PCA is an unsupervised analytical pattern recognition tool that provides an overview of complex data through an examination of the covariance structure. The multivariate data can be displayed in a few principal components as a set of “scores” that highlight general trends and outliers.<sup>[31]</sup>

Partial least squares discriminant analysis and OPLS-DA were performed using a unit variance scaled approach. PLS-DA is a supervised regression method to maximize the covariance between the predictor space and the response space. It can predict responses in the population using the predictor matrix. OPLS-DA was performed with the NMR data to facilitate interpretation of loading. The model coefficients were back-calculated from the coefficients incorporating the weight of the variables and plotted with color-coded coefficients to enhance interpretability of the model.<sup>[31]</sup> The color-coded correlation coefficients indicate the significance of the metabolite contribution in predicting the response. In this research, the correlation coefficient cut-off values depended on number of samples needed in each group to obtain statistical significance based on the discrimination significance of the Pearson product-moment correlation coefficient at the level of  $P < .05$ .<sup>[32]</sup> Two parameters— $R^2Y$  and  $Q^2$ —were used for evaluation of the models.  $R^2Y$  explains the latent variables of the sums of squares of all Xs and Ys.  $Q^2$  reflects the cumulative cross-validated percentage of the total variation that can be predicted by the current latent variables. High coefficient values of  $R^2Y$  and  $Q^2Y$  ( $>0.5$  is acceptable) showed good discrimination and the predictive ability.<sup>[15]</sup> PLS-DA models were cross-validated using permutation tests (200 times) to observe whether there was an overfitting, whereas the permutation test was evaluated by using cross validation, with  $R^2$  and  $Q^2$  as correlation coefficients of the cross validation. It is generally believed that the intercept of the  $Q^2$  regression line on the Y axis (permutation plot) being 0 or less than 0 indicates a reliable and effective model, without overfitting.<sup>[33]</sup>

#### 2.5. Potential biomarkers selection and metabolic pathway analysis

According to correlation coefficients, variable importance for the projection (VIP) values and  $P$  values of all metabolites obtained from multidimensional statistical analysis, the most critical metabolites were selected as the potential biomarkers for GCT. The criteria for selection were: absolute value of correlation coefficients of metabolites  $>$  cut-off values or VIP value  $>$  1.0, and  $P < .05$  at the same time.<sup>[21,31]</sup> Then, based on these potential biomarkers, KEGG, HMD, and related literature review were retrieved to identify related metabolic pathways associated with these key metabolites. Meanwhile, combined with the analysis of the pathogenesis of GCT, screened metabolic pathways were plotted eventually.

#### 2.6. Statistical methods

The data were expressed as the means  $\pm$  SDs. The differences between the 2 groups were analyzed using Student 2-sided  $t$  test, and differences involving more than 2 groups were analyzed using 1-way analysis of variance. The level of significance was set at  $P < .05$ . Statistical analysis was performed by using the Statistical Package for the Social Sciences, version 20.0 (SPSS, Chicago, IL).

### 3. Results

#### 3.1. Participants

At our invitation, 26 GCT patients and 28 healthy volunteers agreed to participate in the study. The clinical information of patients and controls was summarized in Table 1. As listed in table, the age, sex, and body mass index value of the control group were basically matched with those of patients with GCT.

#### 3.2. <sup>1</sup>H NMR spectra of samples

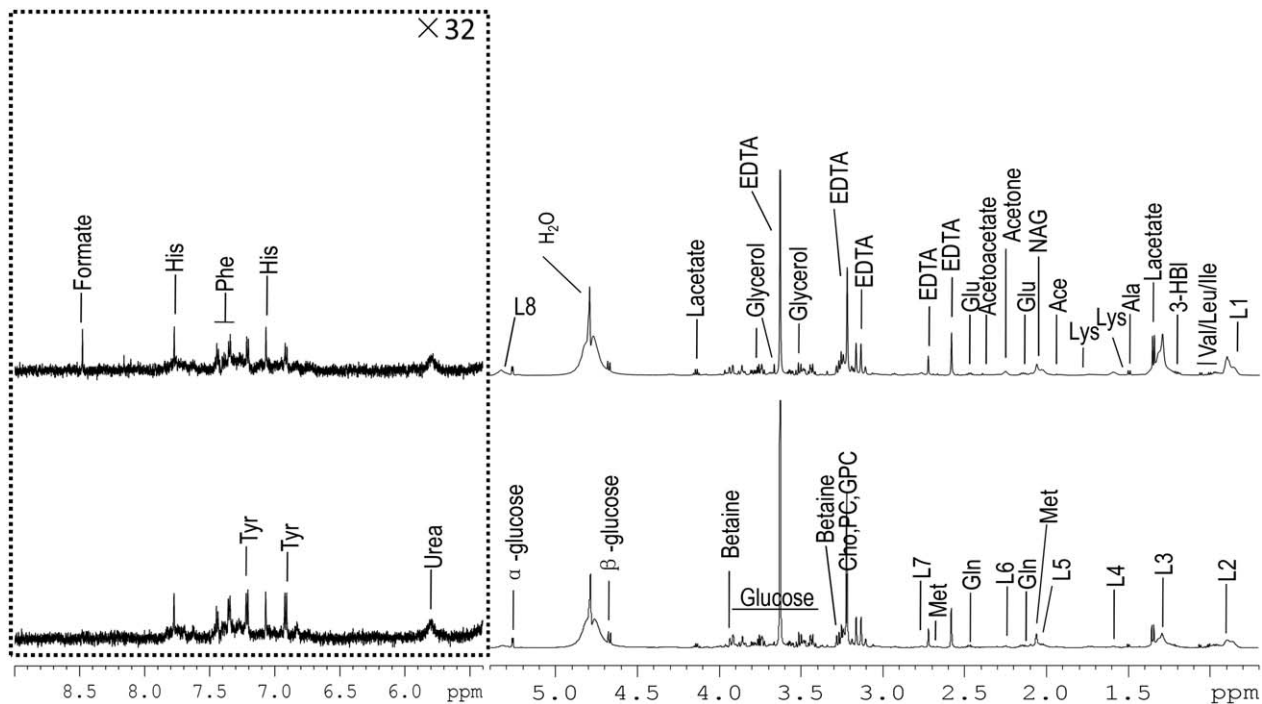
Figure 1 shows typical <sup>1</sup>H NMR spectra of the plasma sample taken from randomly selected people in the GCT and control groups. NMR signals were assigned to the <sup>1</sup>H resonances of specific metabolites (Table 2). The plasma samples mainly contained a series of amino acids, glucose metabolites and lipid metabolites.

**Table 1**  
The participant characteristics for plasma metabolomics analysis.

Characteristics	GCT patients	Healthy controls	$P^*$
Number of participants	28	26	—
Age (y, mean $\pm$ SD)	40.6 $\pm$ 11.3	38.0 $\pm$ 11.5	.405
Range	24–60	20–58	
Sex (n, %)			1.000
Female	18 (35.7%)	17 (34.6%)	
Male	10 (64.3%)	9 (65.4%)	
BMI (kg/m <sup>2</sup> , mean $\pm$ SD)	21.2 $\pm$ 3.2	22.5 $\pm$ 4.0	.199
Campanacci stages			
I	5	—	—
II	13	—	—
III	10	—	—
Tumor site			
Distal femur	13	—	—
Proximal humerus	6	—	—
Sacrum	9	—	—

BMI = body mass index, GCT = giant cell tumor of bone.

\* Calculated by Student's  $t$ -tests for continuous variables and  $\chi^2$  tests for categorical variables between AS patients and healthy controls.



**Figure 1.** Representative 600 MHz  $^1\text{H}$  NMR spectra of plasma from the GCT group (upper layer) and healthy controls (next layer). 3-HB=3-hydroxybutyrate, EDTA=ethylene diamine tetra-acetic acid, GCT=giant cell tumor of bone, Gln=glutamine, GPC=glycerophosphocholine, HC=healthy controls, His=histidine, Ile=isoleucine, L1= $\text{CH}_3(\text{CH}_2)_n$ , L2= $\text{CH}_3\text{CH}_2\text{CH}_2\text{C}=\text{C}$ , L3= $-(\text{CH}_2)_n-$ , L4= $\text{CH}_2\text{CH}_2\text{CO}$ , L5= $-\text{CH}_2\text{C}=\text{C}-$ , L6= $\text{CH}_2\text{CO}$ , L7= $\text{C}=\text{CCH}_2\text{C}=\text{C}$ , L8= $-\text{CH}=\text{CH}-$ , Leu=leucine, Met=methionine, NAG=N-acetyl-glycoprotein, NMR=nuclear magnetic resonance, OAG=O-acetyl-glycoprotein, Phe=phenylalanine, TG=triglyceride, Tyr=tyrosine, Val=valine.

### 3.3. Multivariate data analysis of NMR data

Figure 2 illustrates the results of PCA analysis of GCT and the control group. There was significant difference regarding the PCA score plots of plasma samples between groups. However,

there was 1 obvious outlier (GCT patients). To prevent outlier from affecting the results of analysis, clinical information of the patient was consulted again. It was confirmed that the patient had no abnormalities and completely met the inclusion and

**Table 2**

**Summary of potential biomarkers of GCT by plasma metabolomics analysis.**

Metabolite	Status*	Chemical shift	Correlation coefficient (GCT vs HC) <sup>†</sup>	VIP value <sup>†</sup>	FC <sup>‡</sup>	P <sup>§</sup>
Lactate	↑	1.33 (d), 4.12 (q)	0.699	2.15	1.6	<.001
GPC	↓	3.23 (s), 3.69 (t), 4.33 (t)	-0.873	2.68	0.315	<.001
Betaine	↓	3.26 (s), 3.91 (s)	-0.903	2.15	0.7	<.001
OAG	↑	2.15 (s)	0.477	1.87	1.21	.028
TG (L2, L3, L5, L7)	↓	0.88 (t), 1.27 (m), 2.01 (m), 2.76 (m)	-0.334	1.27	0.76	.017
Acetoacetate	↑	2.29 (s)	0.499	1.47	1.31	.018
3-HB	↑	1.20 (d), 2.32 (dd), 2.42 (dd), 4.16 (m)	0.439	1.47	1.10	.041
Methionine	↓	2.14 (s), 2.16 (m), 2.65 (t), 3.87 (t)	-0.398	1.37	0.75	.015
Lysine	↓	1.49 (m), 1.74 (m), 1.92 (m), 3.03 (t), 3.76 (t)	-0.399	1.30	0.71	.004
Leucine	↑	0.96 (d), 0.97 (d), 1.72 (m), 1.72 (m), 3.73 (t)	0.431	1.26	1.19	.030
Glutamate	↑	2.07 (m), 2.35 (m), 3.75 (m)	0.334	1.24	1.12	.039
Glycerol	↑	3.65 (dd), 3.56 (dd), 3.77 (m)	0.709	1.19	1.13	.037
Valine	↑	1.00 (d), 1.05 (d), 2.28 (m), 3.62 (d)	0.353	1.18	1.17	.032
Isoleucine	↑	0.94 (t), 1.02 (d), 1.99 (m), 1.26 (m), 1.47 (m), 3.68 (d)	0.348	1.16	1.08	.046
Acetate	↓	1.92 (s)	-0.318	1.15	0.87	.035
Formate	↓	8.47 (s)	-0.835	1.10	0.294	<.001
Glucose	↓	3.42 (dd), 3.54 (dd), 3.71 (dd), 3.78 (m), 3.84 (m), 5.26 (d)	-0.467	0.99	0.77	.018
Acetone	↑	2.23 (s)	0.44	0.58	1.22	.026

3-HB=3-hydroxybutyrate, FC=fold change, GCT=giant cell tumor of bone, GPC=glycerophosphocholine, HC=healthy control, L2= $\text{CH}_3\text{CH}_2\text{CH}_2\text{C}=\text{C}$ , L3= $-(\text{CH}_2)_n-$ , L5= $-\text{CH}_2\text{C}=\text{C}-$ , L7= $\text{C}=\text{CCH}_2\text{C}=\text{C}$ , OAG=O-acetyl-glycoprotein, TG=triglyceride, VIP=variable importance for projection.

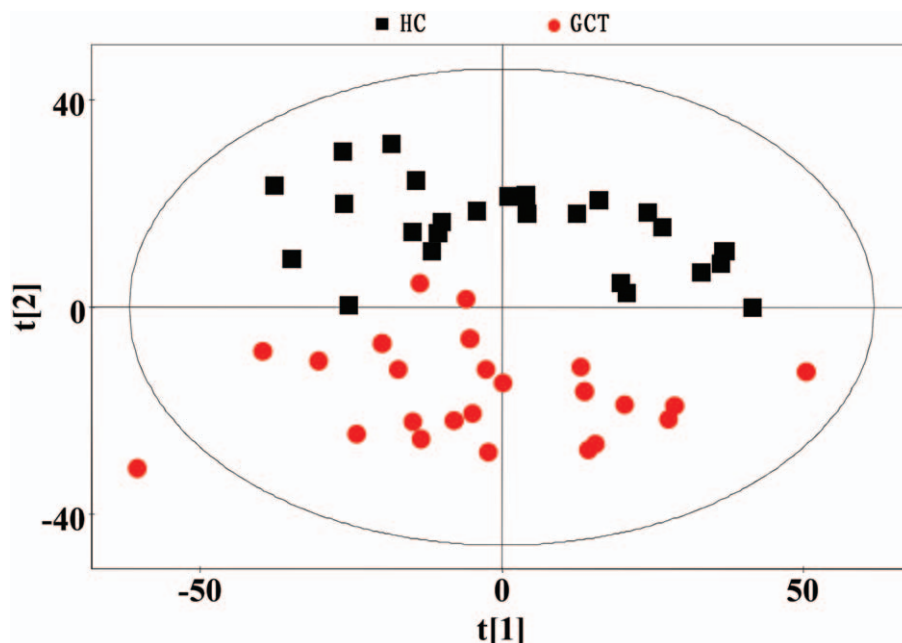
\* Relative concentrations compared to healthy controls: ↑=up-regulated, ↓=down-regulated.

<sup>†</sup> Correlation coefficient and VIP value were obtained from OPLS-DA analysis.

<sup>‡</sup> Fold change between GCT patients and healthy controls.

<sup>§</sup> P value determined from Student *t* test.





**Figure 2.** Principal component analysis (PCA) score plots based on the  $^1\text{H}$  NMR spectra of plasma metabolites obtained from the patients of giant cell tumor of bone (GCT, red circles) and healthy controls (HC, black squares). NMR=nuclear magnetic resonance.

exclusion criteria. The patient was thus involved in the study eventually.

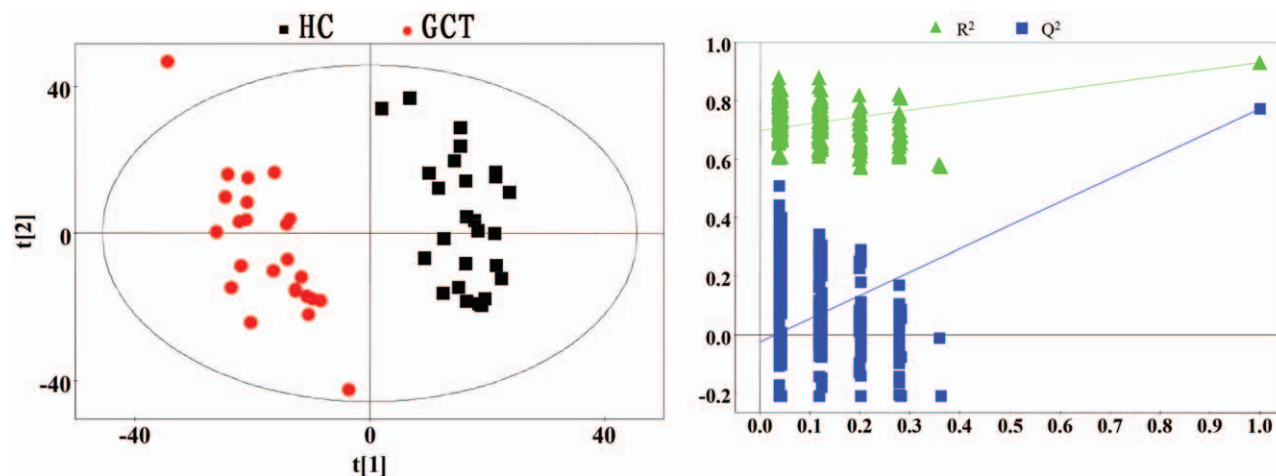
The PLS-DA result is present in Fig. 3. The score plots highlighted 2 clusters corresponding to the GCT and control groups. In the PLS-DA model, the parameters of  $R^2Y$  and  $Q^2$  were 0.931 and 0.819. This result revealed good discrimination and predictive ability in this model. Model cross-validation through permutation tests (200 times) generated intercepts of  $R^2$  (0.699) and  $Q^2$  (-0.020). The low values of the intercepts that the model was not overfitted.

The OPLS-DA analytical results and the correlation coefficient loading plots are shown in Fig. 4. The parameters of  $R^2Y$  and  $Q^2$

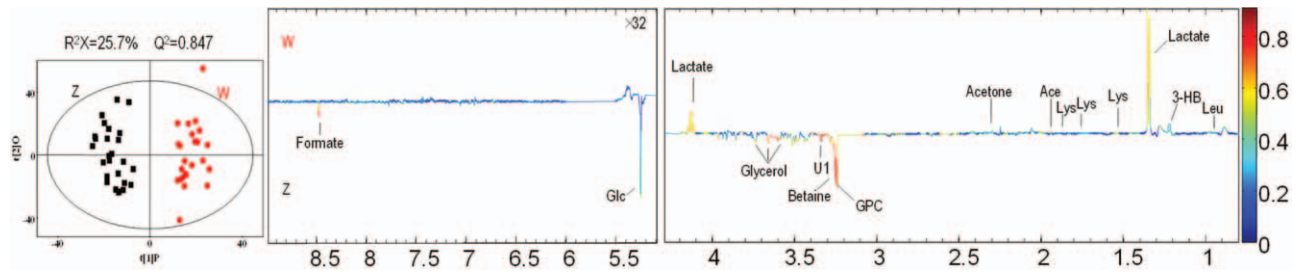
were 0.931 and 0.847, which also revealed very good discrimination and predictive ability in this model. In addition, based on our results from the correlation coefficient loading plots, correlation coefficient cut-off value for potential markers was set at 0.312.

### 3.4. Potential biomarkers and pathway analysis

According to the obtained correlation coefficients, VIP values, and  $P$  values of all metabolites during multivariate analysis, 18 metabolites were screened as potential biomarkers for GCT (Table 2). The box-and-whisker plots of these potential



**Figure 3.** Partial least squares discriminant analysis (PLS-DA) score plots (left panel) and statistical validation of the corresponding PLS-DA model by permutation analysis (right panel) based on the  $^1\text{H}$  NMR spectra of plasma metabolites obtained from the patients of giant cell tumor of bone (GCT, red circles) and healthy controls (HC, black squares). NMR=nuclear magnetic resonance.

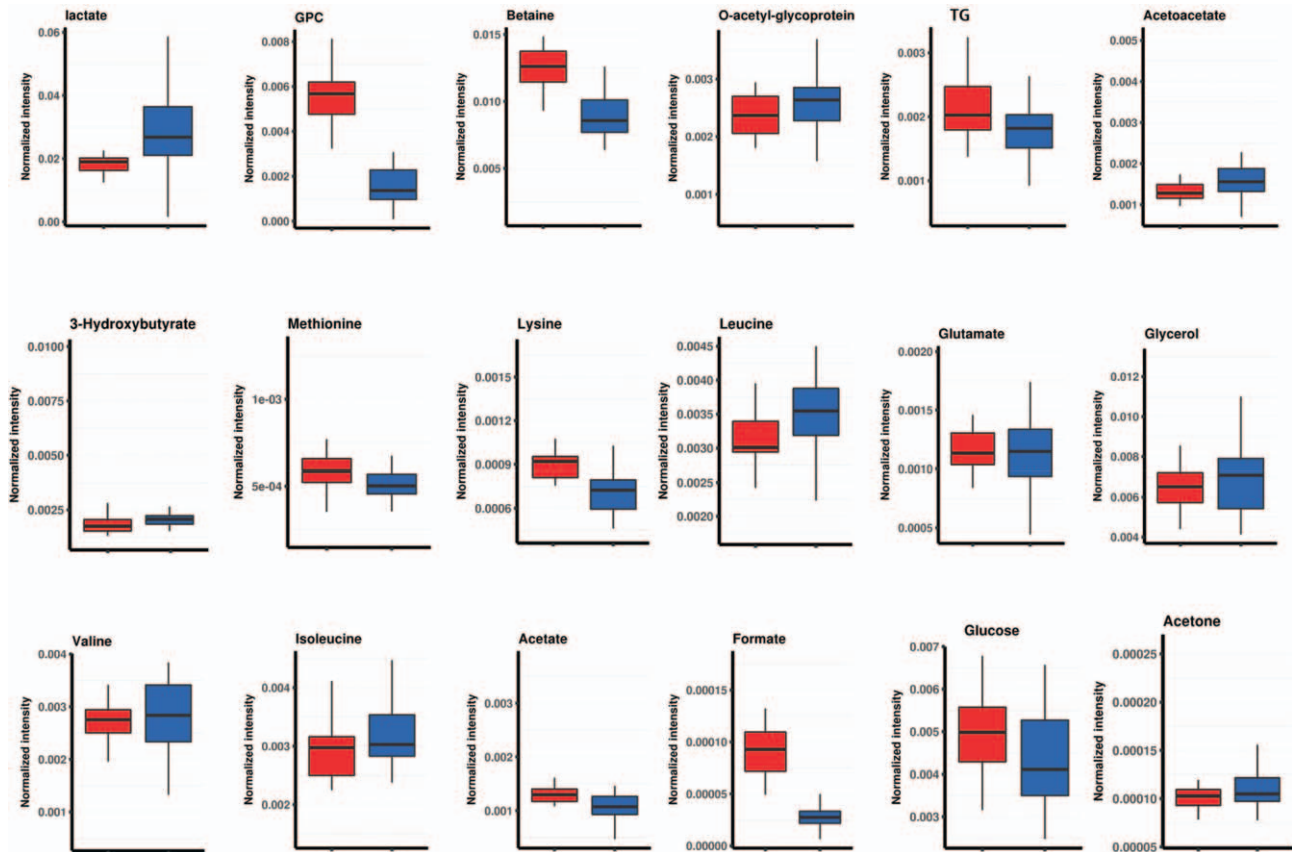


**Figure 4.** Orthogonal projection to latent structure with discriminant analysis (OPLS-DA) score plots (left panel) and the corresponding coefficient loading plots (right panel) based on the  $^1\text{H}$  NMR spectra of plasma metabolites obtained from the patients of giant cell tumor of bone (GCT, red circles) and healthy controls (HC, black squares). NMR = nuclear magnetic resonance.

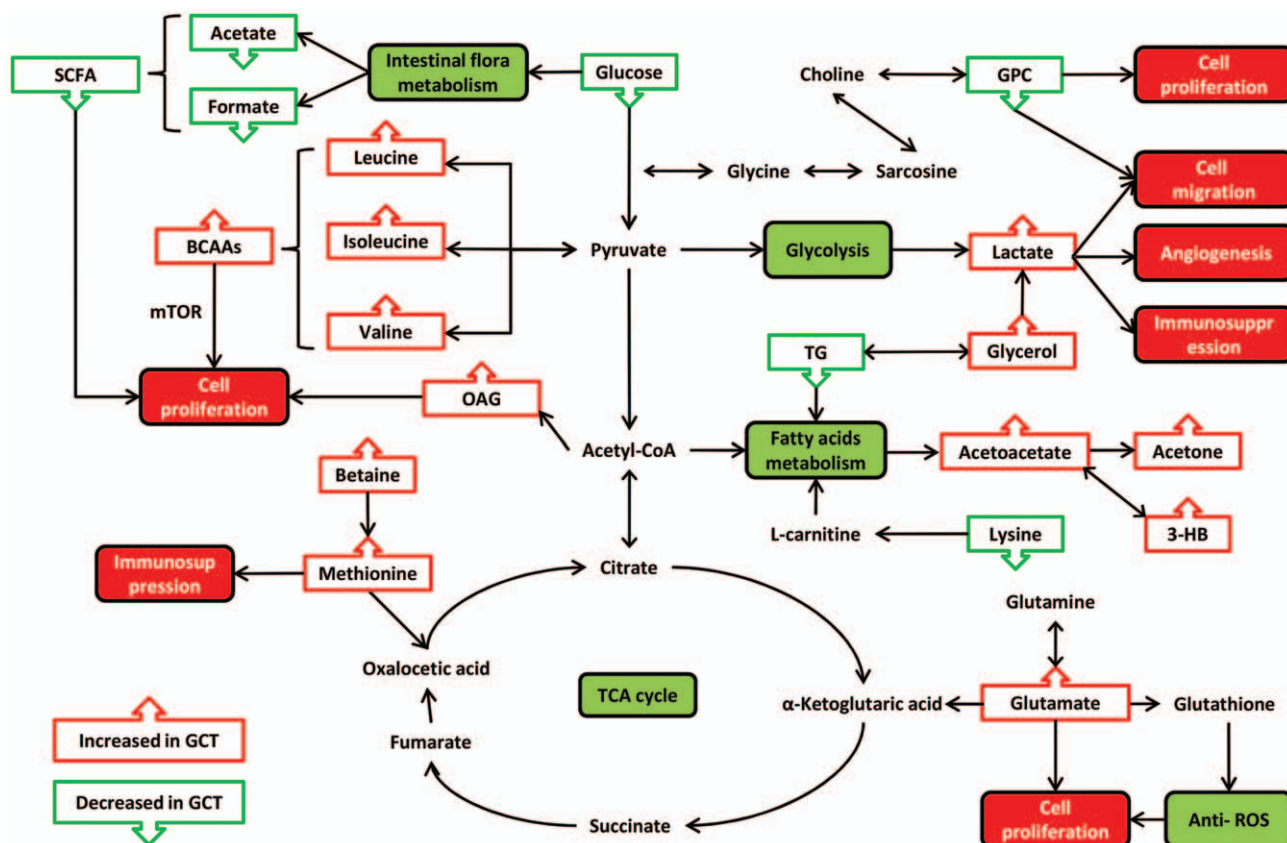
biomarkers also suggested obvious difference between groups (Fig. 5). Based on this, it was found that these metabolites mainly involved glucose metabolism, lipid metabolism, amino acid metabolism, and intestinal microbial metabolism through database searching (KEGG, HMD) and literature review. The disturbance of these metabolic pathways might be closely related to changes in tumor microenvironment such as tumor cells proliferation, migration, angiogenesis, and immune escape. Combined with these results, a metabolic pathway map (Fig. 6) was plotted to exhibit the relationship between these metabolites more intuitively.

#### 4. Discussion

The concept of metabolomics was first proposed in 1999 by Nicholson et al. Metabolomics is more concerned with the phenotype of organisms than genomics, transcriptomics, and proteomics.<sup>[34,35]</sup> Metabolomics analysis can reflect the function and state of the organism within a certain period of time because cells are metabolized at the time of maintaining their function. In such a way, tiny changes of gene and protein expression will be magnified on metabolites in a certain disease state. Several changes that cannot be reflected from these 2 substances can be detected in metabolomics.<sup>[24]</sup> Numerous studies have shown that the



**Figure 5.** Box-and-whisker plots showing the relative levels of selected potential biomarkers for GCT. The red box on the left represents the HC group and the blue box on the right represents the GCT group. Horizontal line in the middle portion of the box, median; bottom and top boundaries of boxes, lower and upper quartile; whiskers, 5th and 95th percentiles. GCT = giant cell tumor of bone, GPC = glycerophosphocholine, HC = healthy controls, TG = triglyceride.



**Figure 6.** Altered metabolic pathways for the most relevant distinguishing metabolites (potential biomarkers) between GCT and HC. The metabolites with green border were up-regulated in GCT, whereas with red border indicated metabolites that were down-regulated. 3-HB=3-hydroxybutyrate, BCAAs=branched-chain amino acids, GCT=glutathione tumor of bone, GPC=glycerophosphocholine, HC=healthy controls, OAG=O-acetyl-glycoprotein, ROS=reactive oxygen species, SCFAs=short-chain fatty acids, TG=triglyceride.

occurrence and progression of tumors are strongly correlated with metabolic abnormalities.<sup>[36]</sup> As early as the 30s of the past century, biochemist Otto Warburg discovered that tumor cells preferred glycolysis. To be specific, even under the condition of sufficient oxygen supply, tumor cells still maintained vigorous glycolysis and consumed a large number of glucose. This abnormal metabolic way was described as “Warburg effect.”<sup>[37]</sup> With the development of isotopic tracing, metabolomics, and other techniques, the complex appearance of tumor metabolism is becoming increasingly clearer. Meanwhile, glycolysis is suggested not to be the only metabolic characteristic of cancer. Multiple molecular mechanisms, both intrinsic and extrinsic, converge to alter core cellular metabolism and provide support for the 3 basic needs of tumor cells: rapid adenosine triphosphate (ATP) generation to maintain energy status; increased biosynthesis of macromolecules; and tightened maintenance of appropriate cellular redox status. To meet these needs, tumor cells acquire alterations to the metabolism of all 4 major classes of macromolecules: carbohydrates, proteins, lipids, and nucleic acids.<sup>[38]</sup> Metabolomics, as an effective tool, has played an important role in the study of searching for potential biomarkers in many tumors.<sup>[39]</sup> However, there is still no metabolomics study on GCT up to now.

Two most powerful and commonly used analytical methods for metabolic fingerprinting are mass spectrum (MS) and NMR spectrometry.<sup>[40,41]</sup> NMR is a non-invasive and non-destructive technique that can provide complete structural analysis of a wide

range of organic molecules in complex mixtures.<sup>[42]</sup> It has a series of advantages compared with MS, including simple sample preparation, not require chromatographic separation, and inexpensive on a consumables basis.<sup>[43]</sup> In this study, the NMR method was used for the first time to study the metabolomics of GCT. Through multivariate data analysis, 18 metabolites with obvious difference were screened and selected as potential biomarkers for GTC.

“Warburg effect” is the earliest discovered and most studied abnormal metabolism phenomenon of tumors. In this study, lactate content was measured to be increased significantly in plasma of GTC patients, whereas glucose content decreased significantly, suggesting that glycolysis might be abnormally active in GTC patients. A large number of lactic acid produced by glycolysis might lead to acidification of tumor cells microenvironment. Acidified microenvironment could further decompose and destroy the surrounding normal cellular matrix, which was conducive to invasion and metastasis of tumor cells.<sup>[44]</sup> At the same time, acidified microenvironment played a protective role in tumor cells; possible reason might be that some endogenous immune cells, immune molecules, and exogenous alkaline anticancer drugs would become invalid under acidified conditions.<sup>[44]</sup> In addition, the increase of lactic acid could promote the release of related factors such as vascular endothelial growth factor (VEGF), which could further promote angiogenesis.<sup>[45]</sup> Previous studies have shown that VEGF was up-regulated in

GCT, and angiogenesis was closely related to proliferation and invasion of GCT tumor cells.<sup>[46]</sup>

Abnormal lipid metabolism is another major feature of tumor metabolism. Tumor cells can proliferate continuously by using the energy produced by fatty acid  $\beta$ -oxidation.<sup>[47]</sup> Prior studies have documented that the expression and activity of key genes in the fatty acid metabolism pathway, including ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC), fatty acid synthase, and stearoyl-coenzyme A desaturase, are observed in various tumors and are closely related to poor prognosis of these patients.<sup>[48–50]</sup> Moreover, the growth of tumor could be inhibited by reducing the expression of these metabolic enzymes or using specific inhibitors to inhibit the activity of metabolic enzymes.<sup>[51]</sup> In our study, the content of triglyceride (TG) was decreased obviously in plasma of patients with GCT, and its metabolite of glycerol increased significantly. More importantly, 3 metabolites of acetoacetate, acetone, and 3-hydroxybutyrate (3-HB) were increased remarkably in plasma of GCT patients. In addition, as a critical precursor of carnitine synthesis, the content of lysine was also decreased evidently in plasma of patients with GCT. Carnitine was critical in lipid metabolism, which could transport long-chain fatty acids from the outer membrane of mitochondria to the inner membrane for  $\beta$ -oxidation to generate energy in the form of acyl carnitine.<sup>[52]</sup> The decrease of lysine content might be the result of the oversynthesis of carnitine. The above results suggested that the lipid metabolism pathway (especially the fatty acid  $\beta$ -oxidation) might be abnormally active in GCT. Apart from this, TG, glycerol, acetoacetate, acetone, 3-HB, and lysine were biomarkers that could reflect the abnormal metabolism.

It was found that glutamate levels were significantly increased in plasma of GCT patients. Glutamate is the metabolite of glutamine, whereas the active metabolism of glutamine is an important form of energy metabolism of tumor cells. Glutamine metabolism provides intermediate products for tricarboxylic acid cycle, which not only offers material for biosynthesis but also supplies energy for cell metabolism, thereby promoting tumor growth.<sup>[53]</sup> Simultaneously, glutamine metabolism participates in a variety of cell metabolic processes and thus inhibits apoptosis. Reactive oxygen species is a by-product of cell growth. The ROS level can be significantly increased due to the proliferation of tumor cells. Extremely high level of ROS can cause oxidative stress in cells, and lead to cell senescence or death. As the metabolite of glutamine, glutamate is an important raw material for the synthesis of glutathione (GSH); apart from this, GSH can against ROS and maintain it at a lower level.<sup>[54]</sup> Therefore, there might be an extremely active glutamine metabolism in GCT, and its metabolite of glutamate could be regarded as potential biomarker of GCT.

Furthermore, there were distinctness increase of all branched-chain amino acids (BCAAs) in plasma of GCT patients, including valine, leucine, and isoleucine. It has been proved that BCAAs can promote the proliferation of tumor cells by activating mammalian target of rapamycin signaling.<sup>[55]</sup> Additionally, glycerophosphocholine (GPC) was found to be significantly lower in plasma of GCT patients, and it has been confirmed in other studies that its declined level is closely related to tumor cells proliferation and migration.<sup>[56]</sup> At the same time, *o*-acetyl-glycoprotein (OAG) was sharply elevated in plasma of patients with GCT. Related research has confirmed that its increased level is associated with the proliferation of tumor cells.<sup>[57]</sup> We also discovered that short-chain fatty acids (SCFAs), which were produced by intestinal

microbial metabolism, including acetate and formate, were significantly decreased in plasma of GCT patients. SCFAs have been demonstrated to be able to inhibit the proliferation and induce the apoptosis of tumor cells, and it has a certain antitumor effect.<sup>[58]</sup> To sum up, BCAAs, GPC, OAG, SCFAs, and other metabolites were tightly related to the proliferation and migration of tumor cells. Therefore, their abnormal changes in GCT might reflect the corresponding pathological process.

Immunosuppression is 1 of the characteristics of tumor microenvironment. Numerous studies have shown that tumor cells can make use of almost all negative regulatory mechanisms of immune system itself to establish an immunosuppressive network for tumor microenvironment.<sup>[59]</sup> In the study, levels of betaine and methionine were both elevated clearly in plasma of GCT patients. Methionine is the precursor of methionine enkephalin (MENK). The latter is involved in the regulation of immune response and the proliferation of cells. The effect of MENK on immune system is mainly manifested by immune stimulation at low concentration and immunosuppression at high concentration.<sup>[60]</sup> In this regard, the increase of methionine in plasma may provide sufficient raw materials for the synthesis of MENK, thereby promoting the formation of tumor microenvironment in the immune suppression in GCT. Because betaine is a precursor of methionine,<sup>[61]</sup> the elevation of both betaine and methionine in GCT may be inter-related.

In general, the metabolomic profiles we have obtained were promising. With good discrimination and predictive ability in multivariate analysis models, the profiles could aid in screening out a series of effective potential biomarkers for the diagnosis and relapse monitoring of GCT. However, some limitations of this research needed to be noticed. First, the sample was limited in size; the results should be validated with a larger number of GCT patients in the future. Second, plasma was the only explored sample in this study; accordingly, metabolomics of GCT in tissues and urine samples can be investigated in the future to provide more accurate complementary information concerning this topic.

## 5. Conclusions

In this study, metabolomics study on GCT is first conducted with the expectation of achieving more comprehensive metabolomic profiles and further screening potential biomarkers that may contribute to the diagnosis of GCT and recurrence monitoring. By multivariate data analysis and metabolic pathway analysis, it is finally speculated that glucose metabolism, lipid metabolism, amino acid metabolism, intestinal microbial metabolism, and other metabolic pathways may be disordered in GCT patients. Apart from this, disorders in these metabolic pathways may reflect essential pathological processes such as proliferation and migration of tumor cells, and immune escape in GCT. In brief, we believe that the 18 metabolites with significant differences in these metabolic pathways can be considered as potential biomarkers for GTC. Still, further validation studies are needed to confirm these results before transition from bench to bedside.

## Acknowledgments

We appreciate for the cooperation all the volunteered patients and the staff working in the outpatient center and in-patient department had given us. Special thanks also to Mr Yao-Yu Chen who helped us to revise the language issue of the manuscript.



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