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Original Article

Evaluation of related abnormal metabolite profiles in patients with bone marrow failure syndrome (BMFS)

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Abstract: Bone marrow failure syndrome (BMFS) is a rare but life-threatening disorder, usually manifesting as (pan) cytopenia. The current study explored abnormal metabolites of patients with BMFS using liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in both positive and negative modes, further investigating changes in metabolites in patients with BMFS. Samples from 20 patients with BMFS (experimental group) and 20 healthy volunteers (normal control, NC) were collected. Metabolite profiles were systematically analyzed by LC-QTOF-MS. A wavelet-based method was used to find and align LC-MS peaks. Substantial differences were detected between metabolite signatures of NC and BMFS groups with partial least-squares discriminant analysis (PLS-DA) score plots. The following metabolites were deemed to be potentially responsible for differences in the metabolic characteristics of the two groups: Phenylalanyl-alanine, tyrosyl-phenylalanine, methionyl-arginine, acetyl-L-tyrosine, *N*-a-acetylcitrulline, *N*-formyl-L-methionine, clupanodonyl carnitine, L-octanoylcarnitine, phosphatidylcholine (PC; 14:0/18:2 and 20:4/16:0), phosphatidylethanolamine (PE; 22:5/16:0 and 18:2/P-16:0), and phosphatidylinositol (PI; 20:4/16:0). These identified abnormal biomarkers in metabolic profiles suggest that there is a substantial metabolite disorder in patients with BMFS. This may be useful for further investigation and diagnosis of BMFS.

Keywords: Metabolomics, bone marrow failure syndrome (BMFS), LC-QTOF-MS, phospholipid

Introduction

Acquired bone marrow failure syndromes (BMFSs) are characterized by hematopoietic stem and (or) progenitor cell damage in the bone marrow, as well as a subsequent decrease in a single blood cell type (cytopenia) or all blood cells (pancytopenia) [1]. A low volume of bone marrow cells and pathological hematopoiesis are the main pathological features. Clinical manifestations include anemia, hemorrhaging, and infections caused by different degrees of three lineages of blood cells [2, 3]. BMFS may be triggered by exposure to environmental compounds, such as chemicals and drugs, viral infections, and endogenous antigens that may be produced by genetically altered bone marrow cells [4]. BMFS diagnosis depends mainly on the morphology of bone marrow cells and cytogenetics detection technology, including routine blood examinations, peripheral blood and bone marrow cell morphology tests, bone marrow biopsies, flow cytometry, and cytogenetic methods [5, 6]. Bone marrow biopsies are generally considered to be the most reliable diagnostic method [7]. However, bone marrow biopsies are invasive. This is not conducive to clinical epidemiological screening. Thus, alternative and more effective methods are needed to improve diagnosis of acquired BMFS.

Metabolomics is a new branch of systems biology developed after genomics, transcriptomics, and proteomics. Metabolomics is the study of life science through examining external stimuli, pathological physiological changes, gene mutations, and levels of metabolites during dynamic response [8]. Compared with other histological techniques for large molecules with similar chemical properties, such as DNA, RNA, and

proteins, metabolomics mainly analyzes low-molecular-weight compounds, in which the molecular weight is < 1000. These include peptides, amino acids, and their derivatives, such as amine substances and lipids. Therefore, metabolomics can reflect the body's physiological and pathological state [9-11]. However, the body is a very complicated organic unit. Metabolite groups are complicated and regulate the metabolic balance of the body, maintaining a healthy state. An abnormal metabolism of specific components can induce disease [12, 13]. Therefore, finding and selecting specific biomarkers closely related to a disease is the end goal of metabolomics research.

Aplastic anemia (AA), myelodysplastic syndrome (MDS), and paroxysmal nocturnal hemoglobinuria (PNH) are common forms of acquired BMFS, closely related in terms of symptoms and characteristics [14, 15]. However, research concerning metabolic profiles associated with acquired BMFS has not been reported. The current study preliminarily analyzed metabolite profiles in the serum of patients with BMFS, comparing the results to those found in healthy people. The aim of this study was to obtain a comprehensive understanding of metabolic changes during the onset of BMF, discovering potential biomarkers that are characteristic and sensitive.

Materials and methods

Patients

A total of 20 patients with BMFS, including 9 patients with MDS and 11 patients with AA, diagnosed at the Second Affiliated Hospital of Qiqihar Medical University, from January 2016 to October 2017, were enrolled in this study. Data associated with these cases was collected to compare with data associated with 20 healthy control subjects (NC). All patients were diagnosed first by routine blood biochemical tests. Standards of diagnosis were in accord with those described by a Chinese expert consensus on the diagnosis and treatment of aplastic anemia (2017) and an expert consensus on the diagnosis and treatment of MDS (2014). Fasting blood samples were collected in the early morning from BMFS subjects and the NC group. All samples were placed in normal blank blood tubes. They were static in an upright position for 2-3 hours at 4°C. The samples were then centrifuged at 3,000 rpm for 20 minutes at 4°C. Serum samples were collected and then frozen at -80°C for further testing. This study was approved by the Institutional Review Board of Qiqihar Medical University. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki (QY2018 [NO. 23]).

Sample preparation

Serum sample preparation for analysis of the BMFS group and control group was based on the following procedures. Briefly, serum was taken from -80°C storage and placed at 4°C for 50 minutes. After the samples were vortexed for 30 seconds, the upper solution (100 μL) from each sample was transferred into a clean 2-ml centrifuge tube, then acetonitrile (400 μL) was added. After the samples were vortexed for 2 minutes, the well-vortexed solutions were centrifuged at 14,000 × g for 15 minutes at 4°C. The upper solution (100 μL) was again extracted and transferred into a clean 2-mL centrifuge tube. It was then evaporated to dryness in a heat block at 35°C under a gentle stream of nitrogen gas. The residue was dissolved in 100 μL of acetonitrile/water (1:3, v/v) via vortexing for 1 minute. It was then centrifuged at 14,000 × g for 15 minutes at 4°C. The supernatant (100 μL) was transferred to the autosampler vials and injected into the LC-QTOF-MS measurement device for analysis. Before LC-QTOF-MS analysis, all samples were randomized. To ensure the stability and repeatability of UPLC/MS systems, pooled quality control (QC) samples were prepared by mixing equal amounts of supernatant samples from 20 BMFS subjects and 20 normal subjects.

Chromatography

A 10-μL aliquot of the pre-treated sample was injected into a 3.0 × 100-mm (1.8 mm) ZORBAX SB-C18 column (Agilent Technologies, Santa Clara, CA, USA) for RRLC (6530 series; Agilent Technologies). As MS was carried out with an electrospray ionization (ESI) source operating under positive-ion (ESI+) and negative-ion (ESI-) modes (see below), two sets of mobile phases were used for chromatography. The mobile phase for ESI+ was a mixture of acetonitrile, containing 0.1% formic acid (A), and water containing 0.1% formic acid (B). The mobile phase for ESI- was a mixture of acetonitrile (A) and water (B). For both modes, a linear mobile phase gradient was used as follows: 2% A, held

Table 1. Subject characteristics

Characteristic	BMFS group (n = 20)	[NC OR Control] group (n = 20)	t
Gender (M/F)	11/9	12/8	
Age	50.4 ± 22.0	48.25 ± 13.23	0.375
WBC	2.91 ± 1.97*	6.79 ± 1.17	7.57
Neu	1.19 ± 1.02*	4.06 ± 1.19	8.289
HGB	68.75 ± 22.56*	140.3 ± 12.2	12.48
PLT	61.8 ± 65.74*	217.7 ± 44.18	8.802

Abbreviations: WBC, white blood cell count; Neu, neutrophil count; HGB, hemoglobin level; PLT, platelet count; *p < 0.01.

for 1 minute; 1-18 minutes, increased to 98% A; 18-21 minutes, held at 98% A; 21-21.1 minutes, decreased to 2% A and 21.1-28 minutes, held at 2% A. The mobile phase flow rate was 0.3 mL/min at 40°C.

Mass spectrometry

MS was performed with an Agilent 6530-QTOF (Agilent Technologies) equipped with ESI+ and ESI- modes. The capillary voltage was set as 4.0 kV for the ESI+ mode and 3.5 kV for the ESI- mode. Nitrogen was used as the desolvation gas at a flow rate of 10 L/minute. Desolvation temperature was set at 350°C. Centroid data were collected in full scan mode from 50 to 1000 m/z.

Data preprocessing and annotation

Raw data were converted into mzdata-format files by Mass-Hunter Qualitative Analysis Software (Agilent Technologies). These files were imported to the XCMS package in R for preprocessing. Default XCMS parameters were used, with the following exceptions: xcmsSet (fwhm = 10); group (minfrac = 0.5, bw = 30). Preprocessing results generated a data matrix that consisted of retention times, mass-to-charge ratio (m/z) values, and peak intensities. CAMERA in R was used for annotation of isotope peaks, adducts, and fragments in the peak lists [16]. After data processing, there were 3,281 ions in the ESI+ mode and 2,011 ions in the ESI- mode for subsequent statistical analysis.

Statistical analysis

Principal component analysis (PCA) was used to detect grouping trends and outliers [17]. Kruskal rank-sum testing was used to determine the significance of different levels for each metabolite ($p < 0.05$). Partial least squares discriminant analysis (PLS-DA) was performed to discriminate the performances of metabolites between NC and BMFS groups [18, 19]. To avoid overfitting, permutation tests with 100 iterations were performed to validate the supervised model. Variable importance in the projection (VIP) for each metabolite was calculated based on the established PLS-DA model. Potential metabolic biomarkers were selected based on *p*-values and VIP values, with thresholds of 0.05 and 1, respectively. The Kruskal rank-sum test was performed in the R platform to determine whether differences in levels of each metabolite were significant [20]. PCA and PLS-DA were performed using SIMCA-P (version 11.5; Umetrics, Malmö, Sweden) [19].

In addition, a paired t-test was used to compare general clinical characteristics between BMFS and NC groups, including average age, white blood cell count (WBC), neutrophil count (Neu), and hemoglobin (HGB) and platelet (PLT) levels. Statistical analysis was performed using Prism 6.0 software (GraphPad, USA). Results are reported as mean ± the standard deviation. Statistical significance is defined as $p < 0.05$ or as $p < 0.01$.

Results

Clinical characteristics of subjects

This study enrolled 20 BMFS patients (F/M = 9/11, average age = 50.4 ± 22.0 years). A total of 9 patients were diagnosed with MDS, while 11 were diagnosed with AA. In addition, 20 healthy individuals (F/M = 8/12, average age = 48.25 ± 13.23 years) were enrolled as the NC group. This study compared serum characteristics between the two groups, including WBC, Neu, HGB, and PLT. Detailed data concerning these patients are listed in **Table 1**. WBC, Neu, HGB, and PLT values were significantly lower in serum samples from patients with BMFS, compared to controls ($p < 0.01$). These changes also matched the clinical diagnostic criteria of BMFS patients.

Quality control evaluation of metabolic data

PCA, which was performed on all samples, revealed that the QC samples were tightly clus-

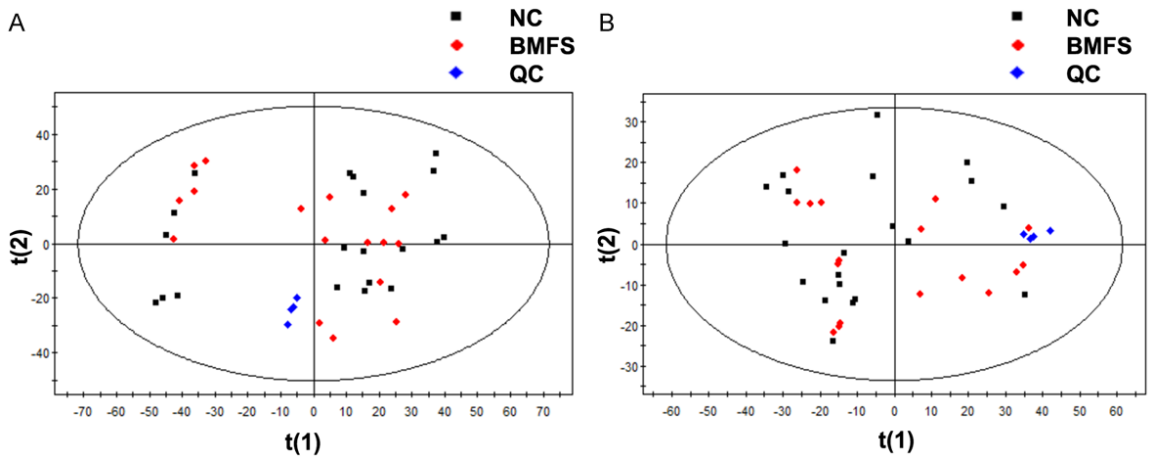

Figure 1. PCA score plots discriminating BMFS and NC. They were used to evaluate the robustness of the metabolic profiling platform and dispersion trends between BMFS and NC. QC samples were tightly clustered, which suggests that the metabolic profiling platform was stable.

tered in PCA score plots. This indicated the robustness of the current metabolic profiling platform. There were no outliers. There were separation trends between NC and BMFS subjects, suggesting differences between these two groups (**Figure 1**).

PLS-DA model analysis

All statistically significant ions ($p < 0.05$ and VIP > 1) based on the ESI+ and ESI- modes were subjected to further analysis. A supervised PLS-DA model was used to determine differences between NC and BMFS groups. The PLS-DA score plot revealed a clear separation between NC and BMFS groups in both the ESI+ mode (**Figure 2A**) and ESI- mode (**Figure 2C**). The PLS-DA models contained two predictive components in the ESI+ mode (R2Ycum = 0.735, Q2cum = 0.375) and two components in the ESI- mode (R2Ycum = 0.702, Q2cum = 0.352). To avoid overfitting, permutation tests with 100 iterations containing two predictive components were performed [4]. Results showed that almost all permuted Q2cum values were lower than the original values (**Figure 2B** and **2D**), assuring the validity of the supervised models.

Abnormal metabolic profile of patients with BMFS

The present study found 13 metabolites exhibiting significant differences between BMFS subjects and healthy individuals (**Table 2**). **Figure 3** shows a heat map demonstrating the dynamic changes in BMFS and NC biomarkers, including expression levels for each metabolite in every sample. Levels of phenylalanyl-alanine, tyrosyl-phenylalanine, methionyl-arginine, acetyl-L-tyrosine, and *N*-a-acetylcitrulline were elevated in the serum samples of patients with BMFS and compared with healthy individuals. In contrast, levels of *N*-formyl-L-methionine, clupanodonyl carnitine, L-octanoylcarnitine, PC (14:0/18:2), PC (20:4/16:0), PE (22:5/16:0), PE (18:2/P-16:0), and PI (20:4/16:0) were lower in the serum of patients with BMFS, compared with that of healthy controls. Bar graphs are presented indicating the mean and standard error measurements for each potential biomarker (**Figure 4**). A clear tendency toward an increase or decrease for each of the metabolic markers was noted in the BMFS group. Phenylalanyl-alanine, tyrosyl-phenylalanine, methionyl-arginine, acetyl-L-tyrosine and *N*-a-acetylcitrulline were higher in BMFS serum samples, while *N*-formyl-L-methionine, clupanodonyl carnitine, L-octanoylcarnitine, PC (14:0/18:2), PC (20:4/16:0), PE (22:5/16:0), PE (18:2/P-16:0), and PI (20:4/16:0) levels were lower in the serum of patients with BMFS.

Discussion

Amino acids, peptides, and proteins are the most essential biomolecules for life processes. Their detection is of enormous importance in many fields, including medical diagnostics. Amino acids, the most important nutrients in

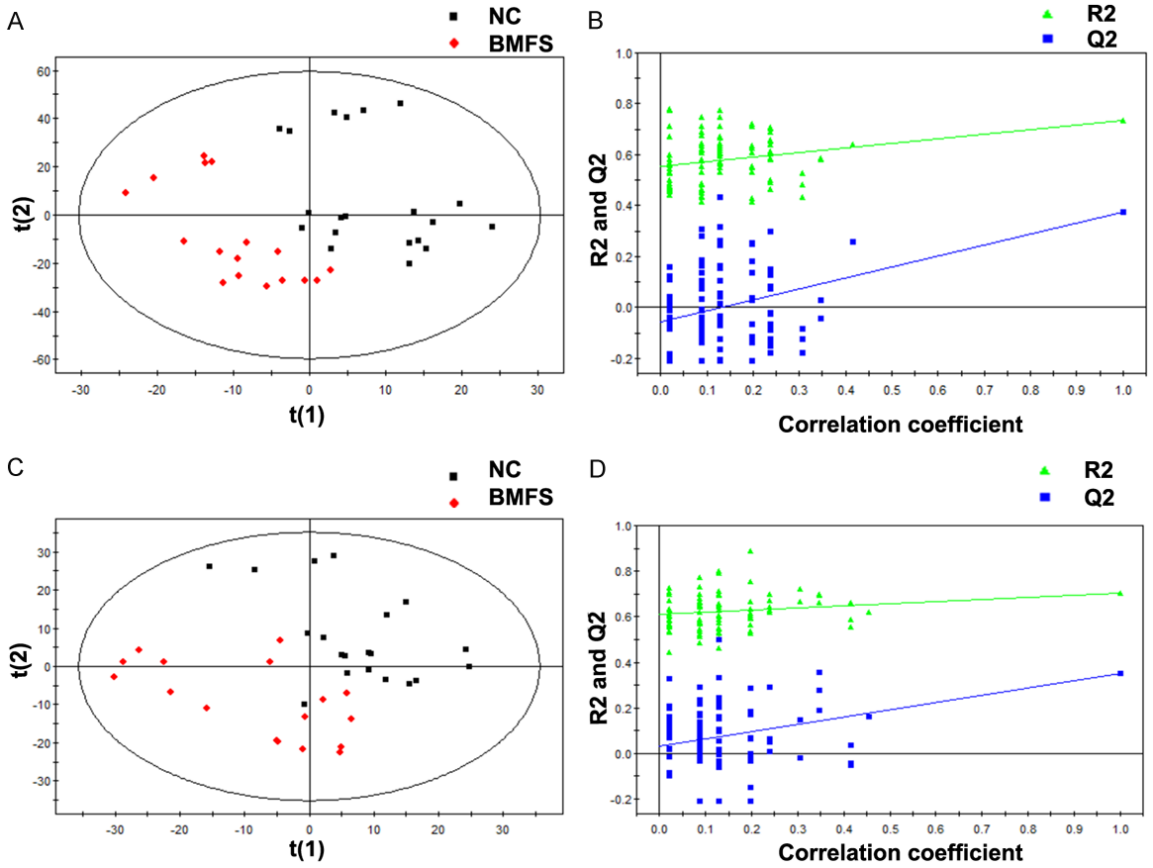

Figure 2. A. PLS-DA score plots discriminating BMFS and NC in the ESI+ mode, which reveals a clear separation between the two groups. B. Validation plot discriminating BMFS and NC in the ESI+ mode, which helps to avoid overfitting. C. PLS-DA score plots discriminating BMFS and NC in the ESI- mode, which also reveals a clear separation between the two groups. D. Validation plot discriminating BMFS and NC in the ESI- mode, which helps to avoid overfitting.

Table 2. Detailed information about 13 plasma metabolites

Num	Metabolite	m/z	RT (min)	*p*	VIP	Mode
1	*N*-Formyl-L-methionine	178.0538	0.91	0.006091	2.47	ESI+
2	Phenylalanyl-alanine	237.1236	1.03	0.000717	2.76	ESI+
3	*N*-a-Acetylcitrulline	240.0966	7.06	0.017448	2.36	ESI+
4	L-Octanoylcarnitine	310.1996	10.90	0.020548	2.25	ESI+
5	Methionyl-arginine	328.1389	1.05	0.00505	2.65	ESI+
6	Tyrosyl-phenylalanine	329.1511	1.05	0.022271	2.03	ESI+
7	Clupanodonyl carnitine	496.337	16.83	0.041162	1.90	ESI+
8	Phosphatidylethanolamine (PE) (18:2/P-16:0)	722.5024	18.30	0.002307	1.84	ESI+
9	Phosphatidylcholine (PC) (14:0/18:2)	752.5145	17.18	0.000123	1.19	ESI+
10	Phosphatidylethanolamine (PE) (22:5/16:0)	766.5338	18.24	0.024119	1.70	ESI+
11	Phosphatidylcholine (PC) (20:4/16:0)	804.5513	22.36	0.041162	1.79	ESI+
12	Phosphatidylinositol (PI) (20:4/16:0)	859.5378	18.10	0.035479	1.63	ESI+
13	Acetyl-L-tyrosine	222.0768	15.82	0.032898	1.12	ESI-

Note: *p*, the *p*-value from the Kruskal rank test; VIP, the variable importance in the projection (VIP) values in fitting PLS model.

the body, are involved in the synthesis of proteins, fatty acids, and ketones. They are vital for important physiological processes, such as glycolysis and tricarboxylic acid circulation [21].

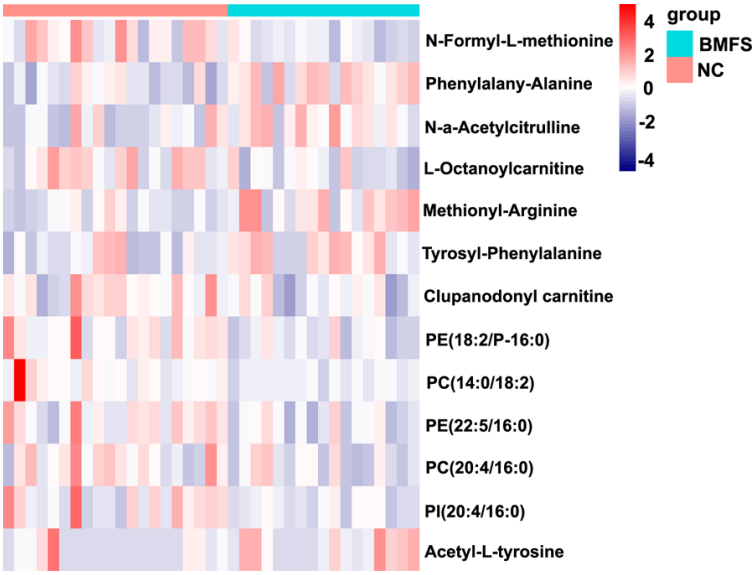

Figure 3. Heat map demonstrating dynamic changes in biomarkers for BMFS and NC groups, which illustrates expression levels of each metabolite in every sample. Each box represents a sample; A box that is a darker shade red indicates higher expression in that sample.

Dipeptides are the simplest active peptides. They undergo rapid enzymatic degradation to amino acids in the body. As the main source of amino acids in the body, their levels are closely related to various physiological activities [16, 22]. Therefore, a change in dipeptide concentrations in the blood can help to predict occurrence, development, and prognosis of possible amino acid metabolic disorders in the body [23].

In the present study, certain metabolite levels, including phenylalanyl-alanine, tyrosyl-phenylalanine, methionyl-arginine, acetyl-L-tyrosine and *N*-a-acetylcitrulline, were higher in the serum of patients with BMFS, compared with those in the serum of healthy people. Additionally, levels of *N*-formyl-L-methionine were significantly lower in BMFS patients than those in healthy controls. Phenylalanyl-alanine is rapidly degraded to phenylalanine and alanine in the body. Alanine is a neutral non-essential amino acid with the highest concentration in blood. It is widely involved in metabolic processing of sugar, fat, and proteins in the body [24, 25]. Phenylalanine is an essential amino acid. It is mainly metabolized to produce tyrosine in liver tissue. Phenylketonuria occurs when phenylalanine metabolism is impaired [26]. Tyrosine is an important amino acid in protein molecular construction. It is involved in the active regulation of various neural signaling pathways [27]. Acetyl-L-tyrosine results from a side chain reaction of tyrosine. It can be converted to tyrosine by an enzymatic reaction. Disordered tyrosine metabolism in the body induces certain diseases, such as uric melanuria [24], tyrosinemia [28-30], Parkinson's disease, and Alzheimer's disease [31]. Methionyl-arginine can be decomposed into methionine and arginine. Arginine is a component of proteins in the body. Its metabolites can supply nitrogen sources and promote cell proliferation [32]. Arginine can also be catalyzed by nitric oxide synthase to produce bioactive nitric oxide, which acts as an antioxidant [33]. *N*-a-acetylcitrulline can generate citrulline by the action of *N*-acetyl-L-citrulline deacetylase [34]. Citrulline can regulate the content of arginine in the body through arginine-citrulline circulation [35, 36]. At the same time, citrulline is an intermediate product of the ornithine cycle, which involves the biosynthesis of various pyrimidines and polyamines in the human body [37]. *N*-formyl-methionine can be converted to methionine by enzymes [38]. Methionine is an essential amino acid that constitutes the human body. It has important physiological functions, providing methyl through methionine circulation for the organism's biological methylation [39]. *N*-formyl-methionine also promotes protein synthesis. As an *N*-formylmethionyl transport RNA, it can activate methionine fragments to enter the ribosome [40]. The current study showed that levels of phenylalanyl-alanine, tyrosyl-phenylalanine, methionyl-arginine, acetyl-L-tyrosine, and *N*-a-acetylcitrulline were increased in the blood of patients with BMFS. This suggests that there may be insufficient amino acid production in the blood of patients with BMFS and there may be related amino acid metabolic disorders. Levels of *N*-formyl-L-methionine were decreased in the blood of patients with BMFS. These observations indirectly suggest that there may be an obstacle of RNA transfer into the ribo-

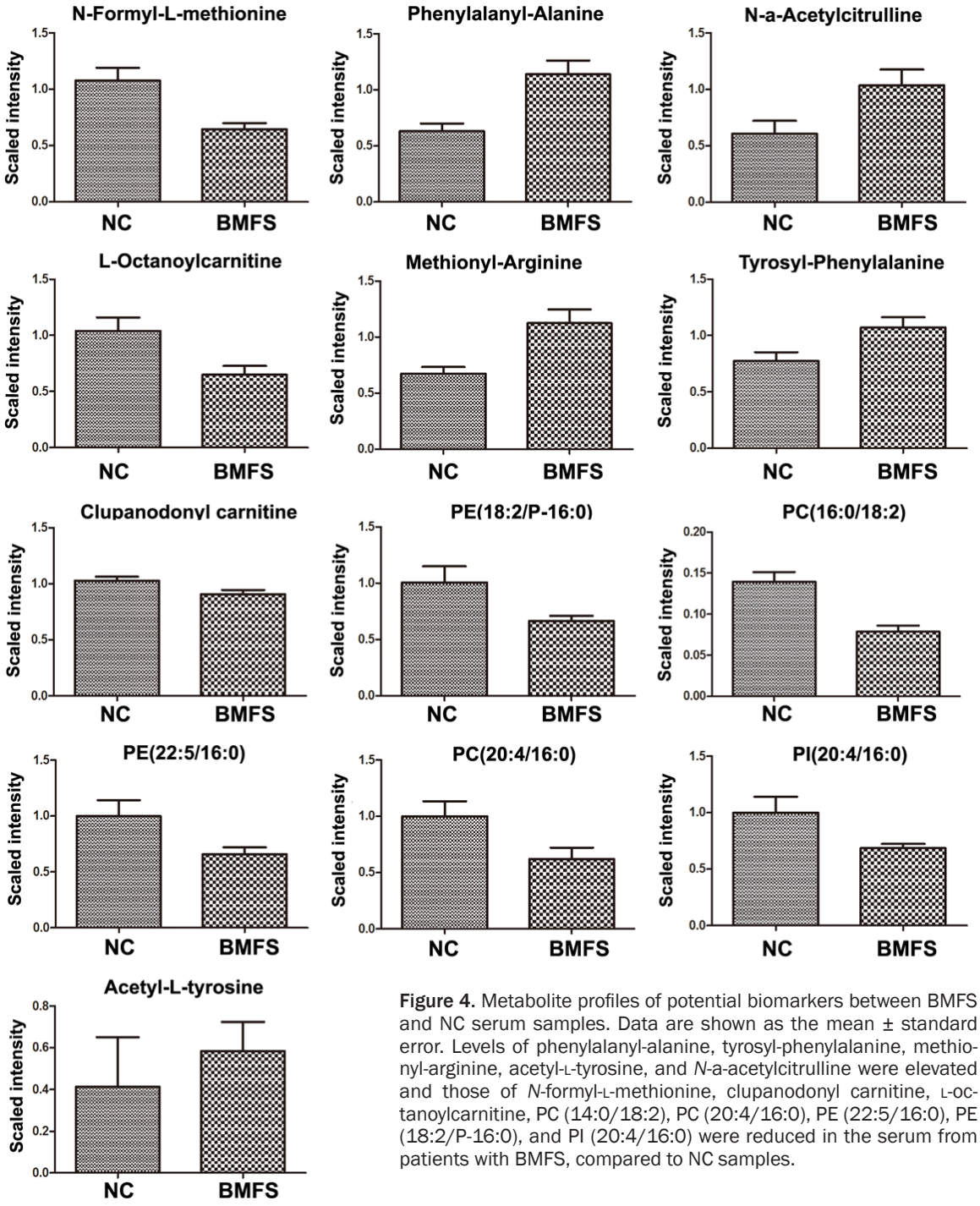

Figure 4. Metabolite profiles of potential biomarkers between BMFS and NC serum samples. Data are shown as the mean ± standard error. Levels of phenylalanyl-alanine, tyrosyl-phenylalanine, methionyl-arginine, acetyl-L-tyrosine, and *N*-a-acetylcitrulline were elevated and those of *N*-formyl-L-methionine, clupanodonyl carnitine, L-octanoylcarnitine, PC (14:0/18:2), PC (20:4/16:0), PE (22:5/16:0), PE (18:2/P-16:0), and PI (20:4/16:0) were reduced in the serum from patients with BMFS, compared to NC samples.

some, leading to the restriction of ribosome protein synthesis. In this study, compared to metabolite levels in the blood of normal people, some metabolite levels, such as levels of L-octanoylcarnitine, clupanodonyl carnitine, PC (14:0/18:2), PC (20:4/16:0), PE (22:5/16:0), PE (18:2/P-16:0), and PI (20:4/16:0), were lower in the serum of patients with BMFS. L-Octanoylcarnitine and clupanodonyl carnitine are acylcarnitine metabolites in the body. They are mainly composed of L-carnitine. Acyl-carnitine is a middle-carrier that can transport free fatty acids in the cytoplasm into mitochondria to promote beta oxidation of fatty acids and regulate lipid metabolism [41]. Present results suggest that decreased levels of acyl-carnitine in

the blood of patients with BMFS can induce fatty acid metabolism disorders. Levels of acylcarnitine were decreased in the blood of patients with BMFS, most likely as a reflection of decreasing levels of L-carnitine. This can increase the number of CD^{4+} T-cells and promote the proliferation of lymphocytes and secretion of antibodies [42]. Carnitine can also effectively remove oxygen free radicals, revealing anti-inflammatory and antioxidant effects. A decrease in its level may lead to a decline in the body's resistance [41]. PC (14:0/18:2) and PC (20:4/16:0) are common isoforms (molecular species) of phosphatidylcholine. PC is composed of glycerol, choline, phosphoric acid, and fatty acid. It is the main component of the membrane lipid bilayer structure [43]. These isoforms of PC play an important role in regulating physiological activities of the body. For instance, PC (16:0/18:1), an endogenous ligand of PPARa, regulates expression of many genes controlling lipid metabolism [44]. In addition, PC also has the function of emulsifying and maintaining the stability of serum glucose concentrations, which is helpful for resisting atherosclerosis. PE (22:5/16:0) and PE (18:2/P-16:0) are common isoforms of phosphatidylethanolamine. PE, also known as brain phospholipid, is rich in brain tissue. It is an important component of brain cell membranes. PE can be hydrolyzed into acetylcholine in the body, which is an important neurotransmitter [45]. Phosphatidylinositol (PI) is a type of phospholipid on the cell membrane that can be hydrolyzed to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3), important second messengers of cells. DAG can regulate the activity of protein kinase (K protein kinase C, PKC), while IP3 can regulate the release of calcium ions. Both metabolites can regulate cell proliferation, differentiation, contraction, secretion, and metabolism through the phospholipase inositol signaling pathway [46]. Inositol groups at different locations in PI can be phosphorylated to produce different phosphatidylinositol metabolites. These metabolites can transport proteins from the Golgi body to the plasma membrane [47]. Therefore, when levels of PC (14:0/18:2), PC (20:4/16:0), PE (22:5/16:0), PE (18:2/p-16:0) and PI (20:4/16:0) decrease, the risk of cardiovascular and cerebrovascular diseases, liver damage, atherosclerosis, and Alzheimer's disease may be increased.

There were some limitations to this study, however. There was a small number of cases included. However, present results demonstrated that differences in amino acids, fatty acid transport, and phospholipid metabolism were found in the blood of patients with BMFS, compared with those from a group of healthy individuals. Changes in these metabolites may be related to the pathogenesis of metabolic pathways in the body. Further studies are necessary to clarify possible detailed pathologic mechanisms of the findings observed in this study. However, current results suggest that these metabolites might be potential markers aiding in the initial diagnosis of BMFS.

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Disclosure of conflict of interest

None.

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