Published in final edited form as:

J Proteome Res. 2013 October 4; 12(10): . doi:10.1021/pr4007359.

Application of ^1H -NMR spectroscopy-based metabolomics to sera of tuberculosis patients

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy is an ideal platform for the metabolic analysis of biofluids, due to its high reproducibility, non-destructiveness, non-selectivity in metabolite detection, and the ability to simultaneously quantify multiple classes of metabolites. Tuberculosis (TB) is a chronic wasting inflammatory disease characterized by multi-system involvement, which can cause metabolic derangements in afflicted patients. In this study, we combined multivariate pattern recognition (PR) analytical techniques with ^1H NMR spectroscopy to explore the metabolic profile of sera from TB patients. A total of seventy-seven serum samples obtained from patients with TB (n=38) and healthy controls (n=39) were investigated. Orthogonal partial least-squares discriminant analysis (OPLS-DA) was capable of distinguishing TB patients from controls, and establishing a TB-specific metabolite profile. A total of 17 metabolites differed significantly in concentration between the two groups. Serum samples from TB patients were characterized by increased concentrations of 1-methylhistidine, acetoacetate, acetone, glutamate, glutamine, isoleucine, lactate, lysine, nicotinate, phenylalanine, pyruvate, and tyrosine, accompanied by reduced concentrations of alanine, formate, glycine, glycerolphosphocholine, and low-density lipoproteins relative to control subjects. Our study reveals the metabolic profile of sera from TB patients and indicates that NMR-based methods can distinguish TB patients from healthy controls. NMR-based metabolomics has the potential to be developed into a novel clinical tool for TB diagnosis and/or therapeutic monitoring, and could contribute to an improved understanding of disease mechanisms.

Keywords

tuberculosis; metabolomics; serum; NMR; systems biology

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Supporting Information

Supplemental figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Over the past fifty years, NMR spectroscopy has become the preeminent technique for the metabolic analysis of biofluids. NMR spectroscopy-based metabolomics has been successfully applied clinically to diagnosis and prognosis of many human diseases such as coronary heart disease, cancer, neurological disease, and diabetes^{1–5}. As an integral component of systems biology approaches encompassing a number of omics technologies, metabolomics has received much attention recently worldwide. Metabolomic strategies have distinct advantages in the identification of low molecular weight catabolites/anabolites in organs or biofluids in response to various pathophysiological events, which can be exploited for the development of diagnostic biomarkers or reliable surrogates for determination of disease or health status.

Tuberculosis (TB) is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent. Roughly more than one-third of the world's population is infected with *M. tuberculosis* (MTB), and new infections occur at a rate of one per second on a global scale. In 2010, there were 8.8 million new cases of TB diagnosed and 1.4 million deaths, most of these occurring in developing countries⁶. In addition, the emergence and rapid spread of multidrug-resistant TB (MDR TB) and extensively drug-resistant TB (XDR TB) present a formidable challenge to global TB control, especially in Asia, Africa, and East Europe^{7, 8}.

Most MTB-infected persons have latent infection, but 5–10% of individuals develop active disease during the course of their lifetime. The classical symptoms of active TB are chronic cough with hemoptysis, fever, night sweats and weight loss. As a chronic wasting disease, TB induces profound changes in whole body energy and protein metabolism⁹. As an intracellular pathogen, MTB strongly influences the metabolism of host cells, potentially inducing metabolic disorders. Since metabolomics analysis enhances the understanding of host-pathogen interactions through a net flow of energy and nutrients between hosts and pathogens¹⁰, study of the metabolic derangements in the host during MTB infection may assist in improving the understanding of TB pathogenesis and the evaluation of treatment response.

Previous metabolomics studies using ¹H NMR in the murine TB model identified dramatic changes in host metabolic profiles during infection^{11, 12}. A recent study by January et al. using GC-MS (Gas chromatography–mass spectrometry) showed differences in metabolic profiles between active TB patients and those without TB¹³, however, relatively little is known about global metabolism in TB patients. The objective of this investigation was to better characterize the metabolism of the host during MTB infection using ¹H NMR spectroscopy. In this study, seventy-seven serum samples obtained from patients with TB (n=38) and healthy controls (n=39) were investigated by ¹H NMR. The orthogonal partial least-squares discriminant analysis (OPLS-DA) was capable of distinguishing TB patients from controls, and establishing a TB-specific metabolite profile.

MATERIALS AND METHODS

Patients and controls

The study included 38 active TB patients from Shanghai Pulmonary Hospital and Shanghai Public Health Clinical Center between March 2010 and August 2011. All patients were diagnosed based on chest X-ray and the biofluids samples obtained from each TB patient were analyzed by Ziehl-Neelsen staining and culture on Lowenstein-Jensen media. Thirty-nine patients without comorbidities, matched for age and sex with experimental subjects, were recruited from the Physical Examination Center at Shanghai Ruijin Hospital and

served as healthy control subjects. Control subjects did not have latent TB infection, as determined by negative tuberculin skin test (TST) and interferon-gamma release assay (IGRA), or other comorbidities. Detailed data about study patients and controls are presented in Table 1 and Table S1. All study participants gave informed consent for the investigation, which was approved by the Ethical Committee of the Shanghai Jiao Tong University School of Medicine.

Sample preparation

Whole-blood samples were drawn from a peripheral vein between 7:00 and 9:00 am. Sera from patients and healthy volunteers was acquired from EDTA-preserved whole blood samples following centrifugation and was stored at -80°C until analysis. Before the NMR experiments, serum samples were defrosted at room temperature for less than 20 minutes and 200 μL aliquots combined with 300 μL of saline (0.9% NaCl in 20% D_2O /80% H_2O) were centrifuged at $12,000 \times g$ for 5 minutes. A 500 μL aliquot of this solution was pipetted into a 5 mm NMR tube and samples were stored at 4°C until further use.

NMR spectroscopy performance

^1H NMR spectra for all serum samples were collected on a Varian Unity INOVA 600 NMR spectrometer (Varian Inc., Palo Alto, CA), at a frequency of 599.93 MHz. Spectra were acquired using a conventional presaturation pulse sequence with solvent suppression NOESYPR1D [RD-90°- t1- 90°- tm-90°- ACQ] (relaxation delay=2.5 s, mixing time=0.1 s solvent presaturation was applied during the relaxation delay and mixing time) at 25°C .

For each sample, one-dimensional-Carr-Purcell-Meiboom-Gill (1D-CPMG) was performed to filter out signals belonging to proteins and other macromolecules, thus obtaining spectra primarily comprising signals from metabolites and small molecules¹⁴.

A line-broadening function of 1 Hz was applied to all acquired FIDs prior to Fourier transformation. NMR spectra were manually corrected for phase and baseline distortion using MestRenova 7.1.0 software (Umetrics, Umeå, Sweden). The baseline was corrected and referenced to the methyl peak signal of lactate at chemical shift (1.33 ppm). NMR spectra were divided into 0.005 ppm-wide regions. After removal of regions containing the residual water signals and buffer signals (6.88-5.35, 4.31-5.50, and 4.09-3.95 ppm), alcohol signals (3.69-3.60 and 1.22-1.12 ppm), and EDTA and its complex with calcium or magnesium signals (3.24-3.06, 3.00-2.88 and 2.62-2.50 ppm), the remaining bins were integrated and normalized to serve for further analysis.

Multivariate statistics

For data reduction and pattern recognition, a series of pattern recognition methods were applied using Simca-P 11.0 software (Umetrics AB, Umea, Sweden). Principle component analysis (PCA) was initially applied to the spectral data to visualize inherent clustering between healthy control and TB patient groups.

After the overview of the NMR data using PCA analysis, the data were subjected to orthogonal partial least-squares discriminant analysis (OPLS-DA) and a model was built and utilized to identify marker metabolites that accounted for the differentiation of all groups¹⁵. OPLS-DA as an extension of PLS-DA featuring an integrated Orthogonal Signal Correction (OSC) can remove variability not relevant to class separation. Both PLS-DA and OPLS-DA were based on unit variance scaling strategy. A 20-fold cross-validation was employed to obtain Q^2 and R^2 values, which represent the predictive ability of the model and the explained variance, respectively. To further validate the quality of the PLS-DA model, permutation tests consisting of a randomly permuting class membership and running 200

iterations were carried out. The sensitivity, specificity, and classification rate (percentage of samples correctly classified) of OPLS-DA models were then depicted ¹⁶.

Metabolite Set Enrichment Analysis

To identify the most significantly affected metabolic pathways, the metabolites affected by MTB infection were analyzed by Metabolite Set Enrichment Analysis (MSEA) ^{17, 18}, defined as an extension of Gene Set Enrichment Analysis ¹⁹, a freely accessible web-based program (<http://www.msea.ca/MSEA/faces/Home.jsp>). After normalization, the data were uploaded and analyzed by Over Representation Analysis (ORA). One-tailed p values are provided after adjusting for multiple testing.

RESULTS

¹H-NMR spectra

¹H Carr-Purcell-Meiboom-Gill (CPMG) superimposed spectra of serum samples from TB patients and healthy controls are shown in Figure 1. More than 30 different metabolites were identified and quantified according to extant literature from each data set of each sera sample, based on their chemical shifts and signal multiplicity ¹⁴. The main differences in peaks between the two groups are concentrated in the area of 0.5–5.6 ppm and 5.6–9.5 ppm (Figure 1).

Multivariate analysis

Principal component analysis (PCA) was performed, and the score plot was obtained with the first two PCs presenting 46.8% and 21.6% variance, respectively (Figure S1. $R^2X=72.9\%$, $Q^2=0.681$). There was no significant difference between control and TB groups. Supervised analysis techniques were then used, including partial least-squares discriminant analysis (PLS-DA) and orthogonal partial least-squares discriminant analysis (OPLS-DA), which can maximize differences among groups and aid in the screening of the metabolite markers responsible for class separation by removing systematic variations unrelated to pathological status ¹⁶.

Based on the PLS-DA model, TB patients and control subjects were discriminated with an $R^2X=25.9\%$, $R^2Y=0.759$, and a $Q^2=0.605$. The goodness-of-fit (R^2 and Q^2) of the original PLS-DA models and cluster of 200 Y -permuted models were visualized in validation plots (Figure S2), which clearly demonstrated that the original PLS-DA model was efficient, as the Q^2 regression line had a negative intercept and all permuted R^2 values on the left were lower than the original point on the right.

The OPLS-DA model was constructed subsequent to PLS-DA analysis, using the first principal component and the second orthogonal component. The quality of the models was described by the cross-validation parameters Q^2 and R^2X , which represented the total variation for the X matrix, and the values are tabulated in Table 2. In OPLS-DA score plots, a significant biochemical distinction between the TB cases and healthy controls was identified (Figure 2).

The metabolic signature associated with each group is derived from model coefficients obtained from the OPLS-DA model segregating TB patients from healthy controls. Seventeen metabolites were detected at significantly different levels between the two groups. Our data reveal up-regulation of 1-methylhistidine, acetoacetate, acetone, glutamate, glutamine, isoleucine, lactate, lysine, nicotinate, phenylalanine, pyruvate, and tyrosine and down-regulation of alanine, formate, glycine, glycerolphosphocholine, and low-density

lipoproteins (LDL) in TB patients relative to healthy controls. These data strongly support the robustness of ^1H NMR to identify metabolic changes in the sera of TB patients.

Metabolite Set Enrichment Analysis

Metabolite Set Enrichment Analysis (MSEA), an extension of the Gene Set Enrichment Analysis (GSEA) was then used to test metabolic pathways enrichment in each group. MSEA indicated that protein biosynthesis and alanine metabolism pathways, as well as phenylalanine and tyrosine metabolism, ammonia recycling, urea cycle, ketone body metabolism, glucose-alanine cycle and valine, leucine and isoleucine degradation pathways are significantly associated with MTB infection (Figure 3).

DISCUSSION

In this study, 35 metabolites were unambiguously identified in every sera sample based on ^1H NMR. The metabolic changes in sera from TB patients produced a distinct pattern, reflecting the interaction between host and pathogen. Seventeen metabolites were altered in TB patients, as compared to those in healthy controls (Table 2). The major group of altered endogenous metabolites in the serum of TB patients contained intermediates of the tricarboxylic acid cycle (TCA cycle), products of glycolysis, amino acids, and molecules related to lipid catabolism. As shown in Figure 3, the metabolic processes found to be most significantly altered between TB patients and healthy controls were protein biosynthesis, followed by alanine metabolism, phenylalanine and tyrosine metabolism and ammonia recycling. These findings are consistent with those of a previous study done by Tailleux et al, who used microarrays to study the transcriptional responses of MTB-infected human macrophages and dendritic cells, and showed that host genes related to TCA cycle, oxidative phosphorylation, glycerolipid metabolism, oxidative stress, and pyruvate metabolism were significantly upregulated²⁰. Although the metabolic profile of TB patients in this study is similar to that of MTB-infected mice, there are significant differences, suggesting that biomarkers identified in the mouse or other mammalian models may not be applied to humans directly^{11, 12}.

Increase in energy consumption

In this study, pyruvate increased significantly in the sera of TB patients compared to those of healthy controls. Pyruvate supplies energy to cells through the TCA cycle. It is well known that TB is a wasting disease, and epidemiological studies have shown that TB patients experience malnutrition, weight loss, and metabolic disorders²¹. The TCA cycle is a key component of the metabolic pathway by which all aerobic organisms generate energy through catabolism of carbohydrates, fats, and proteins. The elevated level of pyruvate in the sera of TB patients suggests increased catabolism of all three major nutrients, as well as increased energy consumption. This result is consistent with previous findings in MTB-infected guinea pigs¹¹ and similar to metabolic changes observed in patients during tumor development²². Furthermore, the increase of pyruvate could be mediated through the methylcitrate cycle (MCC), which oxidizes propionyl-CoA generated by α -oxidation of odd-chain fatty acids to pyruvate²³, which then feed into the TCA cycle^{24, 25}. The MCC might be required for the detoxification of propionate²⁵.

Increase in glycolysis

Pyruvate and lactate are intermediate and end products of anaerobic glycolysis, respectively. Levels of pyruvate and lactate were elevated in the sera of TB patients, consistent with increased anaerobic glycolysis. Anaerobic glycolysis increases as a result of tissue hypoxia, lung injury, and ischemia²⁶, and MTB infection is known to induce granulomatous inflammation in the lung with central necrosis and tissue hypoxia²⁷. Lactate has been found

to be associated with the progression of malignancy through the formation of tumor necrosis²⁸. Therefore, the accumulation of lactate could be an index of tissue hypoxia and extent of necrosis as the infection progresses. In addition, more pyruvate was converted into lactate rather than entering into the TCA cycle pathway, likely as a result of insufficient oxygen supply. A previous study by Zhuang et al showed that glycolysis is elevated in granulomatous inflammation, primarily in macrophages and neutrophils, and the elevation is associated with an affinity of glucose receptors for deoxyglucose, which is increased by various cytokines and growth factors²⁹.

Amino acid metabolism

Our data showed an increase in 5 amino acids, including glutamate, glutamine, isoleucine, lysine, and phenylalanine, and a decrease in glycine, and alanine in sera from TB patients relative to those of healthy controls, suggesting alterations in protein metabolism during active TB. Amino acid metabolism is complex, involving a large number of metabolites. Proteolysis, gluconeogenesis, and oxidative catabolism contribute to amino acid balance. As important precursors for gluconeogenesis, amino acid levels might increase when they are not utilized for protein anabolism but are oxidized by impairment of protein synthesis^{9, 30, 31}. Previous studies have reported proteolysis into amino acids during TB and malnutrition⁹. 1-methyl-histidine (1M-His) is present in skeletal muscles as a precursor of anserine (-alanyl-N-methylhistidine). The increase in 1M-His in TB patient sera may reflect accelerated muscle protein degradation. Moreover, vitamin E deficiency can lead to 1-methylhistidinuria from increased oxidative effects in skeletal muscle. These results suggest that a greater amount of ingested amino acids may be oxidized rather than utilized for new protein synthesis. Our findings are consistent with several reports indicating malnutrition and wasting in TB patients^{9, 30–32}.

Enhanced lipid degradation

Low-density lipoproteins (LDL) are one of the five major groups of lipoproteins, which facilitate transport of multiple different lipid molecules, including cholesterol, triglyceride, and glycosphingolipids, and mediate lipid catabolism. The decrease of LDL in TB patients might be related to upregulation of host lipid metabolism. This result is consistent with an earlier study reporting correlation of LDL with caseation of human TB granulomas and pathogen-mediated dysregulation of host lipid metabolism³³. Moreover, we found that acetoacetate and acetone were increased in TB patient sera. Together with -hydroxybutyric acid, acetoacetate and acetone constitute ketone bodies, which are important by-products of fatty acid oxidation in the liver and serve as a major source of energy in the heart and brain³⁴. In this study, the metabolic profile of increased acetoacetate and acetone and decreased LDL is consistent with enhanced lipid degradation in TB patients relative to healthy controls.

Increase in nucleotide biosynthesis

Formate was markedly decreased in the sera of TB patients in this study. As the simplest carboxylate anion, formate is an alternative single-carbon unit for the production of 5,10-methylenetetrahydrofolate (THF), which is required for purine and pyrimidine biosynthesis. The reduced serum levels of formate might reflect an increased requirement for nucleotide biosynthesis in an inflammatory condition³⁵. These results suggest that nucleotide metabolism increases after MTB infection, likely reflecting active host inflammatory cell division. These results are consistent with the report stating that nucleotide metabolism increased in MTB-infected mice¹².

Metabolomics is a newly developed approach, which has received much attention. Recently, NMR and GC-MS combined with multivariate statistical technique has been used to study

the metabolic profile of MTB infection^{11–13}. A recent metabolomics study based on GC-MS identified more than 400 metabolites in human serum, 20 of which were sufficient to discriminate TB patients from healthy individuals. The authors also identified changes in amino acid, lipid, and nucleotide metabolic pathways in TB¹³, which are similar to our data. Although the number of metabolites profiled in this study was significantly lower and the metabolic variation of TB patients was different from the previous study, the altered metabolic pathways are congruent with our study, including histidine metabolism, bile acid metabolism, glutathione metabolism, urea cycle, phenylalanine and tyrosine metabolism. These results indicate that NMR can be used to differentiate TB cases from healthy controls based on metabolic changes in serum. Although GC-MS is more sensitive than NMR, with the ability to detect more molecules, the latter is a relatively inexpensive technique, which is simple in data analysis and sample treatment.

Technical components

Although Mass spectrometry (MS) is more sensitive for the detection of low concentration small molecular endogenous metabolites, ¹H NMR spectroscopy has been widely used to the metabolomics research for its minimal sample preparation, low cost and robustness^{36–40}. In addition, ¹H NMR is a stable and repeatable approach, and nearly all major classes of metabolites have characteristic NMR spectra, which makes this technique very useful for metabolite fingerprinting.

OPLS, as an extension of PLS, is a well-accepted statistical analysis method, which has been applied in many fields. Later extensions of OPLS has been upgraded to OPLS-DA in 2005, thus making it appropriate for use for discriminant analysis along with prediction purposes⁴¹. Recently, OPLS-DA has been widely used in metabolomics research^{42–48}. In this study, OPLS-DA was used for the Multivariate analysis, and the metabolite differences between two groups were derived from the OPLS-DA model. OPLS-DA regression coefficients (*r*) show the contribution of each variable to the model classification, and a higher *r* indicates a greater contribution to the model classification. However, since the data do not represent absolute concentrations, the fold change of a given metabolite between two groups cannot be determined.

In conclusion, this study illustrates the successful application of ¹H NMR spectroscopy-based metabolomics for investigating the metabolic changes in the sera of TB patients. Our results indicate significant dysregulation of metabolic pathways in TB patients. Specifically, we found that TB disease was associated with amino acid and lipid catabolism and enhanced glycolysis. Our results partially validate previous reports on metabolomic profiling of MTB-infected murine models^{11, 12} and active TB patients¹³ and suggest that global metabolic profiling could provide insight into TB pathogenesis in relevant hosts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Jinghan Wang Ph.D from Shanghai Institute of Hematology for his helpful discussions and comments. This work was supported by grants from the National Natural Science Foundation of China (No. 31070114, No.31200109), Shanghai Rising-Star Program, Science and Technology Commission of Shanghai Municipality (No.12QH1401300), the State Key Development Programs for Basic Research of China (973 Program No.2009CB552605), the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning, and HL106786 (NIH).

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Figure 1. Representation of 600 MHz ^1H NMR CPMG spectrum (0.5–5.6 and 5.6–9.5) of serum obtained from (A) TB patients and (B) control subjects

The region of 5.6–9.5 (in the box) was magnified 16 times compared with the corresponding region of 0.5–5.6 for the purpose of clarity. Keys: 1-MH: 1-Methylhistidine; AA: Acetoacetate; Ace: Acetate; Act: Acetone; Ala: Alanine; Cr: Creatine; Eth: Ethanol; For: Formate; Gln: Glutamine; Glu: Glutamate; Gly: Glycine; GPC: Glycerolphosphocholine; Ileu: Isoleucine; L1: LDL, $\text{CH}_3\text{-(CH}_2\text{)}_n\text{-}$; L2: VLDL, $\text{CH}_3\text{-(CH}_2\text{)}_n\text{-}$; L3: LDL, $\text{CH}_3\text{-(CH}_2\text{)}_n\text{-}$; L4: VLDL, $\text{CH}_3\text{-(CH}_2\text{)}_n\text{-}$; L5: VLDL, $\text{-CH}_2\text{-CH}_2\text{-C=O}$; L6: Lipid, $\text{-CH}_2\text{-CH=CH-}$; L7: Lipid, $\text{-CH}_2\text{-C=O}$; L8: Lipid, $\text{=CH-CH}_2\text{-CH=}$; L9: Lipid, -CH=CH- ; Lac: Lactate; Leu: Leucine; Lys: Lysine; MA: Methylamine; NAG: N-acetyl glycoprotein signals; PC: Phosphocholine; Phe: Phenylalanine; Py: Pyruvate; Tyr: Tyrosine; Val: Valine; -Glc: -Glucose; -Glc: -Glucose.

Figure 2. OPLS-DA scores plots derived from ^1H NMR spectra of sera (A) and corresponding coefficient loading plots (B) obtained from control and TB groups

The color map shows the significance of metabolite variations between the two groups. Peaks in the positive direction indicate metabolites that are more abundant in the control group. Consequently, metabolites that are more abundant in the TB group are presented as peaks in the negative direction. Keys of the assignments are shown in Figure 1.

Figure 3. Metabolic differences between TB patients and controls

Differences in metabolic pathways noted between TB patients and healthy controls are shown in this map. The horizontal bar graph summarizes the most significant metabolite sets identified during the analysis. The most significant change is protein biosynthesis according to fold enrichment (>15) followed by alanine metabolism and phenylalanine tyrosine metabolism.

Table 1

Characteristics of TB patients and healthy controls

	Controls	Patients
Total individuals (n)	39	38
Age (years) *	42 ± 12.83	44.4 ± 17.87
Age range (years)	10–62	10–77
Gender (F/M)	26/13	20/18

* Data are presented as mean ± SD. There was no significant difference in demographic data between control and TB patients.

Table 2

OPLS-DA coefficients derived from the NMR data of metabolites in serum obtained from TB and health controls

Metabolites	r^a
1-Methylhistidine: 7.06 (s ^b), 7.78 (s)	-0.694
Acetoacetate: 2.28 (s)	-0.353
Acetone: 2.23 (s)	-0.363
Alanine: 1.48 (d)	0.474
Formate: 8.46 (s)	0.779
Glutamate: 2.12 (m), 2.35 (m), 2.78 (t)	-0.347
Glutamine: 2.14 (m), 2.45 (m), 2.78 (t)	-0.571
Glycine: 3.56 (s)	0.483
Glycerolphosphocholine: 3.36 (s)	0.715
Isoleucine: 0.94 (t), 1.01 (d)	-0.500
L1: LDL, CH ₃ - (CH ₂) _n -: 0.85 (br)	0.494
Lactate: 1.33 (d), 4.11 (q)	-0.361
Lysine: 1.73 (m), 1.91 (m), 3.01 (m), 3.77 (t)	-0.440
Nicotinate: 8.18 (s), 8.20 (s), 8.69 (m), 8.75 (m), 8.81 (m), 9.19 (s)	-0.489
Phenylalanine: 7.32 (d), 7.37 (t), 7.42 (dd)	-0.491
Pyruvate: 2.37 (s)	-0.375
Tyrosine: 6.90 (d), 7.19 (d)	-0.470

^aCorrelation coefficients; positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The correlation coefficient of each metabolite refers to the relative intensities of ¹H NMR spectra following spectral normalization. The correlation coefficient of $|r| > 0.325$ was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $p=0.05$ and df (degree of freedom) = 37.

^bMultiplicity: s, singlet; d, doublet; t, triplet; q, quartet; br, broad resonance; dd, doublet of doublets; m, multiplet.