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ORIGINAL RESEARCH

^{1}H-NMR-based metabolic profiling of healthy individuals and high-resolution CT-classified phenotypes of COPD with treatment of tiotropium bromide

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Background: Heterogeneity of COPD results in different therapeutic effects for different patients receiving the same treatment. COPD patients need to be individually treated according to their own characteristics. The purpose of this study was to explore the differences in different CT phenotypic COPD by molecular metabolites through the use of metabolomics.

Methods: According to the characteristics of CT imaging, 42 COPD patients were grouped into phenotype E (n=20) or phenotype M (n=24). Each COPD patient received tiotropium bromide powder for inhalation for a therapeutic period of 3 months. All subjects were assigned into phenotype E in pre-therapy (EB, n=20), phenotype E in post-therapy (EA, n=20), phenotype M in pre-therapy (MB, n=22), phenotype M in post-therapy (MA, n=22), or normal control (N, n=24). The method of metabolomics based on ^{1}H nuclear magnetic resonance (^{1}H-NMR) was used to compare the changes in serum metabolites between COPD patients and normal controls and between different phenotypes of COPD patients in pre- and post-therapy.

Results: Patients with COPD phenotype E responded better to tiotropium bromide than patients with COPD phenotype M in terms of pulmonary function and COPD assessment test scores. There were differences in metabolites in COPD patients vs normal control people. Differences were also observed between different COPD phenotypic patients receiving the treatment in comparison with those who did not receive treatment. The changes of metabolites involved lactate, phenylalanine, fructose, glycine, asparagine, citric acid, pyruvic acid, proline, acetone, ornithine, lipid, pyridoxine, maltose, betaine, lipoprotein, and so on. These identified metabolites covered the metabolic pathways of amino acids, carbohydrates, lipids, genetic materials, and vitamin.

Conclusion: The efficacy of tiotropium bromide on COPD phenotype E is better than that of phenotype M. Metabolites detected by ^{1}H-NMR metabolomics have potentialities of differentiation of COPD and healthy people, discrimination of different COPD phenotypes, and giving insight into the individualized treatment of COPD.

Keywords: COPD, metabolomics, tiotropium bromide, CT phenotyping, individualized treatment

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Introduction

COPD is the fourth leading cause of death in the world and is projected to be the third leading cause of death by 2020. Many people suffer from the disease or its complications for many years and die prematurely.[1] Public health challenges brought by COPD are of vital importance. COPD is a heterogeneous disease with different subtypes that have entirely different clinical presentations and disease progression. The definition of

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"phenotype" proposed of "a single or combination of disease attributes that describe differences between individuals with COPD as they relate to clinically meaningful outcomes (symptoms, exacerbations, response to therapy, rate of disease progression, or death)."[2] Therefore, studies aiming at different phenotypes of COPD are the current areas of research interest. This study aimed to make a breakthrough in this area.

High-resolution computed tomography (HRCT) scan provides an objective method for the quantitative evaluation of emphysema, which has a good correlation with the pathological changes and can predict the degree of expiratory airflow obstruction. Classifying COPD into morphological phenotypes by HRCT might help in differentiating patients who would respond to individualized treatment and in determining different pathophysiological phenotypes.[3] HRCT measurement of emphysema and small airway disease is advantageous in reflecting pulmonary physiology and functional status.[4] A combination of CT density with clinical parameter such as FEV_1 is a more appropriate measurement in the studies of emphysema.[5] Whether or not the existence of particular pulmonary structural abnormalities such as emphysema, airway wall thickening, and/or bronchiectasis predicts significant clinical meaning is of current research interest. The treatment and prognosis of COPD patients with different CT phenotypes are different, but the underlying molecular mechanism remains unclear. In recent years, with the development of metabolomics, finding molecular biomarkers from metabolites to identify the risk factors and prognoses of diseases has become the hottest research.

Metabolomics is the scientific study of chemical processes involving metabolites. Metabolomics is well-known as a competent and reproducible technology, capable of catching relevant metabolic changes of diseases and biomarkers of disease progression.[6] ^{1}H nuclear magnetic resonance (^{1}H-NMR) is an attractive technology, as it not only provides qualitative and quantitative measurements but can also simultaneously study multiple compounds in the same biological fluid samples. ^{1}H-NMR has already presented considerable potential as an auxiliary diagnostic technique for the identification of respiratory phenotypes of asthma,[7] and discrimination of patients with cystic fibrosis (CF) from healthy people and between patients with unstable and stable CF.[8] At present, there are few experiments on the study of COPD patients with different CT phenotypes using ^{1}H-NMR metabolomics.

Therefore, in the present study, we aimed to investigate whether differences exist in molecular metabolites of different phenotypes of COPD which are classified by HRCT and treated with tiotropium bromide, whether different COPD phenotypes have different therapeutic efficacy of tiotropium bromide, and whether the method of metabolomics could discriminate well between the phenotypes of COPD. Our previous study showed that bronchial wall thickening in COPD may be an indicator for better response to the treatment with budesonide–formoterol.[9] Nevertheless, the present study explains how the metabolites have changed in COPD patients with different phenotypes undergoing treatment with tiotropium bromide from a completely brand-new perspective. We expect that our study will be an investigation to provide information and understanding in discrimination of phenotypes and personal optimized treatment in COPD.

Materials and methods

Study subjects

Diagnostic criteria for patients with COPD were based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD 2016). COPD should be considered in any patients who have a medical history, appropriate symptoms, and exposure history (≥40 years old). Spirometry was required for diagnosis, the presence of a post-bronchodilator FEV_1/FVC <0.70 confirmed the presence of irreversible airflow limitation. Seventy-nine patients with stable COPD with post-bronchodilator FEV_1/FVC <70% (moderate-to-severe COPD) and FEV_1 30%–80% predicted, who visited the Department of Respiratory and Critical Care Medicine of the First Affiliated Hospital of Kunming Medical University as outpatients or inpatients between September 2016 and November 2017, were selected. Thirty-one healthy subjects whose age, gender, and body mass index matched the cases of COPD were chosen by the Physical Examination Center of the First Affiliated Hospital of Kunming Medical University at the same time. Forty-four patients and 24 healthy subjects were finally enrolled to this study. COPD patients with exacerbation of an airway disease or an infection of respiratory tract in the previous 4 weeks were excluded. Use of inhaled corticosteroids (ICSs) in fixed combination with long-acting beta2-agonists, ICS/short-acting beta2-agonists (SABA), short-acting antimuscarinic antagonists/SABA, or phosphodiesterase type 4 inhibitors, injected and oral steroids, 4 weeks before study and during the study was not permitted. Antileukotrienes, antihistamines, theophyllines, and mucolytics were allowed. Exclusion criteria also include metabolic diseases, systemic diseases, desmosis, tumor, psychiatric disorders, cognitive dysfunction, and other chronic bronchial or pulmonary diseases (such as pulmonary tuberculosis, bronchiectasis, bronchial asthma, and sleep

apnea hypopnea syndrome). Seven controls were excluded due to systemic and/or respiratory infection in the past four weeks, psychiatric disorders and participant withdrawal (Figure 1). This protocol was approved by the medical ethics committee of Kunming Medical University, and all the participants have signed the informed consents.

Study design

According to the imaging features of HRCT in the chest, patients with COPD were divided into phenotype E and phenotype M. They were further grouped into phenotype E pre-therapy (EB, n=20), phenotype M pre-therapy (MB, n=22), phenotype E post-therapy (EA, n=20), phenotype M post-therapy (MA, n=22), and normal control (N, n=24). Tiotropium bromide powder for inhalation (18 μg × 10 capsule + 1 hand inhaler, Spiriva; Boehringer Ingelheim, Ingelheim am Rhein, Germany), one inhalation once daily, was used by the patients with COPD for a therapeutic period of 3 months. Fasting serum specimens of COPD patients from EB, EA, MB, MA, and normal control groups were collected before and after treatment. Information was collected and compared among groups: lung function (FEV_1/FVC%, FEV_1 predicted), serum samples, medical history, age, sex, smoking, physical examination, and scores of COPD assessment test (CAT).

HRCT examination and classification of COPD patients

All the selected patients were scanned with SOMATOM Definition Flash CT (Siemens Healthcare, Forchheim, Germany) in the CT Department of the First Affiliated Hospital of Kunming Medical University. Workstation of Picture Archiving and Communication Systems was used to review the CT images by two radiologists independently. The three slices were observed and measured at the upper (close to the upper edge of 1 cm of aortic arch), middle (1 cm under the carina), and lower lung field (3 cm superior right diaphragm) to determine the extent of

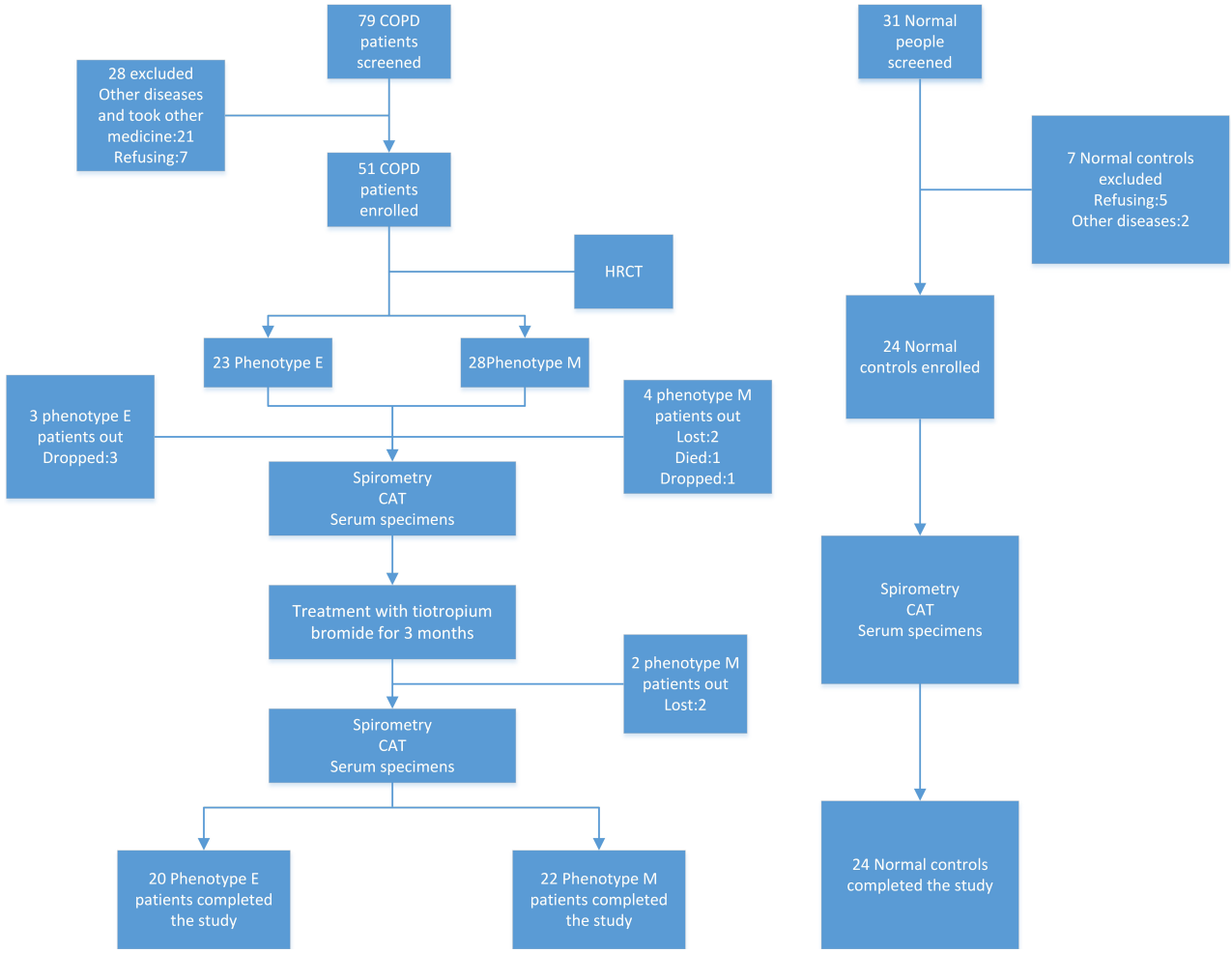

Figure 1 Flowchart for the recruited patients.
Abbreviations: HRCT, high-resolution computed tomography; CAT, COPD assessment test.

emphysema and the thickness of bronchial tube wall. Severity of emphysema was scored visually through low attenuation areas in each bilateral lung field by the method of Geddard.[10] The extent of emphysema was graded, and the scores in every level are calculated in Table 1. Assessment of bronchial wall thickness (observing the morphology of the small bronchi of 2–4 mm in diameter of the pulmonary subsegments outside the hilus pulmonis) and the results of CT classification of COPD patient are as shown in Table 1.

Investigation of CAT assessment questionnaire

CAT is a simple instrument for use in everyday clinical practice, and it is possible to relate its scores to scenarios descriptive of impaired health status in COPD.[11] CAT questionnaire was applied to score the untreated COPD patients and normal subjects, meanwhile ensuring that all subjects were not affected by the opinions of their families, friends, and also the staffs. After 3 months of treatment, the patients were evaluated by CAT survey again at the follow-up visit.

Sample preparation and ^{1}H-NMR experiment

A sample of 5 mL of fasting venous blood for each person was collected and centrifuged at 3,000 rpm for 10 minutes. The supernatant (≥300 μL) was transferred to another tube and then stored and frozen at −80°C. For the NMR experiment, blood samples were thawed and centrifuged at 13,000 rpm for 10 minutes at 4°C. About 100 μL of deuterium oxide of 2,2,3,3-D4-3-(trimethylsilyl)propionic acid sodium salt (1 mg/mL), 200 μL of phosphate buffer solution (0.2 M), and 300 μL of serum were added sequentially to tubes and mixed thoroughly, then centrifuged at 13,000 rpm for 10 minutes again. Finally, 550 μL of supernatant for every sample was extracted into a 5 mm NMR tube. Using Carr–Purcell–Meiboom–Gill pulse sequence to collect the serum samples, small molecular metabolites in the samples were observed. The detention period of relaxation is 2 seconds, during which the low-power pulse was used to presaturate the peak of water. The data of free induction decay signal were filled with zero, designating δ1.33 for the left peak of the hydroxyisobutyric acid signal doublet. The ^{1}H-NMR spectra were then obtained by Fourier transform after the linewidth broadening enhancement factor of 0.5 Hz was added.

NMR data processing and pattern recognition analysis

The phase and baseline of ^{1}H-NMR spectra were modulated and corrected by the software of Mest Re Nova (version 9.0.1.13255; Mestrelab Research, Santiago de Compostela, Spain). The spectra in the range of δ0.4–4.4 were integration segmented according to 0.01 ppm per section and the spectra between δ4.6–5.0 were excluded. Obtained data were collected and converted to Microsoft Excel 2010 after normalizing the integral based on the total integrated intensity of each spectrum. The data in the Excel format were submitted to

Table 1 HRCT classification of COPD

Emphysema		**Bronchial wall thickness (bronchial wall thickness/adjacent pulmonary artery diameter, B/A)**	
Score	**LAA/each bilateral lung field**	**Grade**	**B/A**
0	LAA <5%	0	B/A <30%
1	5% ≤ LAA <25%	1	30% ≤ B/A <50%
2	25% ≤ LAA <50%	2	B/A ≥50%
3	50% ≤ LAA <75%		
4	LAA ≥75%		
Total score (sum of scores taken from six dimensions)	**Grade**		
0	0		
1–6	1		
7–12	2		
13–18	3		
19–24	4		

Phenotype	**Severity of emphysema**	**Degree of bronchial wall thickness**
E	LAA ≥ grade 2	Bronchial wall thickness = grade 0
M	LAA ≥ grade 2	Bronchial wall thickness ≥ grade 1

Abbreviations: HRCT, high-resolution computed tomography; LAA, low attenuation area.

SIMCA-Package software package (version 11.0; Umetrics, Umea, Sweden) for multivariate analysis. After the data were preprocessed with Pareto scaling, the method of principal component analysis (PCA) was applied to analyze the separation trend between the groups. If the PCA is not good enough to separate the metabolic profiles between different groups, the method of partial least square discriminant analysis (PLS-DA) and/or orthogonal partial least square discriminant analysis (OPLS-DA) could be used to further analyze the differences between groups. PLS-DA analysis was carried out to maximize this observed difference between the groups. On the basis of PLS-DA, OPLS-DA could be performed to filter out signals that were irrelevant to the model. Modeling parameters of R^2X, R^2Y, and Q^2 were used to evaluate the validity of the model. Using variable importance for projection (VIP) for analysis, the variables with VIP >1.0 were considered the metabolites contributing the most to the group difference. Further screening of metabolites was performed by univariate analysis. First, Student's *t*-test was carried out first, then the metabolites with a concentration difference of more than twofold were analyzed and screened by fold change (FC). Finally, metabolites of statistical significance and FC ≥2 were screened. The chemical shifts corresponding to the metabolites were finally obtained. The chemical compounds were identified by chenomx NMR suite software (http://www.chenomx.com/software/), and the metabolic markers were finally obtained.

Statistical analysis

Statistical analyses were performed with SPSS software (version 22.0; IBM Corporation, Armonk, NY, USA). It was divided into normal distribution and abnormal distribution after normal testing of measurement data. Independent-sample *t*-test and paired-sample *t*-test were applied when data satisfied normal distribution; otherwise, non-parametric test (Mann–Whitney *U* analysis or Wilcoxon analysis) would be used. Data are presented as mean ± SD. A *P*-value of <0.05 was considered statistically significant.

Results

Characteristics of all subjects

The clinical characteristics of the study population are presented in Table 2. There was only one set of data of the normal control group in FEV_1/FVC%, FEV_1/predicted %, and CAT scores respectively because the drug (tiotropium bromide powder for inhalation) was not used in the normal control group. The three groups were statistically similar with respect to gender, age, and body mass index ($P>0.05$). Smoking history of packs per year in phenotype E and phenotype M was more than normal control ($P<0.05$), but no statistical difference was found between phenotype E and phenotype M ($P>0.05$). The data of phenotype E and phenotype M in FEV_1/FVC% and FEV_1/predicted % were both increased significantly post-therapy than in pre-therapy ($P<0.05$). The CAT scores of phenotype E and phenotype M were obviously decreased in post-therapy than in pre-therapy ($P<0.05$). FEV_1/FVC%, FEV_1/predicted %, and CAT scores of both phenotype E and phenotype M were statistically different compared with the normal control in pre- and post-therapy ($P<0.05$). FEV_1/FVC% in pre- and post-therapy, FEV_1/predicted % in pre-therapy, and CAT scores in pre-therapy

Table 2 Characteristics of all subjects

Characteristics	COPD phenotype E (n=20)	COPD phenotype M (n=22)	Normal control (n=24)	*P*-value
Gender (M/F)	9/11	14/8	14/10	0.459
Age (years)	60.6±12.5[b,e]	62.0±11.9[e]	61.5±9.6	0.084
BMI (kg/m²)	19.1±1.8[b,e]	19.8±1.6[e]	20.1±2.50	0.072
Current smoker	13 (65.00%)[b,e]	16 (72.73%)[e]	16 (66.67%)	0.847
Smoking history, pack-years	47.22±26.40[b,c]	51.08±31.57[c]	21.80±33.23	0.002
FEV_1/FVC% in pre-therapy	57.73±8.51[a,b,c]	59.34±8.39[a,c]	92.37±5.81	<0.01
FEV_1/FVC% in post-therapy	66.00±5.46[b,c]	65.31±7.53[c]	92.37±5.81	<0.01
FEV_1/predicted % in pre-therapy	62.89±17.65[a,b,c]	70.25±8.28[a,c]	94.72±5.27	<0.01
FEV_1/predicted % in post-therapy	82.35±13.55[d,c]	79.67±6.97[c]	94.72±5.27	<0.01
CAT scores in pre-therapy	26.15±4.92[a,b,c]	25.18±4.87[a,c]	0.63±0.71	<0.01
CAT scores in post-therapy	8.55±2.86[d,c]	15.59±4.51[c]	0.63±0.71	<0.01
Change of FEV_1/FVC% in pre- vs post-therapy	8.28±3.64[d]	5.97±2.86	–	0.027
Change of FEV_1/predicted % in pre- vs post-therapy	18.97±5.02[d]	9.34±2.23	–	<0.01
Change of CAT scores in pre- vs post-therapy	17.60±3.19[d]	9.59±1.44	–	<0.01

Notes: Data are presented in mean ± SD. [a]*P*<0.05 vs post-therapy. [b]*P*>vs phenotype M. [c]*P*<0.05 vs normal control. [d]*P*<0.05 vs phenotype M. [e]*P*>0.05 vs normal control.
Abbreviations: BMI, body mass index; CAT, COPD assessment test.

of phenotype E had no significant difference compared with phenotype M ($P>0.05$), but FEV_1/predicted % in post-therapy of phenotype E was higher than that of phenotype M ($P<0.05$). CAT scores of COPD phenotype E in post-therapy were lower than phenotype M in post-therapy ($P<0.05$). The change of FEV_1/FVC%, FEV_1/predicted %, and CAT scores of phenotype E in pre- vs post-therapy was significantly higher than that in phenotype M ($P<0.05$).

NMR experimental spectra

The ^{1}H-NMR spectra were obtained by Fourier transform after the processing of the original NMR signal data. Different peak heights and peak areas in the ^{1}H-NMR spectra of the five groups were found. It is indicated that the metabolites of serum molecules have changed in these five states, so they had different metabolic profiles (different ^{1}H-NMR spectra are shown in Figure 2).

Results of data processing

Data were preprocessed with Pareto scaling and then imported into MetaboAnalyst 4.0 software (http://www.metaboanalyst.ca/) for PCA, PLS-DA, and OPLS-DA analysis. The graphic presentation of the quality of separation between groups in the model is shown by their respective OPLS-DA scores plots (Figure 3). The quality of the model is tested by cross-validation, and the obtained parameters R^2X, R^2Y, and Q^2 (Table 3) which respectively represent explanatory variables of model X, interpretable variables of Y, and predictable variables of model were used to evaluate the validity of the model. Using VIP for analysis, the variables with VIP >1.0 were considered the metabolites contributing the most to the group difference. Further operation of metabolites was performed by univariate analysis, and *t*-test ($P<0.05$) and FC ≥2 were used to screen metabolites.

Concentration changes of metabolites

COPD phenotypes vs healthy control

In Table 4, we could see the changing trends of 30 identified metabolites. In the group of EB and N, the concentration of COPD patients of phenotype E with pre-therapy had increased levels of lactate, fructose, glycine, creatine, citric acid, pyruvic acid, pyruvate, proline, acetone, L-glutamine, L-proline, ornithine, lipid CH_2CH_2CO, 2-hydroxyisobutyrate, threonine, isopropyl alcohol, maltose, L-threonine, L-valine, glutamic acid, beta-alanine, cyclopentane, and 2-aminoisobutyric acid compared to the normal control group, whereas the levels of

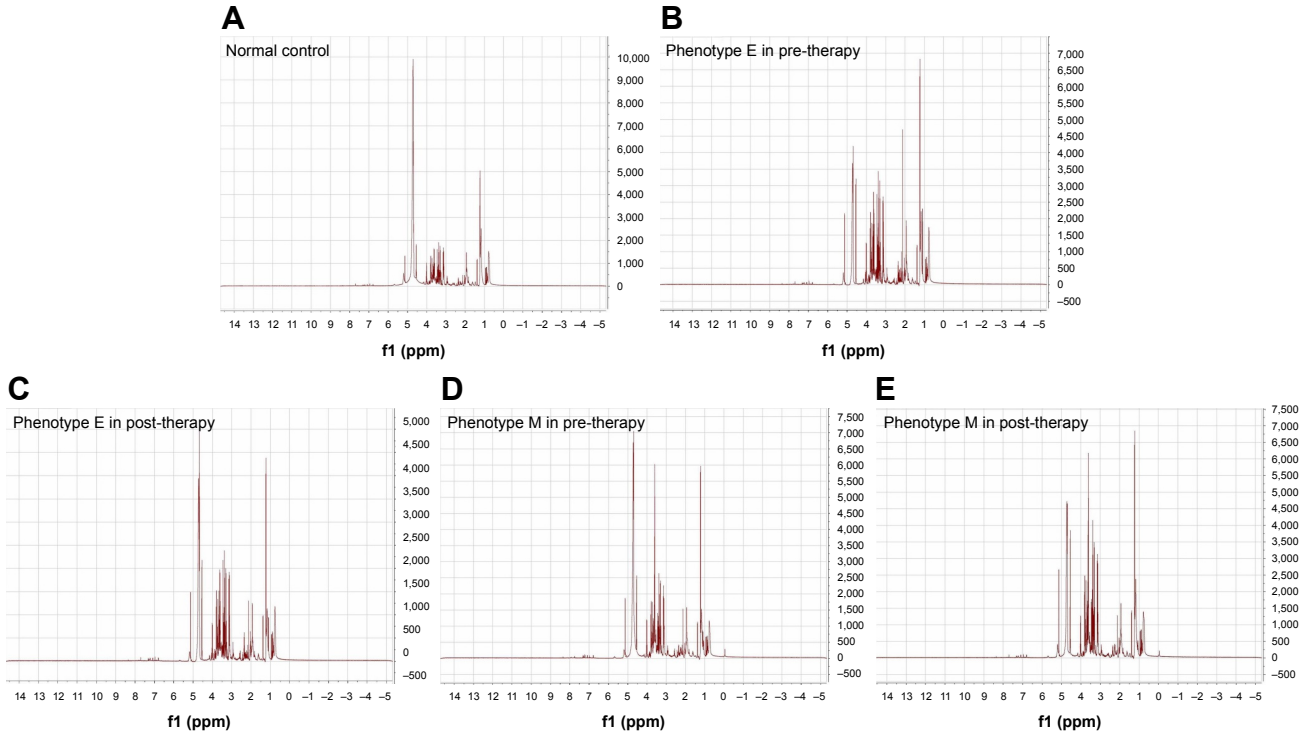

Figure 2 We chose one spectrum from each experimental group.
Note: Spectra **A**, **B**, **C**, **D**, and **E** were selected from normal controls, phenotype E of pre-therapy, phenotype E of post-therapy, phenotype M of pre-therapy, and phenotype M of post-therapy, respectively.
Abbreviations: EB, phenotype E in pre-therapy; EA, phenotype E in post-therapy; MB, phenotype M in pre-therapy; MA, phenotype M in post-therapy; N, normal control.

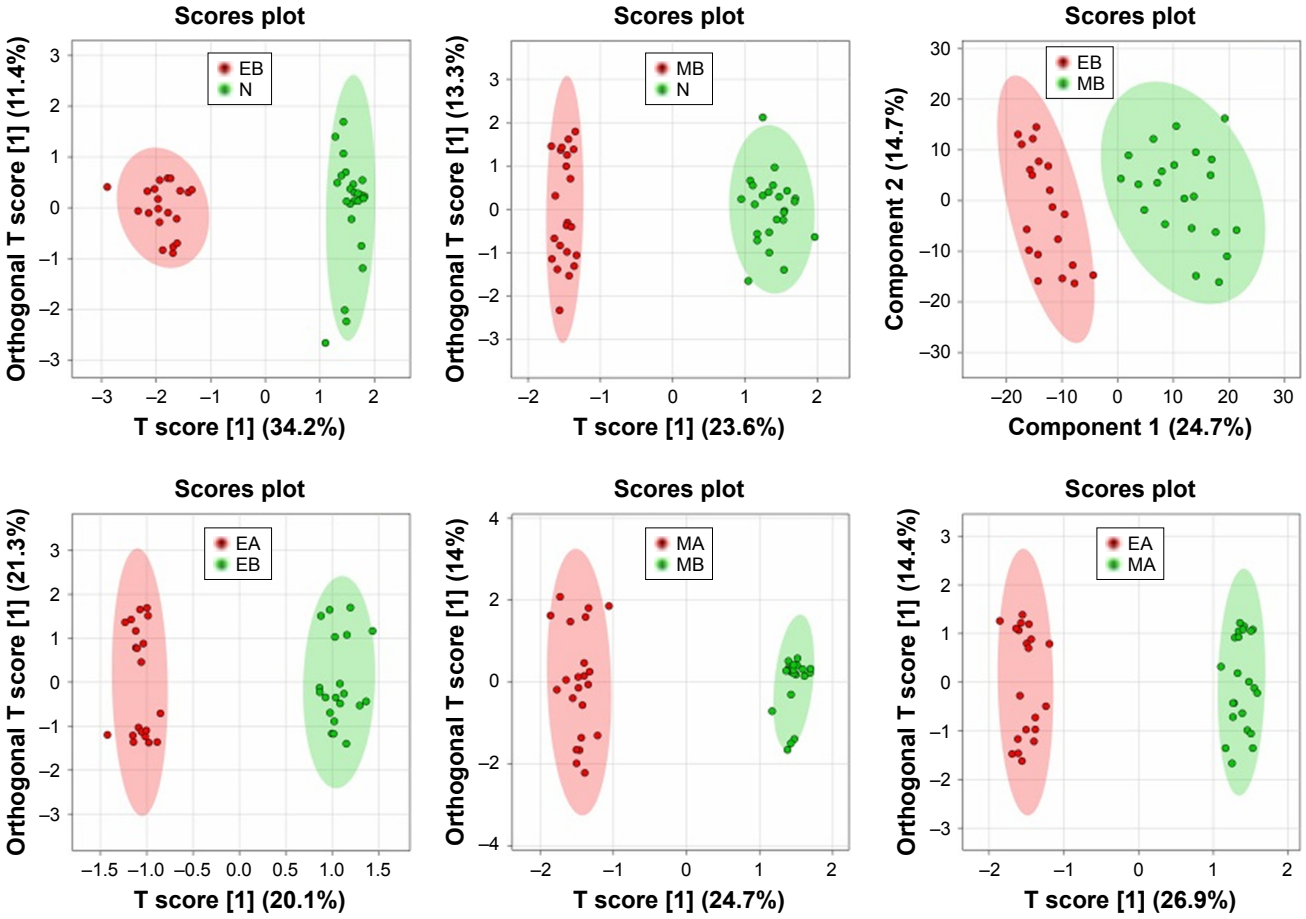

Figure 3 Six OPLS-DA score plots are selected from six comparative groups.
Abbreviations: OPLS-DA, orthogonal partial least square discriminant analysis; EB, phenotype E in pre-therapy; EA, phenotype E in post-therapy; MB, phenotype M in pre-therapy; MA, phenotype M in post-therapy; N, normal control.

asparagine and pyridoxine were decreased. Between groups of MB and N, the concentration of COPD patients with phenotype M in pre-therapy had increased levels of fructose, glycine, pyruvic acid, pyruvate, proline, acetone, L-proline, lipid CH_2CH_2CO, threonine, isopropyl alcohol, betaine and N-acetylcysteine (NAC) compared to normal controls, but the levels of ornithine, guanosine, and lipoprotein were decreased. In groups of phenotype E and phenotype M both in pre-therapy, L-glutamine and L-alanine were increased in EB compared to MB.

Table 3 Parameters for the evaluation of OPLS-DA model

Model	R^2X	R^2Y	Q^2
EB-N	0.342	0.903	0.883
MB-N	0.236	0.943	0.909
EB-MB	0.233	0.949	0.727
EB-EA	0.201	0.959	0.882
MB-MA	0.247	0.974	0.752
EA-MA	0.269	0.967	0.742

Notes: R^2X explains variability of X-variables. R^2Y explains variability of Y-variables and Q^2 represents the predictive capability of model.
Abbreviations: OPLS-DA, orthogonal partial least square discriminant analysis; EB, phenotype E in pre-therapy; EA, phenotype E in post-therapy; MB, phenotype M in pre-therapy; MA, phenotype M in post-therapy; N, normal control.

COPD phenotypes pretreatment vs posttreatment

Eight metabolites were identified as seen in Table 5. Between phenotype E COPD patients, the levels of glycine, asparagine, and citric acid were downregulated in posttreatment compared to pretreatment, whereas the level of choline was upregulated. In the groups of COPD patients of phenotype M, the level of creatinine was upregulated in posttreatment compared to pretreatment. After treatment of inhaling tiotropium bromide for 3 months, the concentrations of phenylalanine, pyruvic acid, and proline were downregulated in phenotype E compared to phenotype M.

Analysis of metabolic pathways

Analysis of metabolic pathways in which all the metabolites may be involved in is shown in Figure 4.

Table 4 Variation trend of the metabolites of COPD and normal controls

Metabolites	EB vs N				MB vs N				EB vs MB			
	ppm	*P*-value	VIP	Trend	ppm	*P*-value	VIP	Trend	ppm	*P*-value	VIP	Trend
Lactate	7.81	0.0148	1.52	↑								
Fructose	3.69	0.0110	1.57	↑	3.69	<0.001	1.57	↑				
Glycine	3.60	0.0130	1.54	↑	3.60	0.0035	1.09	↑				
Creatine	3.06	0.0168	1.50	↑								
Asparagine	2.94	0.0049	1.68	↓								
Citric acid	2.64	0.0471	1.30	↑								
Pyruvic acid	2.47	0.0037	1.01	↑	2.35	<0.001	1.52	↑				
Pyruvate	2.38	0.0043	1.69	↑	2.38	0.0021	1.22	↑				
Proline	2.34	0.0021	1.77	↑	3.33	0.0219	1.55	↑				
Acetone	2.22	<0.001	1.87	↑	2.22	0.0023	1.48	↑				
L-glutamine	2.17	0.0056	1.66	↑					2.17	0.0192	2.04	↑
L-proline	2.02	<0.001	2.16	↑	2.02	<0.001	1.81	↑				
Ornithine	1.98	<0.001	2.16	↑	1.83	0.0212	1.46	↓				
Lipid CH_2CH_2CO	1.58	<0.001	1.95	↑	1.30	0.0290	1.13	↑				
2-Hydroxyisobutyrate	1.34	0.0044	1.69	↑								
Threonine	1.31	<0.001	1.89	↑	1.31	0.0348	1.09	↑				
Isopropyl alcohol	1.16	0.0354	1.36	↑	1.16	<0.001	1.56	↑				
Pyridoxine	7.69	0.0142	1.53	↓								
Maltose	4.63	0.0193	1.36	↑								
L-threonine	4.24	0.0427	1.32	↑								
L-valine	2.52	0.0027	1.74	↑								
Glutamic acid	2.11	<0.001	2.09	↑								
Beta-alanine	1.52	0.0266	1.42	↑								
Cyclopentane	1.51	0.0448	1.31	↑								
2-aminoisobutyric acid	1.48	0.0116	1.56	↑								
Guanosine					7.95	0.0280	1.13	↓				
Betaine					3.37	0.0099	1.07	↑				
NAC					2.06	<0.001	1.45	↑				
Lipoprotein					1.10	0.0019	1.20	↓				
L-alanine									1.46	0.0445	1.83	↑

Notes: The variation trend of all metabolites in comparative groups is the trend of the former compared to the latter. ↑ means concentration increased. ↓ means concentration decreased.

Abbreviations: NAC, N-acetylcysteine; EB, phenotype E in pre-therapy; MB, phenotype M in pre-therapy; N, normal control; ppm, chemical shift; VIP, variable importance for projection.

Table 5 Variation trend of the metabolites of COPD phenotypes

Metabolites	EA vs EB				MA vs MB				EA vs MA			
	ppm	*P*-value	VIP	Trend	ppm	*P*-value	VIP	Trend	ppm	*P*-value	VIP	Trend
Choline	3.18	0.0257	1.47	↑								
Asparagine	2.84	0.0467	1.38	↓								
Citric acid	2.53	0.0327	1.04	↓								
Glycine	2.51	0.0210	1.75	↓								
Creatinine					3.10	0.0249	3.22	↑				
Phenylalanine									7.38	0.0496	1.72	↓
Pyruvic acid									2.35	0.0283	1.81	↓
Proline									2.34	0.0256	1.82	↓

Notes: The variation trend of all metabolites in comparative groups is the trend of the former compared to the latter. ↑ means concentration increased. ↓ means concentration decreased.

Abbreviations: EB, phenotype E in pre-therapy; EA, phenotype E in post-therapy; MB, phenotype M in pre-therapy; MA, phenotype M in post-therapy; ppm, chemical shift; VIP, variable importance for projection.

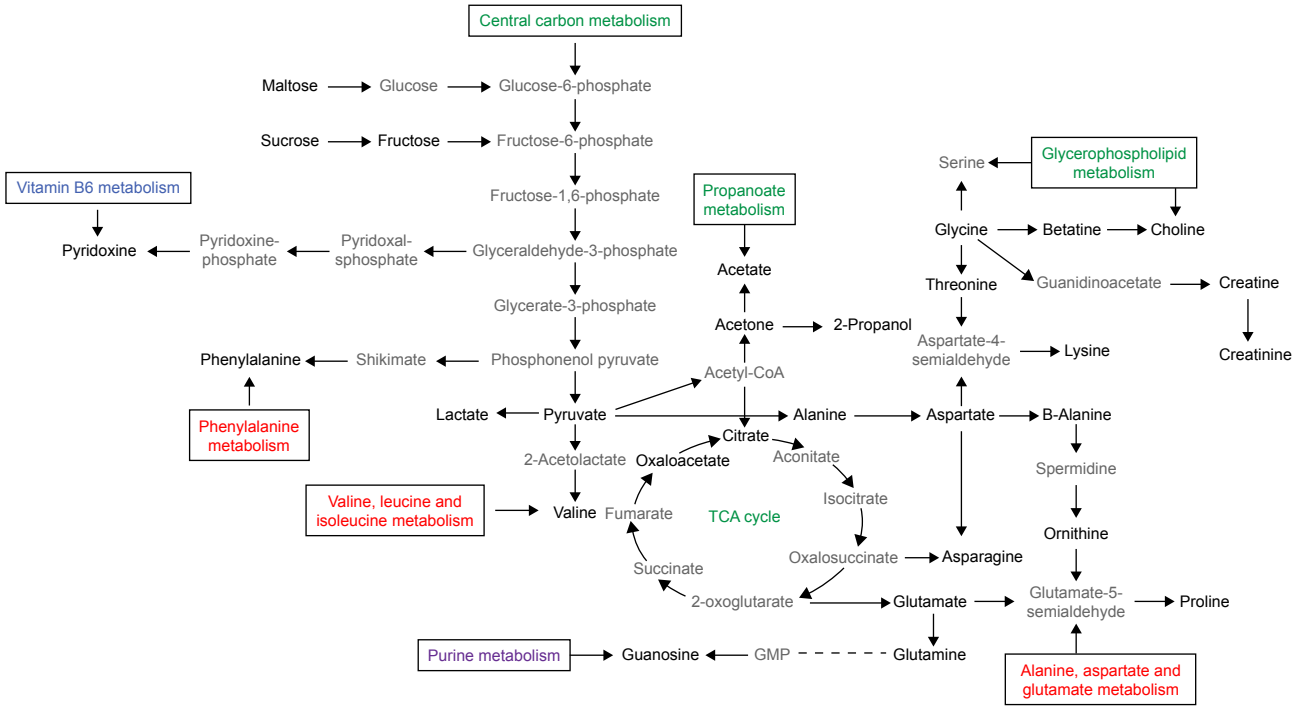

Figure 4 Analysis of metabolic pathways.
Notes: Gray represents undetected metabolites. Black represents the metabolites that have significant difference. Red is amino acid metabolic pathway. Green is carbohydrate and lipid metabolic pathway. Purple is the metabolic pathway of genetic material. Blue is vitamin metabolic pathway.
Abbreviations: TCA cycle, tricarboxylic acid cycle; GMP, guanosine monophosphate.

Discussion

Our experiment is the first to classify COPD patients into new phenotypes by using HRCT. Our study is also the first in treating different phenotypes of COPD with tiotropium bromide powder for inhalation, using the method of metabolomics to compare the serum metabolites between different CT phenotypes and evaluating therapeutic efficacy through lung function and CAT scores.

Phenotype E and phenotype M could be distinguished through pulmonary function and CAT scores after treatment with tiotropium bromide. The visible difference between phenotype E and phenotype M patients is the extent of the thickness of bronchial tube wall. The M phenotype group with increased airway wall thickening in the small airway, but not in the large airway, mainly results in airflow limitation in COPD. On the other hand, the E phenotype group's airway wall thickening in the small airway may be mild and the airflow limitation may be caused by the decreased elastic recoil. However, the pathological changes of both the types of airways ultimately contribute to airflow limitation; therefore, it is difficult to discriminate between the two types from the measured values of lung function. Only after using drugs we can see that there are certain changes between the two types. The subtle difference between phenotype E and phenotype M is the airway remodeling. We speculated that the difference in efficacy of tiotropium bromide might be due to the differences in muscarinic receptors between two phenotypes of COPD patients. It seems likely that patients with emphysema but without bronchial wall thickening (or with mild bronchial wall thickening) may be more sensitive to tiotropium bromide. The most widespread disease-specific health status questionnaires such as St George's Respiratory Questionnaire[12] and Chronic Respiratory Questionnaire[13] are too complicated to perform. As such, simpler measures such as The COPD Control Questionnaire (The CCQ) and CAT have been applied. In China, the CAT scores correlated well with the measurements of quantitative CT. Not only can it assess status of health and disease, but it can also associate with the extent of emphysema and thickness of bronchial tube wall in Chinese COPD patients.[14] In any subtypes of patients, the CAT scores after treatment were significantly lower than that before the treatment, but phenotype E was lower than that of phenotype M. This shows that symptomatic improvement of patients with phenotype E with treatment of tiotropium bromide is superior to that of phenotype M. Fujimoto et al[15] also obtained similar result in 2011. In addition to

emphysema, phenotype M shows more severe airway remodeling of tube wall thickening in pathological morphology and increased airway inflammation; after using the same medicine, phenotype E may have better improvement of pulmonary function and symptom than phenotype M. Interestingly, our previous study[9] found that phenotype M in response to therapeutic effects of budesonide–formoterol were significantly greater compared with phenotype E, and bronchial wall thickening may be a better predictor for the response to treatment with bronchodilator and corticosteroid. However, further experiments will be needed to study the reason why different phenotypes of COPD have different therapeutic efficacy to tiotropium bromide and what the mechanism is. It could guide better and individualized treatment of COPD in the future. Metabolomics is a comprehensive assessment of endogenic metabolites and aims to methodically quantify and identify metabolites from specimens in a global and targeted way. We chose NMR-based metabolomics because NMR requires little specimen. It is also fast, non-invasive and nondestructive. At present, samples of metabolomics in COPD are limited to blood, urine, and exhaled breath condensate (EBC). Urinary metabolic profiling may correlate with lung function[16] and could become a useful clinical assessment to distinguish asthma from COPD.[17] The metabolic fingerprint of EBC could be used to discriminate the α1-antitrypsin-deficient patients from healthy people,[18] and serum metabolic changes have also been detected in COPD and normal people.[19] We chose to analyze the blood of the subjects by the ^{1}H-NMR spectroscopy because the blood sample is more accurate and less affected.

Increased energy consumption, increased catabolism, reduced intake of nutrients, impaired gastrointestinal function, and altered endocrine hormones led to malnutrition in COPD. COPD patients are accompanied by energy metabolism disorders, which are characterized by abnormal glucose metabolism, increased lipid oxidation, and reduced protein supply. Glucose and fructose are the most important monosaccharides to the human body. Like glucose, maltose is a reducing sugar, which can be broken down to glucose by maltase. In COPD patients, the concentration of all carbohydrate metabolites was increased. When the energy supply to the body is insufficient, the body will decompose the glycogen in the form of energy storage and increase the concentration of the sugar in the blood for metabolism. The increased carbohydrate metabolism indicates increased uptake and utilization of glucose in the COPD patients.[20] These results suggest that patients with COPD produce more glucose that enter glycolytic pathways and form more pyruvate. Hence, we found higher levels of pyruvate in COPD patients than normal controls. Pyruvate can also be converted to fatty acids through a reaction with acetyl-CoA and can be used to construct alanine and be converted into ethanol or lactate through fermentation. Increased lactate and alanine suggest that pyruvate is non-oxidized to lactate and alanine rather than being oxidized through the citric acid cycle. Hypoxia and respiratory muscle fatigue are the causes of lactic acidosis in patients with COPD.[21] Lipids exert multiple effects in the body, many of which may be related to the pathogenesis of COPD. Different species of lipids have dynamic balance conditions in the course of disease development. Change in lipids indicates the presence of its catabolism in COPD patients. The resting energy expenditure (REE) of COPD patients was higher than that of the same-age normal people due to airway obstruction, increased working load of respiratory muscles, and decreased blood oxygen saturation. The abnormality of lipid metabolism in COPD patients is mainly caused by a decrease in dietary intake and increase in REE. Related studies[22,23] have shown that fatty acid residues extracted from lipids could serve as energy substrates for glycolysis and upregulation of oxidation. Elevated levels of acetate, acetone, and pyruvate have been found in urine samples of COPD patients compared to normal controls.[19] Acetate is catalyzed by lipometabolism, and its increase may indicate the accelerated lipid catabolism to fulfill the energy requirements caused by poor nutritional status related to COPD patients. Acetone is produced from acetyl-CoA when energy is acquired from decomposed fatty acids because of the insufficient carbohydrate. Increased acetone can interpret the utilization of stored lipids as an alternative energy resource for patients with COPD. Amino acids are another large category of energy metabolism in human body. Valine is one of the branched-chain amino acids (BCAAs). Pouw et al reported that BCAAs in plasma do not differ between COPD patients and healthy individuals.[24] However, more studies found that the concentration of BCAAs is reduced in COPD patients[19] and models.[25] The elevated level of valine in our experiment is consistent with the previous phenotyping study.[26] The reason for the different results is that previous metabolomics experiments did not use CT classification as a criterion for screening patients with COPD. Proline is a significant metabolite related to environmental stress, and under stress, proline accumulation may increase cell viability by scavenging reactive oxygen species and stabilizing proteins and the cell membrane.[27] It is well known that the pathogenesis of COPD is related to oxidative stress, and the elevated proline just demonstrates this. Glycine can act on inflammatory cells, inhibiting the activation of transcription factors and the formation of free radicals and inflammatory

cytokines.[28] Inflammatory reactions in COPD patients are associated with impaired energy metabolism,[29] and the levels of inflammatory factors and inflammatory responses in COPD are higher than those in normal people. The increment of glycine may be related to the reactive suppression of inflammation. An elevated level of ornithine may enhance nitrogen metabolism and cellular activity in COPD patients.[30] Choline and betaine are two related metabolites obtained from food. Betaine was increased in the subjects of phenotype M compared with normal subjects. Choline is needed to synthesize membrane phospholipids and can be converted to betaine.[31] This conversion can proceed through bacterial enzymes, which may be a unique feature of the airways in patients with COPD. Betaine serves as an organic osmolyte and plays an important role in DNA methylation and homocysteine production. Previous studies on these metabolites were targeted in asthma patients.[32–34] This is the first time that these two metabolites have been observed in COPD patients with different phenotypic classification (Figure 4). The reduction of guanosine may be related to the mechanism of cell damage. Few studies have evaluated the association of vitamin B6 with COPD patients. Our experiment is the first to find the presence of metabolism of vitamin B6 and NAC in the serum of patients with COPD by means of metabolomics. Pyridoxine, of vitamin B6 metabolism, was found to be reduced in COPD phenotype E compared with healthy subjects. This result is in line with the previous experimental results[35] and, furthermore, notes that the vitamin B6 values were related to FEV_1.[36] The mechanism of poor vitamin B6 status in patients with COPD is still unknown, but chronic inflammatory coexistence may account for this. We have previously researched NAC and COPD at the genetic level.[37,38] It has been shown previously that antioxidant NAC could reduce acute exacerbations and moderately improve the health of COPD patients.[39,40] In this study, concentration of NAC increased in COPD patients of phenotype M compared with normal subjects. After the treatment of tiotropium bromide, the NAC metabolism was not detected in the phenotype M. The mechanism of metabolic change of NAC in COPD patient blood requires further studies.

Patients with phenotype M have more severe small airway lesions than those with phenotype E, although there was no significant difference between them in terms of lung function and CAT scores. Glutamine is the most abundant naturally occurring, nonessential amino acid in the human body.[41] However, patients with emphysema and airway thickening have more severe respiratory symptoms and more frequent hospitalizations than those with emphysema alone.[42] We can speculate that patients with phenotype M have more severe inflammatory reactions in airway and body than those with phenotype E. Glutamine level is reported to be higher in asthmatic patients compared with normal people.[32] Glutamine augment of phenotype E may be related to the transportation of interleukins and increased protein synthesis.[43] Asparagine is the main participant in the α-helix and beta-pleated sheet in the protein configuration. The decrease of the content of asparagine of phenotype E after treatment may be related to the damage of the corresponding inflammatory protein. As asparagine is at the junction of glycine and citric acid (convertible to citric acid and glycine) (Figure 4), it indicates that tiotropium may interfere with the phenotype E by altering the metabolic pathway of asparagine. Creatinine is the product of muscle metabolism in the body and its clinical detection is one of the most commonly used methods in estimating the functions of the kidney. Reduced urinary creatinine has been reported in COPD patients.[19] Blood creatinine is closely related to the total amount of muscle in the body and is not easily affected by diet. The concentration of creatinine of phenotype M was increased after treatment. Although creatinine is a marker of many inflammations, it has not yet been found in relevant reports in lung disease models. For phenylalanine, its concentration in phenotype E after treatment was decreased than that in phenotype M. The decreased phenylalanine is consistent with a study conducted in China[19] but differs from earlier reports.[44,45] Explanation for this inconsistency may be different human species and lifestyle factors, because the earlier studies had been performed with Caucasian participants. Another possible explanation for the controversy is that none of their subjects were based on the classification of CT subtypes in COPD. Before treatment, patients with phenotype E had higher levels of glutamine and alanine than phenotype M. After treatment, the difference between the two subtypes was reflected by the two amino acid metabolites of proline and phenylalanine and pyruvic acid, and the three metabolites were lower in patients with phenotype E than those with phenotype M. Phenylalanine is closely associated with increased systemic inflammation[44] and the production and actions of pyruvic acid could be defective in the conditions of anti-inflammation and anti-oxidation.[46] After treatment with the same medicine, the concentrations of these two metabolites are lower in phenotype E than those in phenotype M. This may indicate that in the case where inflammation in phenotype M is severer than phenotype E, since E-type is more sensitive to tiotropium efficacy, the inflammatory markers of phenotype E after treatment are reactively decreased than that of phenotype M. The metabolic pathways that the metabolites involved in and the transformations of metabolites can be seen in Figure 4.

Limitations

As all the selected COPD patients and normal subjects came from the Southwest of China, the low number of subjects was limited. There are geographical limitations in these samples. The influence of race, lifestyle, living environment, and eating habits could not be excluded. In the process of filling out the CAT questionnaires, the subjects might have subjective bias due to the degree of education and the difference of understanding. We need further multicenter, multi-area, large-sample, long-term, and dynamic research for the identification of different CT phenotypes of COPD patients, ^{1}H-NMR analysis, and their therapeutic response of anticholinergic drugs in the future.

Conclusion

COPD patients and healthy subjects before and after treatment can be distinguished well by the method of metabolomics based on ^{1}H-NMR. Simultaneously, the differences in serum metabolites can also result in different diagnoses between the two phenotypes of COPD patients before and after treatment. COPD patients with different phenotypes who were classified through HRCT have different therapeutic efficacy on tiotropium bromide. Changes in amino acids, carbohydrates, lipids, genetic materials, and vitamin metabolic pathways were involved in the classification of COPD and its treatment with tiotropium bromide. Combined with CAT scores and results of lung function, the therapeutic efficacy of tiotropium bromide on phenotype E was better than that of phenotype M. The present study found that these metabolites have potentialities of differentiation of COPD and healthy people, COPD phenotyping, and therapeutic efficacy evaluation. Thus, it could scientifically guide the individualized and optimized treatment of COPD.

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Disclosure

The authors report no conflicts of interest in this work.

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