A pilot data analysis of a metabolomic HPLC-MS/MS study of patients with COPD

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Conflict of interest

None declared

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Abstract

Background. Chronic obstructive pulmonary disease (COPD) is a heterogeneous condition with multiple clinical faces. Metabolomic profiling studies small molecules present in biological samples by combined use of chromatography with mass spectrometry.

Objectives. The goal of our work was to perform a high performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) metabolomic study to compare the concentrations of metabolites in COPD patients and in controls.

Material and methods. Participants were recruited at the University Hospital, Hradec Králové, Czech Republic, with the approval of the ethics committee. The analysis of blood samples was performed at Health Sciences Center (HSC) in Kuwait. The blood samples were analyzed for concentrations of acylcarnitines and amino acids by high performance liquid chromatography (Waters 2690 HPLC; Waters, Milford, USA) and a triple-quadrupole tandem mass spectrometer (Quattro LC, Micromass, Manchester, United Kingdom).

Results. Groups of 10 subjects with COPD and 10 healthy controls were analyzed. Carnitine analysis showed that the free carnitine to acylcarnitine ratio (CO/AC ratio) was significantly lower in COPD (0.58 μM/L) compared to the controls (0.73 μM/L; $p = 0.002$). The mean C8/C2 ratio in the COPD group was significantly higher (0.03 μM/L) – in the control group it was 0 μM/L ($p = 0.03$). Amino acid analysis showed lower levels of phenylalanine in the COPD group (22.05 μM/L) compared to the controls (30.05 μM/L; $p = 0.008$). The alanine concentrations were significantly lower in the COPD group (173 μM/L) than in the control group (253 μM/L; $p = 0.001$). The pyroglutamate levels were higher in COPD (1.58 μM/L) than in the controls (1 μM/L; $p = 0.040$).

Conclusions. The carnitine and acylcarnitine levels in COPD subjects in this study possibly indicate a predisposition to atherosclerosis as a result of inadequate β-oxidation of fatty acids and show the presence of oxidative stress. Furthermore, the high sensitivity to changes in circulating amino acid levels may allow us to detect subclinical malnutrition and take early preventative interventions such as nutritional supplementation and patient education.

Key words: amino acids, chronic obstructive pulmonary disease, liquid chromatography-tandem mass spectrometry, carnitine, metabolomics

Introduction

Chronic obstructive pulmonary disease (COPD) is a term referring to a heterogeneous group of chronic lung diseases marked by restricted airflow. Lung damage in COPD is nonreversible and progressive, but may be halted through proper treatment. The two most prevalent conditions characteristic for COPD are emphysema and chronic bronchitis, but new, not yet fully elucidated phenotypes such as asthma-COPD overlap syndrome (ACOS), pulmonary cachexia and frequent exacerbators are emerging.^{1–3} COPD has a severe impact on patients' health and quality of life.^{4,5}

Metabolomic studies represent a modern trend in biological and clinical scientific work.⁶ Metabolomic profiling studies small molecules (molecular weight <900 Da) present in biological samples. Originally, mainly nuclear magnetic resonance (NMR) was applied for this purpose. Presently, the combination of chromatographic methods with mass spectrometry (combinations of gas or liquid chromatography coupled to mass spectrometry – GC-MS or LC-MS) has become a widely applied analytical method for global profiling of metabolites.⁷

Only a limited number of metabolomic profiling studies was performed in patients with COPD. A limited number of papers are devoted to analyzing concentrated samples of exhaled air in patients with COPD using chromatographic methods or NMR spectroscopy.^{8–11}

A metabolomic study based on the use of NMR spectroscopy was able to pinpoint the differences between serum of healthy individuals and COPD patients; particularly, decreased concentrations of lipoproteins and amino acids and increased concentrations of glycerolphosphocholine, acetate, ketone bodies, carnosine, m-hydroxyphenylacetate, phenylacetylglycine, pyruvate, and α -ketoglutarate.¹² Another NMR metabolomic study demonstrated the method by investigating plasma and urine samples from 197 COPD patients and 195 adults without COPD.¹³ The findings were not conclusive and experiments need to be repeated and validated. On the other hand, the authors found this approach suitable for the identification of novel biomarkers, useful for determining COPD therapeutic outcomes.¹³ A metabolomic approach for studying COPD patients was applied to a defined cohort from the Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points (ECLIPSE) study.¹⁴ Serum from healthy subjects and Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage II, III and IV patients with COPD was investigated using NMR spectrometry. The findings were confirmed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) and correlated with other data. NMR spectrometry helped in identifying decreased concentrations of lipoproteins and N,N-dimethylglycine. On the other hand, increased concentrations of glutamine, phenylalanine, 3-methylhistidine, and ketone bodies were shown in GOLD stage IV COPD patients who also had decreased branched-chain

amino acids (BCAAs) levels. There were found negative correlations between BCAAs, their degradation products, 3-methylhistidine, ketone bodies, and triglycerides on one side and cachexia on the other side. These concentrations positively correlated with systemic inflammation.¹⁴

In the ECLIPSE study, the concentrations of amino acids and dipeptides were measured by LC-MS/MS in groups of patients divided according to the GOLD characteristics. Differences in the levels of several amino acids and other small molecules were confirmed in the groups of COPD patients.¹⁵ The study showed differences among various groups of COPD patients (emphysema and no emphysema; cachectic and non-cachectic). Additionally, COPD patients were distinguished from 'healthy' smokers used as a control group. These results are in agreement with the findings of another study that concentrated on the differentiation of subjects with or without emphysema.¹⁶

High performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) may be used for the identification of subtle differences in biomarkers between COPD patients, healthy controls, smokers and non-smokers, etc. This method proved to be useful not only in metabolomic studies, but also in various proteomic investigations identifying proteins that could help differentiate healthy subjects from diseased groups of patients.^{17–19} Some proteins present at very low concentrations show different expression in various COPD patients, reflecting the possible pathogenic mechanisms and the extent and severity of lung remodeling. This has a potential in COPD diagnosis and disease activity tracking by biomarkers for the benefit of patients by improving the disease management.

The goal of our work was to perform a HPLC-MS/MS metabolomic study to compare the abundance of several small molecules in COPD patients and healthy volunteers.

Material and methods

Diagnostic criteria for chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease was diagnosed on the basis of an evaluation of patients' lung functions, symptoms and history of exacerbations. All patients in this study performed a spirometry test and met the COPD diagnostic criteria of post-bronchodilator values of forced expiratory volume in 1 s/forced vital capacity (FEV1/FVC) <0.70.^{1,2}

Patients

Blood samples from COPD patients for this study were obtained from the participants registered in the Czech Multicenter Research Database of Chronic Obstructive Pulmonary Disease (ClinicalTrials.gov identifier NCT01923051). Blood samples from patients with coronary artery disease and healthy controls were obtained

from the Department of Cardioangiopathy and the Transfusion Department of the University Hospital in Hradec Králové (Czech Republic), respectively. The ethics committee of the hospital approved the study. All participants signed an informed consent form. The analysis of blood samples was performed at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kuwait University (Kuwait).

The inclusion criteria for this study were: non-smokers or ex-smokers of at least 6 months, patients without acute exposition to carbon monoxide (CO) due to smoke or pollution inhalation (measured by smokerlyzer) and COPD patients with post-bronchodilator values of $FEV_1 < 60\%$.

Patients were excluded upon the following criteria: current smoker or ex-smokers of less than 6 months (CO levels > 10 ppm), a known metabolic disease including diabetes type I and II, kidney disease with moderate to severe creatinine clearance, and the presence of coronary artery disease.

Venous blood was collected by standard venipuncture into BD Vacutainer® Heparin Tubes (Becton Dickinson, Mississauga, Canada). Heparinized blood was spotted onto a filter paper cards and left to dry at room temperature for at least 24 h.

We analyzed 2 groups of subjects (Table 1). They included 10 subjects with COPD and 10 healthy controls. The mean age of the COPD group was 61.5 years with a male to female ratio (m:f) of 1. The mean age of the control group was 55 years with m:f of 1. Although the healthy control group was younger, this difference was not significant ($p = 0.052$). The mean body mass index (BMI) was matched in the COPD group (25.3 kg/m^2) and in the control group (27.10 kg/m^2), with $p = 0.496$. The COPD patients had an average fat free mass index (FFMI) of 18.79 kg/m^2 ; FEV_1 of 33 %; and FEV_1/FVC of 0.52. Spirometric data and FFMI were not available for the control group.

HPLC-MS/MS instrumentation

The blood samples were analyzed for acylcarnitines and amino acids by a triple-quadrupole tandem mass spectrometer (Quattro LC, Micromass, Manchester, UK) with a positive electrospray ionization probe. High performance liquid chromatography (Waters 2690 HPLC) with an autosampler (Waters, Milford, USA) was used for automatic injection. A designed tandem mass spectrometry (MS/MS) program for automatic profiling of acylcarnitines and amino acids was used. The NeoLynx program (Quattro LC, Micromass, Manchester, United Kingdom) was used to determine the concentration of the diagnostic metabolites.

HPLC-MS/MS method

Using a BSD 400 Puncher (BSD Biosample punchers, Brendale, Australia), 2–3-mm disks were punched from the center of dried blood spots. The disks were placed in a capped glass for extraction of the blood with $200 \mu\text{L}$

Table 1. Descriptive statistics

	COPD (n = 10)	Control (n = 10)	p-value
Sex (male), n (%)	5 (50.0%)	5 (50.0%)	0.999
Age [years]	61.50 (28.00; 70.00)	55.00 (53.00; 61.00)	0.052
BMI [kg/m^2]	25.30 (19.47; 32.95)	27.10 (24.40; 29.40)	0.496
FFMI [kg/m^2]	18.79 (14.96; 25.79)	–	–
FEV_1 [%]	33.00 (26.00; 56.00)	–	–
FEV_1/FVC	0.52 (0.30; 0.74)	–	–
C0	24.75 (15.40; 44.20)	33.40 (19.10; 47.60)	0.140
C2	12.45 (8.10; 35.30)	10.95 (6.20; 14.10)	0.076
C3	0.43 (0.20; 0.67)	0.26 (0.00; 0.61)	0.140
C4	0.00 (0.00; 0.22)	0.00 (0.00; 0.19)	0.351
C5	0.00 (0.00; 0.22)	0.04 (0.00; 0.23)	0.525
C5-OH	0.04 (0.00; 0.39)	0.02 (0.00; 0.36)	0.716
C8	0.07 (0.00; 1.34)	0.00 (0.00; 0.16)	0.264
C14	0.00 (0.00; 0.09)	0.00 (0.00; 0.10)	0.564
C14:1	0.00 (0.00; 0.18)	0.00 (0.00; 0.31)	0.551
C16	0.56 (0.00; 5.56)	0.16 (0.00; 2.85)	0.412
C16-OH	0.00 (0.00; 0.04)	0.00 (0.00; 0.12)	0.842
C18:1	0.42 (0.00; 0.61)	0.00 (0.00; 0.74)	0.090
AC total	37.67 (26.99; 81.04)	45.44 (26.90; 61.48)	0.450
C0/AC	0.58 (0.53; 0.68)	0.73 (0.57; 0.86)	0.002
C3/C16	0.22 (0.00; 0.61)	0.07 (0.00; 0.84)	0.589
C3/C2	0.10 (0.00; 0.16)	0.22 (0.00; 0.82)	0.074
C5/C2	0.00 (0.00; 0.06)	0.01 (0.00; 0.09)	0.648
C8/C2	0.03 (0.00; 0.50)	0.00 (0.00; 0.06)	0.030
C14:1/C16	0.01 (0.00; 0.44)	0.00 (0.00; 0.34)	0.183
C16-OH/C16	0.00 (0.00; 0.06)	0.00 (0.00; 0.20)	0.829
Glutaryl	0.00 (0.00; 0.36)	0.00 (0.00; 0.29)	0.402
Valine	85.95 (57.20; 166.00)	97.40 (2.80; 134.00)	0.910
Pyg	1.58 (0.00; 3.43)	1.00 (0.00; 1.96)	0.040
Met	0.00 (0.00; 41.30)	0.00 (0.00; 0.00)	0.317
Phe	22.05 (17.20; 34.20)	30.05 (21.10; 43.70)	0.008
Tyr	21.75 (0.80; 171.00)	27.85 (22.20; 50.00)	0.130
Cit1	13.20 (0.00; 25.70)	12.40 (0.00; 26.40)	0.940
Glyc	90.40 (77.20; 163.00)	104.00 (67.00; 146.00)	0.496
Alanine	173.00 (141.00; 215.00)	253.00 (173.00; 346.00)	0.001
Leu/Ile	58.85 (37.30; 69.40)	64.65 (51.40; 96.00)	0.104
Phe/Tyr	0.00 (0.00; 3.43)	0.00 (0.00; 2.69)	0.487

BMI – body mass index; FFMI – fat free mass index; FEV_1 – forced expiratory volume in 1 s; FVC – forced vital capacity; C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

The concentrations of metabolites are in units $\mu\text{M/L}$; categorical variable is described by absolute (relative) frequency and tested by Fisher's exact test; continuous variable is described by median (5th and 95th percentiles) and tested by the Mann-Whitney test.

of methanol, containing known concentrations of a mixture of isotopically labeled internal standards of diagnostic acylcarnitines and amino acids. After 20 min of shaking, the solvent was gently evaporated, and the residue was mixed with 80 μ L of butanolic HCl and heated at 65°C for 15 min in a capped glass tube. The solvent was evaporated again and the residue was finally reconstituted in 80 μ L of the mobile phase acetonitrile/water (80/20 v/v). A 20 μ L aliquot of each sample was directly injected into the mobile phase flowing to the ionization probe of the tandem mass spectrometer at a flow rate of 0.1 mL/min. A Waters 2690 HPLC with an autosampler was used for automatic injection. The run cycle time for each sample was 2–3 min

from injection to injection. A designed MS/MS program for automatic profiling of acylcarnitines and amino acids was used. The NeoLynx program for Neonatal Screening (Quattro LC, Micromass, Manchester, United Kingdom) was used for automatic detection of abnormal samples. The values obtained for COPD patients and patients with coronary heart disease were compared to those of healthy controls.

Statistical methods

Standard descriptive statistics was applied for the analysis: absolute and relative frequencies for categorical

Table 2. Influence of sex on the results

Carnitines and amino acids	COPD			Control		
	female (n = 5)	male (n = 5)	p-value	female (n = 5)	male (n = 5)	p-value
C0	24.60 (18.70; 44.20)	24.90 (15.40; 25.50)	0.602	24.60 (19.10; 35.70)	42.70 (20.10; 47.60)	0.117
C2	12.10 (9.70; 35.30)	12.90 (8.10; 19.50)	0.917	11.00 (7.69; 14.10)	10.90 (6.20; 12.70)	0.754
C3	0.47 (0.20; 0.67)	0.41 (0.34; 0.64)	0.917	0.16 (0.00; 0.61)	0.36 (0.00; 0.54)	0.916
C4	0.00 (0.00; 0.20)	0.07 (0.00; 0.22)	0.290	0.00 (0.00; 0.10)	0.00 (0.00; 0.19)	0.881
C5	0.00 (0.00; 0.22)	0.00 (0.00; 0.20)	0.999	0.03 (0.00; 0.09)	0.04 (0.00; 0.23)	0.832
C5OH C5-OH	0.00 (0.00; 0.18)	0.07 (0.00; 0.39)	0.577	0.00 (0.00; 0.04)	0.11 (0.00; 0.36)	0.034
C8	0.06 (0.00; 1.34)	0.10 (0.00; 0.51)	0.746	0.00 (0.00; 0.14)	0.00 (0.00; 0.16)	0.368
C14	0.00 (0.00; 0.09)	0.00 (0.00; 0.05)	0.638	0.00 (0.00; 0.10)	0.00 (0.00; 0.04)	0.521
C14:1	0.00 (0.00; 0.18)	0.00 (0.00; 0.01)	0.881	0.00 (0.00; 0.03)	0.00 (0.00; 0.31)	0.368
C16	0.29 (0.00; 1.56)	0.89 (0.00; 5.56)	0.245	0.00 (0.00; 0.31)	0.81 (0.00; 2.85)	0.034
C16-OH	0.00 (0.00; 0.03)	0.00 (0.00; 0.04)	0.366	0.00 (0.00; 0.06)	0.00 (0.00; 0.12)	0.881
C18:1	0.40 (0.00; 0.52)	0.44 (0.00; 0.61)	0.916	0.00 (0.00; 0.40)	0.00 (0.00; 0.74)	0.521
ACTotal	36.53 (33.42; 81.04)	38.81 (26.99; 46.62)	0.917	35.01 (26.90; 50.33)	49.39 (35.56; 61.48)	0.076
C0/AC	0.59 (0.55; 0.68)	0.57 (0.53; 0.66)	0.347	0.71 (0.65; 0.74)	0.77 (0.57; 0.86)	0.117
C3C16	0.15 (0.00; 0.61)	0.28 (0.00; 0.34)	0.675	0.00 (0.00; 0.65)	0.23 (0.00; 0.84)	0.147
C3C2	0.09 (0.00; 0.15)	0.11 (0.00; 0.16)	0.287	0.07 (0.00; 0.67)	0.25 (0.21; 0.82)	0.075
C5C2	0.00 (0.00; 0.06)	0.00 (0.00; 0.06)	0.906	0.01 (0.00; 0.05)	0.00 (0.00; 0.09)	0.911
C8C2	0.02 (0.00; 0.50)	0.04 (0.00; 0.19)	0.829	0.00 (0.00; 0.00)	0.00 (0.00; 0.06)	0.317
C14:1/C16	0.00 (0.00; 0.44)	0.01 (0.00; 0.12)	0.737	0.00 (0.00; 0.34)	0.00 (0.00; 0.00)	0.136
C16-OH/C16	0.00 (0.00; 0.00)	0.00 (0.00; 0.06)	0.136	0.00 (0.00; 0.20)	0.00 (0.00; 0.15)	0.881
Glutaryl	0.00 (0.00; 0.36)	0.00 (0.00; 0.00)	0.136	0.00 (0.00; 0.14)	0.03 (0.00; 0.29)	0.290
Valine	67.40 (57.20; 103.00)	95.30 (80.90; 166.00)	0.076	80.70 (2.80; 110.00)	103.00 (77.10; 134.00)	0.117
Pyg	1.23 (0.00; 3.43)	2.27 (1.18; 2.50)	0.251	1.18 (0.00; 1.96)	0.00 (0.00; 1.40)	0.281
Met	0.00 (0.00; 41.30)	0.00 (0.00; 0.00)	0.317	0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.999
Phe	21.80 (17.20; 34.20)	24.00 (19.70; 24.50)	0.463	29.60 (21.10; 36.20)	30.50 (23.10; 43.70)	0.754
Tyr	26.30 (13.40; 171.00)	19.50 (0.80; 31.50)	0.251	28.00 (22.20; 50.00)	27.80 (22.20; 44.60)	0.675
Cit1	8.61 (0.00; 14.30)	18.90 (5.96; 25.70)	0.172	12.60 (0.00; 26.40)	9.26 (0.00; 20.40)	0.530
Glyc	88.40 (77.20; 163.00)	92.40 (78.30; 120.00)	0.754	113.00 (82.00; 146.00)	101.00 (67.00; 119.00)	0.465
Alanine	147.00 (141.00; 193.00)	176.00 (157.00; 215.00)	0.251	276.00 (197.00; 307.00)	230.00 (173.00; 346.00)	0.917
Leulle Leu/Ile	57.10 (37.30; 65.50)	59.50 (51.40; 69.40)	0.175	55.50 (51.40; 91.60)	77.50 (62.40; 96.00)	0.175
PhETyr Phe/Tyr	1.09 (0.00; 3.43)	0.00 (0.00; 0.00)	0.054	0.00 (0.00; 0.92)	0.00 (0.00; 2.69)	0.881

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

The concentrations of metabolites are in units μ M/L; continuous variable is described by median (5th and 95th percentiles) and tested by the Mann-Whitney test.

variables, and median supplemented by 5th–95th percentile range for continuous variables. Statistical significance of differences was tested using Fisher's exact test for categorical variables and the Mann-Whitney test for continuous variables. Spearman's correlation coefficient was used for the analysis of the relationship between continuous variables. Statistical analysis was computed using SPSS 22 (IBM Corporation, New York, USA; 2013).

Results

In the analysis of the concentrations of free carnitine and acylcarnitines, small variations between the 2 groups were noted. Most notably, the free carnitine to acylcarnitine ratio (C0/AC ratio) was significantly lower in the COPD group compared to the control group – COPD 0.58 $\mu\text{M/L}$ (0.53; 0.68) and control group 0.73 $\mu\text{M/L}$ (0.57; 0.86) with $p = 0.002$. The mean C8/C2 ratio in the COPD group was significantly higher, 0.03 $\mu\text{M/L}$ (0; 0.50), whilst it was 0 $\mu\text{M/L}$ in the control group ($p = 0.03$).

The analysis of amino acids showed significantly lower levels of phenylalanine in the COPD group compared to the controls. The phenylalanine level in the COPD group was 22.05 $\mu\text{M/L}$ (17.20; 34.20) and in the control group, it was 30.05 $\mu\text{M/L}$ (21.10; 43.70) with $p = 0.008$; the alanine levels were significantly lower in the COPD group, 173 $\mu\text{M/L}$ (141; 215), than in the control group – 253 $\mu\text{M/L}$ (173; 346); $p = 0.001$. The pyroglutamate levels were significantly higher in COPD patients, 1.58 $\mu\text{M/L}$ (0; 3.43), than in the control group – 1 $\mu\text{M/L}$ (0; 1.96); $p = 0.040$.

Gender had no influence on the metabolomic profile of COPD patients, and only a small effect in the control group (Table 2). In the control group, males had significantly higher levels of C5OH (0.11 $\mu\text{M/L}$) compared to females (0 $\mu\text{M/L}$); $p = 0.034$. Also, the levels of C16 were significantly higher in males (0.81 $\mu\text{M/L}$) than in females (0 $\mu\text{M/L}$); $p = 0.034$.

Increased age (Table 3) positively correlated with increased levels of free carnitine (Spearman's coefficient 0.695; $p = 0.026$) and the phenylalanine/tyrosine ratio (Spearman's coefficient 0.818; $p = 0.004$) in COPD patients. In the COPD group, a negative correlation with age was found in C5-OH (Spearman's coefficient -0.644 ; $p = 0.044$) and C14:1 (Spearman's coefficient -0.688 ; $p = 0.028$). In the control group, age positively correlated with the levels of C14 (Spearman's coefficient 0.765; $p = 0.010$) as well as the phenylalanine/tyrosine ratio (Spearman's coefficient 0.694; $p = 0.026$) and the phenylalanine level (Spearman's coefficient 0.671; $p = 0.034$).

In the COPD group, BMI (Table 4) positively correlated with C0/AC ratios (Spearman's coefficient 0.758; $p = 0.011$), whilst there was a negative correlation with C5/C2 ratios (Spearman's coefficient -0.809 ; $p = 0.005$). In the control group, the leucine/isoleucine ratio

Table 3. Influence of age on the results

Carnitines and amino acids	COPD (n = 10)		Control (n = 10)	
	Spearman's coefficient	p-value	Spearman's coefficient	p-value
C0	0.695	0.026	0.419	0.229
C2	0.494	0.147	0.345	0.329
C3	0.335	0.343	0.287	0.422
C4	0.052	0.888	0.501	0.140
C5	-0.017	0.962	-0.069	0.850
C5-OH	-0.644	0.044	0.033	0.928
C8	-0.157	0.664	-0.174	0.630
C14	0.498	0.143	0.765	0.010
C14:1	-0.688	0.028	0.182	0.615
C16	-0.642	0.045	-0.210	0.560
C16-OH	-0.328	0.355	-0.092	0.800
C18:1	0.136	0.707	0.008	0.983
AC total	0.591	0.072	0.443	0.200
C0/AC	0.134	0.712	0.086	0.813
C3/C16	0.294	0.410	0.223	0.535
C3/C2	-0.205	0.570	-0.052	0.886
C5/C2	-0.224	0.534	-0.118	0.745
C8/C2	0.237	0.510	-0.530	0.115
C14:1/C16	-0.202	0.575	0.004	0.990
C16-OH/C16	-0.544	0.104	0.004	0.990
Glutaryl	0.017	0.962	0.180	0.618
Valine	-0.305	0.392	-0.203	0.574
Pyg	0.146	0.687	0.083	0.821
Met	0.292	0.413	–	–
Phe	-0.260	0.468	0.671	0.034
Tyr	0.250	0.486	0.364	0.301
Cit1	-0.310	0.384	0.321	0.366
Glyc	-0.280	0.432	0.499	0.142
Alanine	-0.104	0.776	0.437	0.207
Leu/Ile	-0.470	0.171	0.443	0.200
Phe/Tyr	0.818	0.004	0.694	0.026

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

positively correlated with BMI (Spearman's coefficient 0.721; $p = 0.019$).

Fat free mass index had no effect on the metabolomic profile of COPD patients (Table 5). In the COPD group, there was a negative correlation of FEV₁ with C3 (Spearman's coefficient -0.634 ; $p = 0.049$), C5 (Spearman's coefficient -0.676 ; $p = 0.032$) and leucine/isoleucine ratios (Spearman's coefficient -0.702 ; $p = 0.024$) (Table 6). There was a negative correlation between FEV₁/FVC and the C4 (Spearman's coefficient -0.635 ; $p = 0.049$) and

tyrosine levels (Spearman's coefficient -0.770 ; $p = 0.009$) in the COPD group (Table 7).

Discussion

The severity of COPD in patients is normally graded based on the degree of obstruction as measured by FEV₁. Today, it is accepted that COPD is a complex disease with systemic effects. Transcriptomics, proteomic and metabolomic investigation have the potential to contribute

to a better understanding of COPD and its natural course. This is paramount, as COPD has been recently recognized as a condition affecting the whole organism. For example, it was shown that COPD patients are very likely to suffer from metabolic syndrome with increased pro-inflammatory markers from the lungs and adipose tissue.²⁰

The development of modern instrumental analytical techniques contributes to the investigation of many pathological states and helps in the elucidation of metabolic and other processes inside the body of a patient. In our work, we investigated the concentrations of various small molecules, mainly various amino acids and free and acylated carnitines with a different length of the alkyl chain, using the HPLC-MS/MS method.

Carnitine is a quaternary ammonium compound, synthesized by the kidneys and liver from the amino acids lysine and methionine. It is also obtained through a diet, particularly through red meat and nuts. Carnitine has a major role in the citric acid cycle by binding and transporting various acyl groups to be metabolized through β -oxidation. Consequently, the disturbance of these processes leads to various pathological states.^{21,22} Genetic disorders, such as primary carnitine deficiency, carnitine palmitoyltransferase deficiency type I and II among others, affect different steps in carnitine biotransformations.²³ The significantly lower ratio of free carnitine/acylated carnitine (C0/AC) in COPD patients, despite the lack of distinct differences in the concentration of free carnitine, and the total and individual acylcarnitine levels, possibly indicates a predisposition to atherosclerosis as a result of inadequate β -oxidation of fatty acids, as well as of being at risk for carnitine deficiency.^{24,25}

Evidence suggests that impaired fatty acid oxidation has some effect on the development of type-2 diabetes.^{26,27} The prevalence of diabetes in COPD patients is significant, and may be one of the factors which cause the progression and worsen the prognosis of COPD.^{28–30} Acylcarnitines have a potential to activate proinflammatory signaling pathways as part of the shared mechanism of COPD and cardiovascular disease.^{27,31} Metabolic heritability may be implicated in aberrant levels of some acylcarnitines, as it was shown that some metabolite levels are inheritable at birth and that some genes are associated with acylcarnitines.³²

The role of inherited fatty acid oxidation deficiency and genetics in COPD may be further implicated by the higher levels of the acylated C8/C2 carnitines ratio in COPD patients. The C8/C2 ratio and the C8 levels are the most accurate markers of medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most common inherited defect in the fatty acid oxidation pathway marked by an inability to break down medium-chain fatty acids during periods of fasting.³³

Additional findings relate to alanine, a nonessential amino acid found in a variety of foods. It may be synthesized by the body itself from pyruvate and a branched-chain

Table 4. Influence of BMI on the results

Carnitines and amino acids	COPD (n = 10)		Control (n = 10)	
	Spearman's coefficient	p-value	Spearman's coefficient	p-value
C0	0.467	0.174	0.624	0.054
C2	-0.370	0.293	0.285	0.425
C3	0.273	0.446	-0.043	0.906
C4	-0.546	0.102	-0.545	0.103
C5	0.027	0.940	0.437	0.207
C5-OH	-0.407	0.243	-0.032	0.929
C8	-0.069	0.850	0.067	0.854
C14	-0.341	0.334	0.470	0.171
C14:1	-0.294	0.409	-0.231	0.521
C16	-0.362	0.304	0.252	0.482
C16-OH	-0.479	0.161	0.528	0.117
C18:1	-0.548	0.101	0.231	0.521
AC total	0.273	0.446	0.612	0.060
C0/AC	0.758	0.011	0.455	0.187
C3/C16	-0.170	0.638	0.614	0.059
C3/C2	-0.278	0.437	0.292	0.413
C5/C2	-0.809	0.005	0.149	0.682
C8/C2	0.038	0.918	0.058	0.873
C14:1/C16	-0.292	0.413	0.199	0.582
C16-OH/C16	-0.389	0.266	0.510	0.132
Glutaryl	-0.450	0.192	-0.191	0.597
Valine	0.624	0.054	0.370	0.293
Pyg	0.394	0.260	0.194	0.592
Met	0.290	0.416	–	–
Phe	-0.498	0.143	0.612	0.060
Tyr	-0.442	0.200	0.511	0.132
Cit1	-0.006	0.987	0.389	0.266
Glyc	-0.152	0.676	0.539	0.108
Alanine	0.176	0.627	0.576	0.082
Leu/Ile	0.018	0.960	0.721	0.019
Phe/Tyr	-0.172	0.636	0.311	0.381

C0 – free or non-acylated carnitine; C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl; AC – acylcarnitines; Pyg – pyroglutamic acid; Met – methionine; Phe – phenylalanine; Tyr – tyrosine; Cit – citrulline; Glyc – glycine; Leu – leucine; Ile – isoleucine.

Table 5. Influence of FFMI on the results

Carnitines and amino acids	COPD (n = 10)	
	Spearman's coefficient	p-value
C0	0.176	0.627
C2	−0.103	0.777
C3	0.030	0.934
C4	−0.171	0.637
C5	−0.191	0.597
C5-OH	−0.226	0.530
C8	−0.144	0.692
C14	−0.178	0.624
C14:1	−0.156	0.668
C16	0.018	0.960
C16-OH	−0.045	0.902
C18:1	−0.363	0.302
AC total	0.188	0.603
C0/AC	0.212	0.556
C3/C16	−0.055	0.881
C3/C2	0.222	0.537
C5/C2	−0.507	0.135
C8/C2	−0.063	0.863
C14:1/C16	0.019	0.957
C16-OH/C16	0.130	0.721
Glutaryl	−0.623	0.054
Valine	0.661	0.038
Pyg	0.624	0.054
Met	0.058	0.873
Phe	−0.140	0.700
Tyr	−0.539	0.108
Cit1	0.299	0.402
Glyc	0.006	0.987
Alanine	0.430	0.214
Leu/Ile	0.152	0.676
Phe/Tyr	−0.455	0.187

C0 – free or non-acylated carnitine;
 C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl;
 AC – acylcarnitines; Pyg – pyroglutamic acid;
 Met – methionine; Phe – phenylalanine;
 Tyr – tyrosine; Cit – citrulline; Glyc – glycine;
 Leu – leucine; Ile – isoleucine.

Table 6. Influence of FEV₁ on the results

Carnitines and amino acids	COPD (n = 10)	
	Spearman's coefficient	p-value
C0	−0.098	0.787
C2	−0.055	0.879
C3	−0.634	0.049
C4	−0.170	0.639
C5	−0.676	0.032
C5-OH	0.158	0.664
C8	−0.410	0.240
C14	0.132	0.717
C14:1	0.439	0.204
C16	0.305	0.391
C16-OH	−0.053	0.884
C18:1	0.194	0.592
AC total	−0.154	0.671
C0/AC	0.037	0.919
C3/C16	0.120	0.740
C3/C2	0.392	0.263
C5/C2	0.097	0.789
C8/C2	−0.628	0.052
C14:1/C16	0.573	0.083
C16-OH/C16	−0.141	0.699
Glutaryl	0.044	0.904
Valine	−0.406	0.244
Pyg	0.579	0.080
Met	0.530	0.115
Phe	−0.096	0.793
Tyr	−0.474	0.166
Cit1	−0.180	0.620
Glyc	−0.031	0.933
Alanine	−0.369	0.294
Leu/Ile	−0.702	0.024
Phe/Tyr	0.083	0.819

C0 – free or non-acylated carnitine;
 C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl;
 AC – acylcarnitines; Pyg – pyroglutamic acid;
 Met – methionine; Phe – phenylalanine;
 Tyr – tyrosine; Cit – citrulline; Glyc – glycine;
 Leu – leucine; Ile – isoleucine.

Table 7. Influence of FEV₁/FVC on the results

Carnitines and amino acids	COPD (n = 10)	
	Spearman's coefficient	p-value
C0	0.248	0.489
C2	−0.285	0.425
C3	−0.248	0.489
C4	−0.635	0.049
C5	−0.478	0.162
C5-OH	0.175	0.630
C8	−0.344	0.331
C14	−0.417	0.231
C14:1	0.372	0.290
C16	0.288	0.419
C16-OH	−0.539	0.108
C18:1	−0.006	0.987
AC total	0.067	0.855
C0/AC	0.358	0.310
C3/C16	−0.261	0.466
C3/C2	−0.198	0.584
C5/C2	−0.343	0.332
C8/C2	−0.496	0.145
C14:1/C16	−0.091	0.803
C16-OH/C16	−0.389	0.266
Glutaryl	0.294	0.409
Valine	0.127	0.726
Pyg	0.370	0.293
Met	0.174	0.631
Phe	0.030	0.934
Tyr	−0.770	0.009
Cit1	−0.439	0.204
Glyc	0.176	0.627
Alanine	−0.406	0.244
Leu/Ile	−0.103	0.777
Phe/Tyr	−0.216	0.548

C0 – free or non-acylated carnitine;
 C2–C18 – carnitine acylated with an acyl containing the indicated number of carbon atoms; -OH – a hydroxyl group on an acyl;
 AC – acylcarnitines; Pyg – pyroglutamic acid;
 Met – methionine; Phe – phenylalanine;
 Tyr – tyrosine; Cit – citrulline; Glyc – glycine;
 Leu – leucine; Ile – isoleucine.

amino acid. Dietary sources include meat, seafood, dairy, eggs, beans, and nuts. It is an important source of muscle energy. Alanine is an amino acid linked to energy metabolism-related pathways of glycolysis, gluconeogenesis, the alanine cycle, and the citric acid cycle.³⁴ Decreased levels of alanine were reported earlier in relation to COPD and muscle wasting because of disturbed metabolism of proteins.³⁵ Our finding of decreased levels of alanine in patients with COPD compared to the control group may indicate a risk of protein

malnutrition as do significantly lower levels of phenylalanine in COPD patients compared to the control group.

Higher levels of pyroglutamate in COPD patients are of unknown significance and should be researched further.

These are important findings, as natural physiological concentrations of substrates related to energy metabolism (alanine, phenylalanine and carnitines), and consequently to malnutrition, are disturbed in COPD patients, while the concentrations of substrates not linked directly

to energy metabolism (other amino acids) are not significantly compromised.

Chronic malnutrition is a common problem in a myriad of chronic diseases, COPD and heart disease being amongst them.^{36,37} It is connected to complications during hospitalizations, a higher rate of readmissions, increased costs of care, and the loss of quality of life.^{36,38} Malnutrition in COPD is a negative predictive factor, being strongly associated with in-hospital mortality and readmittance 30 days after discharge.^{39,40} Skeletal muscles are affected by inactivity, unbalanced nutrition, age, and inflammation, leading to sarcopenia, which involves both muscle loss and muscle dysfunction with contractile impairment and metabolic anomalies. These responses are poorly understood (and are beyond the scope of this article), but 2 groups of patients may be identified: cachectic and with normal or increased BMI. Malnutrition may be present in cachectic patients as well as in patients with normal or increased BMI, i.e., sarcopenia and sarcopenic obesity.⁴¹ Nutritional intervention is beneficial for malnourished patients, combined with exercise or a rehabilitation program, which, in the long term, may be associated with lower health care costs.^{36,38,42} This is already being echoed by some national guidelines.²

Metabolomic profiling using the HPLC-MS/MS method is a new application in the investigation of COPD, and as such it serves as a source of essential information on processes that occur in COPD patients' bodies. Our results were obtained in the limited number of patients. We intend to continue our research in this direction, as it is highly desirable that future studies involve a higher number of subjects to investigate a varying role of individual comorbidities and physiological COPD parameters in the long term to elucidate further COPD pathology.

Limitations

This was a pilot study conducted on a small number of patients. The control group was younger than the COPD test subjects, although not statistically significantly. This was due to the strict exclusion criteria, namely smokers or ex-smokers of less than 6 months, and the presence of metabolic diseases including diabetes mellitus types 1 and 2. There is a high prevalence of diabetes mellitus in the Czech Republic, which is 8%, compared to the estimated worldwide prevalence of 2.8–4.4%.^{43,44} As the incidence of diabetes mellitus increases with age, the set criteria excluded most of our older control group candidates from the study.⁴⁴ Further studies on significantly larger cohorts are needed.

Patients with chronic obstructive pulmonary disease are commonly afflicted with comorbidities, particularly coronary artery disease. As there is no data available on the metabolomic effects of cardiovascular diseases on the metabolism of COPD patients, future investigation on whether a cardiovascular disease concomitant with

COPD further contributes to metabolomic changes may be beneficial.

As this pilot study already shows, there are metabolic differences between COPD patients and the control group, which makes future investigations of this kind rational.

Conclusions

Our study shows that the HPLC-MS/MS method is able to show minute changes in the metabolism of patients shown on a molecular level. The significance of many of these differences is not yet understood. The carnitine and acylcarnitine levels in COPD subjects in this study possibly indicate a predisposition to atherosclerosis as a result of inadequate β -oxidation of fatty acids and show the presence of oxidative stress. Furthermore, the high sensitivity to changes in circulating amino acid levels may allow us to detect subclinical malnutrition and take early preventative interventions such as nutritional supplementation and patient education.

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