

Disruption of histidine and energy homeostasis in chronic obstructive pulmonary disease

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Background: Chronic obstructive pulmonary disease (COPD) is a systemic condition that is too complex to be assessed by lung function alone. Metabolomics has the potential to help understand the mechanistic underpinnings that contribute to COPD pathogenesis. Since blood metabolomics may be affected by sex and body mass index (BMI), the aim of this study was to determine the metabolomic variability in male smokers with and without COPD who have a narrow BMI range.

Methods: We compared the quantitative proton nuclear magnetic resonance acquired serum metabolomics of a male Chinese Han population of non-smokers without COPD, and smokers with and without COPD. We also assessed the impact of smoking status on metabolite concentrations and the associations between metabolite concentrations and inflammatory markers such as serum interleukin-6 and histamine, and blood cell differential (%). Metabolomics data were log-transformed and auto-scaled for parametric statistical analysis. Mean normalized metabolite concentration values and continuous demographic variables were compared by Student's *t*-test with Welch correction or ANOVA with post-hoc Tukey's test, as applicable; *t*-test *p*-values for metabolomics data were corrected for false discovery rate (FDR). A Pearson association matrix was built to evaluate the relationship between metabolite concentrations, clinical parameters and markers of inflammation.

Results: Twenty-eight metabolites were identified and quantified. Creatine, glycine, histidine, and threonine concentrations were reduced in COPD patients compared to non-COPD smokers (FDR $\leq 15\%$). Concentrations of these metabolites were inversely correlated with interleukin-6 levels. COPD patients had overall dampening of metabolite concentrations including energy-related metabolic pathways such as creatine metabolism. They also had higher histamine levels and percent basophils compared to smokers without COPD.

Conclusion: COPD is associated with alterations in the serum metabolome, including a disruption in the histidine-histamine and creatine metabolic pathways. These findings support the use of metabolomics to understand the pathogenic mechanisms involved in COPD.

Trial registration www.clinicaltrials.gov, NCT03310177.

Keywords: China, metabolomics, histidine, energy homeostasis, inflammation, chronic obstructive pulmonary disease

Background

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung condition with considerable mortality and morbidity worldwide.¹ The grading of COPD severity using the GOLD (Global initiative for chronic Obstructive Lung Disease) classification is based on lung function, which does not accurately capture the breadth of COPD heterogeneity. As such, there is a large gap in our understanding of the mechanisms behind the various phenotypes and clinical manifestations associated

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with COPD, including emphysema, chronic bronchitis, systemic inflammation and muscle dysfunction.² Disruptions in metabolism have been previously reported in patients with COPD,^{3–7} which supports the notion that metabolomics, the identification and measurement of small molecules in biological samples, may provide insight into potential links between metabolic features and pathological mechanisms in COPD.⁸ Furthermore, testing the associations between metabolite concentrations and other markers of COPD phenotypes, such as inflammatory cytokines (eg, interleukin-6) and blood cell differential, may further corroborate these links.

Previous COPD metabolomics studies have found metabolic changes related to sex and body mass index (BMI).^{4,5} BMI is frequently in the normal range and diet is most often regionally uniform among the Chinese Han population.⁹ A population-based study showed that COPD is two times more prevalent in men compared to women in China.¹⁰ This presents a unique opportunity to assess disease-related metabolic alterations with minimal bias from BMI and diet. To this end, we measured and compared serum metabolites using quantitative proton nuclear magnetic resonance (¹H-NMR)-based metabolomics in male Chinese Han patients with COPD, smokers without COPD and non-smoker controls.

Methods

Subjects

The study (www.clinicaltrials.gov, NCT03310177) was approved by the Peking University (PUIRB) and the University of Michigan Institutional Review Boards (IRBMed). Written informed consent was acquired from all subjects before enrollment into the study in accordance with the principles of the Declaration of Helsinki.

Individuals with COPD were enrolled in the clinic of Peking University Third Hospital and Shougang Hospital in China from December 2015 to July 2017. The inclusion criteria for male COPD subjects have been previously described.¹¹ Briefly, subjects had to: 1) be male of 40–80 years of age; 2) have a smoking history ≥ 10 pack-year; 3) carry the diagnosis of COPD according to the GOLD definition;^{1,12} 4) not have experienced a respiratory exacerbation in the past 3 months; 5) be free of severe hepatic, cardiovascular, mental or renal dysfunction; and 6) not have other pulmonary diseases (eg, asthma). The control groups included male non-COPD smokers (NCS) and never smokers (NS) without COPD, who met the

above-mentioned 1st, 5th and 6th inclusion criteria of COPD patients. This paper represents a report of the NMR metabolomics portion and secondary outcomes of the study NCT protocol.

Spirometry and high-resolution computed tomography

Spirometry (SensorMedics, Yorba Linda, CA, USA) was performed in all subjects according to American Thoracic Society/European Respiratory Society guidelines.¹³ The predicted percent of forced expiratory volume in the first second (FEV1%pred) was used to evaluate lung function in order to categorize subjects as follows: GOLD I, FEV1%pred $>80\%$; GOLD II, $80\% > FEV1pred \geq 50\%$; GOLD III, $50\% > FEV1pred \geq 30\%$; GOLD IV, FEV1pred $<30\%$. Chest high resolution computed tomography (HRCT) with continuous slices of 0.625 mm was performed at the time of blood sample collection. Emphysema extent was assessed by calculating the percent of lung volume with a low attenuation area (LAA%) defined as less than -950 Hounsfield Units (HU) (AW 4.5 software, GE healthcare, Fairfield, CT, USA).¹⁴

Blood sample collection and management

Blood sample collection was conducted in the morning (9:00 AM to 12:00 PM) for which study participants were required to be fasting and to have stopped smoking for at least 12 hrs. Two tubes of blood were collected from each patient one for EDTA-preserved plasma and one for serum. The serum sample remained at room temperature (25 °C) for at least 30 mins until clotted. Both samples were centrifuged ($3000 \times g$, 4 °C, 15 min) and serum or plasma was aliquoted (500 μ L) and stored (-80 °C) until assay or the time of shipment to the University of Michigan (serum). At the time of shipment, serum samples were placed on dry ice and packaged in accordance with the requirements of World Courier (www.worldcourier.com). The dry ice supply was maintained during shipping so samples remained frozen during transit. Upon arrival to Ann Arbor, MI, they were inventoried and immediately stored (-80 °C) until the time of assay.

Quantitative 1-dimensional (D) proton (¹H)-nuclear magnetic resonance (NMR)

At the time of the assay, samples were randomized and thawed in an ice-water bath. Pre-chilled methanol was added to the volume of each sample (2:1) to precipitate

macromolecules as a modification of previously described protocols.^{15–18} The samples were dried on a FreeZone 4.5 –105 °C lyophilizer (Labconco Corporation, Kansas City, MO, USA) and ultrafiltered (3 kDa MWCO, Pall Nanosep, Westborough, MA, USA) prior to the addition of a known amount of formate that was used as an internal standard.

The 1-D ¹H-NMR spectrum of each sample was acquired on a 500 MHz spectrometer (Agilent Inc., Santa Clara, CA) with host software VNMRJ 4.0. The resulting spectra were processed and profiled to identify and quantify metabolites using Chenomx NMR Suite 8.3 (Chenomx Inc., Edmonton, AB, Canada) and its reference library that contains 338 compounds. Details of the ¹H-NMR protocol and pulse sequence can be found in the [Supplemental materials](#).

Measurement of blood leukocytes, analytes and free hemoglobin concentrations

Blood leukocytes were counted and sorted via a routine laboratory protocol, and plasma fibrinogen concentration was measured by the Clauss method¹⁹ within 6 hrs of sample collection. Technical replicate serum samples were used to measure levels of histamine, tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), compounds known to be associated with inflammation and COPD.^{20–22} Serum histamine was extracted with methanol (1:1) and was subsequently assayed with a commercial ELISA kit (Abcam, Cambridge, UK). Serum TNF- α and IL-6 were arrayed with Aimplex[®] Human Custom 9-plex kit (Quantobio, Beijing, China) on a NovoCyte D1040 flow cytometer (ACEA Biosciences, Hangzhou, China). Serum free hemoglobin was detected using a colorimetric kit (Cayman Chemical, Ann Arbor, MI, USA) on a 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA) using residuals from the serum samples assayed by NMR at the University of Michigan. All assays were performed according to the manufacturers' instructions.

Statistical analysis

Only metabolites present in at least 70% of the samples were considered for statistical analysis. Missing values were replaced with the half of minimum value of the data set. The metabolomics concentration data were log-transformed and auto-scaled to achieve a normal distribution. Mean normalized metabolite concentration values and continuous demographic variables were compared by unpaired Student's *t*-test with Welch

correction or ANOVA with post-hoc Tukey's test, as applicable. Categorical variables were compared with an exact Chi-square test. For metabolomics data, *t*-test *p*-values were corrected for false discovery rate (FDR) using the modified Benjamini-Hochberg method reported by Storey.²³ A Pearson association matrix was built to evaluate the relationship between metabolite concentrations, clinical parameters and markers of inflammation. In this preliminary study, for metabolite concentration comparisons, FDR-corrected *p*-values ≤ 0.15 were viewed as potentially differentiating. For other comparisons, *p*-values of less than 0.05 were considered statistically significant; all statistical analyses were performed in R v3.4.0.

Results

Subject demographics

A total of 166 subjects were enrolled in the study of which 9 were excluded; samples from the remaining 157 subjects were shipped to the University of Michigan for assay. Another 12 subjects were excluded due to insufficient sample volume, poor quality NMR spectra, or high hemoglobin concentrations,¹⁶ leaving metabolomics data from 145 subjects for final analysis ([Figure S1](#)). The demographic data of the final cohort are summarized in [Table 1](#). Age and BMI were similar across all groups, and smoking history was not different between smokers with and without COPD. However, fibrinogen, IL-6, TNF- α , neutrophils and basophils were all significantly higher in patients with COPD compared to NCS ($p < 0.05$, *t*-test).

Serum 1-D ¹H-NMR metabolomics differentiates COPD patients from smokers without COPD

A total of 28 serum metabolites were identified and quantified ([Table S1](#)). The metabolomics data set and associated NMR spectra can be found at the NIH's metabolomics repository (<http://www.metabolomicsworkbench.org/>). While there were differences in metabolite concentrations between NCS and COPD ([Figure 1A](#)), the data did not differentiate GOLD I/II versus GOLD III/IV in the COPD group ([Figure S2A](#) and [B](#)). No discriminating metabolites were observed between GOLD stage A-D. In general, mean normalized metabolite concentrations were lower in COPD subjects compared to NCS ([Figure 1A](#)). Specifically, concentrations

Table 1 Demographic and clinical data of analyzed study cohort

	Non-Smokers (NS, n=7)	Non-COPD Smokers (NCS, n=59)	COPD (n=79)	ANOVA <i>p</i> value	T-test <i>p</i> value
Demographics					
Age (years)	57.4±11.6	56.8±10.7	58.8±9.9	0.532	0.269
BMI (kg/m ²)	24.9±3.2	24.6±3.2	24.6±3.8	0.967	0.991
Pack-years	0±0	35±21.1	39.3±22.7	<0.001	0.256
Current smoker	Never	59 (100%)	43 (54.4%)	<0.001 [#]	
Respiratory burden and emphysema					
FEV ₁ (%pred)	95.7±12.1	91.4±13.5	45.3±16.8	<0.001	<0.001
LAA (%)	0.49±0.83	0.46±0.85	8.02±10.16	<0.001	<0.001
Inflammatory biomarker					
Fibrinogen (g/L)	2.7±0.50	2.7±0.51	3.2±0.72	<0.001	<0.001
TNF- α (pg/ml)	9.8±12.6	3.9±2.4	7.1±11.9	0.138	0.03
IL-6 (pg/ml)	23.6±20.2	10.9±11.3	27.8±34	0.007	<0.001
Leukocytes					
Neutrophil (%)	57.8±4.4	60.1±7.8	63.9±8	0.014	0.012
Eosinophil (%)	2.1±1.4	2.2±1.7	2.9±2.5	0.201	0.082
Monocyte (%)	5.4±1.6	5.9±1.3	6.3±1.6	0.159	0.107
Lymphocyte (%)	34.2±2.8	38.1±49.6	26.3±7.5	0.149	0.098
Basophil (%)	0.39±0.16	0.37±0.21	0.5±0.27	0.012	0.003
GOLD Classification					
	NA	NA	I: n=2		
	NA	NA	II: n=21		
	NA	NA	III: n=40		
	NA	NA	IV: n=16		
	NA	NA	A: n=42		
	NA	NA	B: n=33		
	NA	NA	C: n=0		
	NA	NA	D: n=4		

Notes: Data are the mean \pm SD. The ANOVA *p*-value represents the comparison between NS, NCS and COPD. The *t*-test *p*-value represents the comparison between COPD and NCS. #: A Chi-square test was used to determine the ratio difference between COPD current smokers and NCS.

Abbreviations: BMI, body mass index; TNF- α , tumor necrosis factor α ; IL-6, interleukin-6.

of creatine, glycine, histidine, and threonine were reduced in COPD compared to NCS subjects (Figure 1B). Figure S3 illustrates the metabolic relationship between threonine, glycine and creatine.

Because 54.4% of the COPD patients were current smokers (Table 1), we investigated whether smoking status influenced the serum NMR-detected metabolome of COPD (Figure S4). The only metabolite concentration that was different (post-hoc Tukey's test $p < 0.05$) between current and former smokers with COPD was

citrate. Additionally, we also investigated whether the serum metabolome was affected by BMI (Figure S5); glucose was the only metabolite that correlated with BMI (Pearson correlation test, $p < 0.05$).

Metabolite concentrations are associated with pulmonary function, emphysema, and markers of inflammation

Creatine, histidine, threonine as well as lactate, proline and serine were positively correlated with FEV₁ (%pred); creatine,

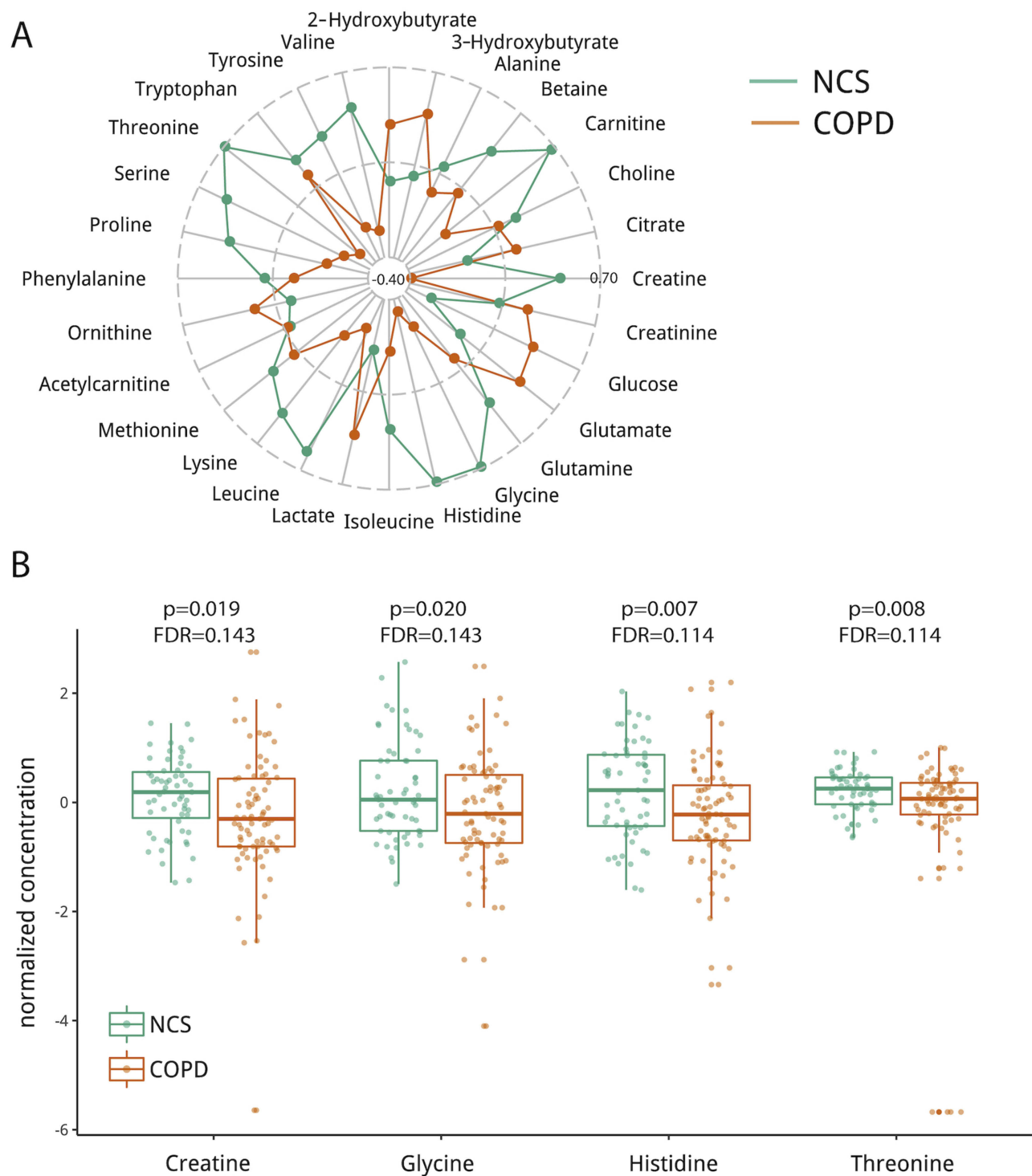


Figure 1 Quantitative 1H-NMR serum metabolomics differentiates patients with COPD from non-COPD smokers (NCS). **(A)** Mean normalized serum metabolite concentrations in NCS (n=59) and COPD (n=79) illustrated in a radar plot. Centroid and maximal dashed circle separately denote the minimal and maximal mean normalized concentration of all metabolites. Overall, the metabolome was dampened in COPD compared to NCS. **(B)** Box and whisker plots of normalized metabolite concentrations with FDR-corrected p -values ≤ 0.15 . Concentrations of creatine, glycine, histidine and threonine were notably lower in COPD compared to NCS.

histidine as well as 3-hydroxybutyrate, betaine, carnitine, glutamine, acetylcarnitine and valine were correlated with LAA (%); creatine, glycine, histidine as well as carnitine, lactate,

lysine, phenylalanine, serine, tyrosine were correlated with IL-6 levels; histidine as well as betaine, glutamine, acetylcarnitine and valine were also negatively correlated with TNF- α levels

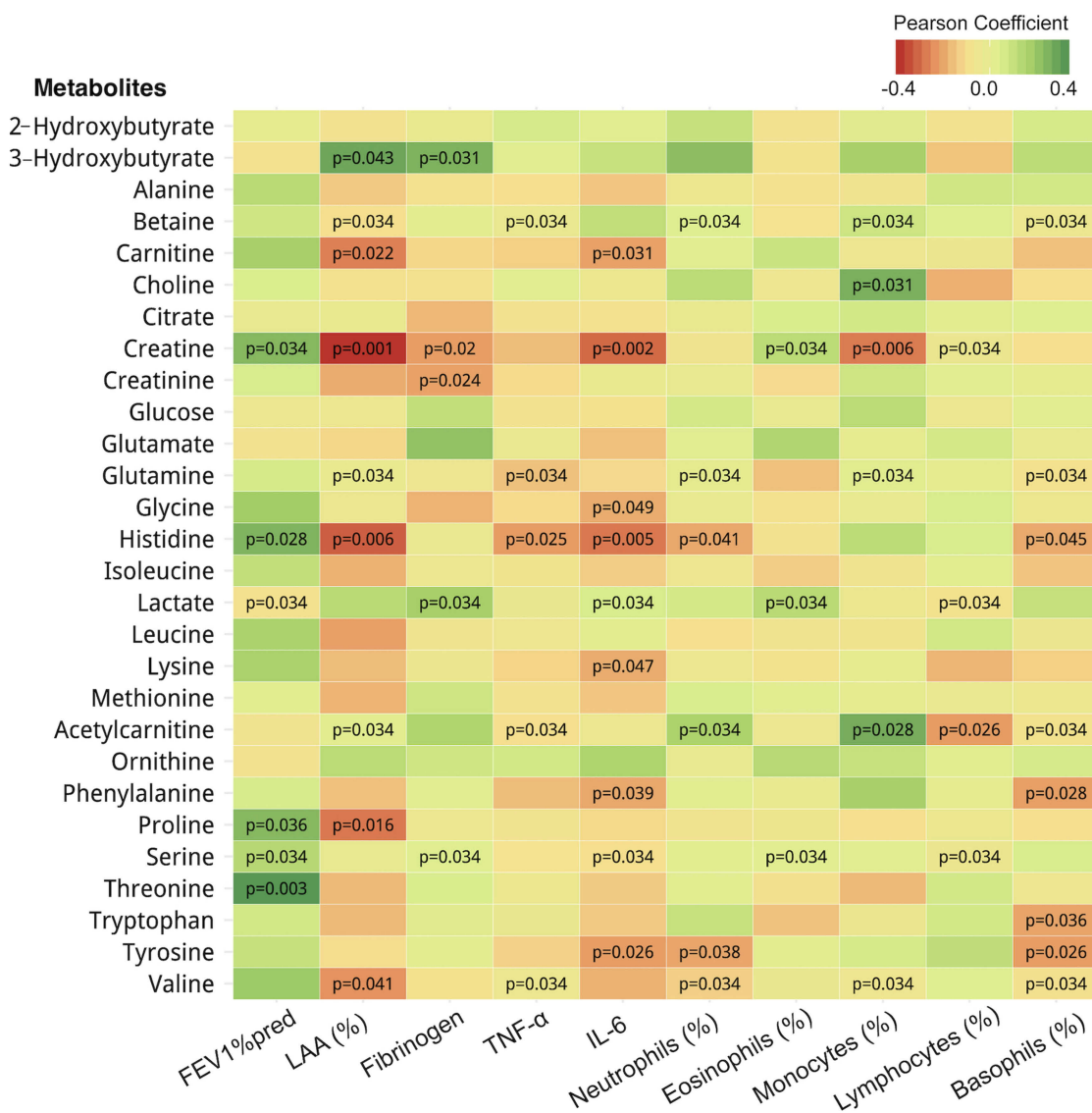


Figure 2 Metabolite concentrations are associated with pulmonary function, emphysema and inflammatory cytokine levels. Association heatmap between metabolomics and clinical data of subjects. Red and green squares reflect the negative and positive correlations, respectively; darker color denotes a higher correlation between metabolites and clinical index. For the statistically significant associations, the *p*-value is labelled in the corresponding square.

Abbreviations: TNF- α , tumor necrosis factor α ; IL-6, interleukin-6.

(Figure 2). Given the relationship between histidine concentration and pulmonary function and inflammation, we assessed whether histamine (a product of histidine metabolism) levels differed between NCS and COPD subjects. Histamine concentration was higher in COPD (Figure 3A) as were basophils (%) (Figure 3B), cells known to produce histamine.²²

Discussion

We identified a broad disruption in energy-related metabolism associated with COPD without BMI bias in a Chinese Han male population. In particular, components of creatine metabolism, including creatine, glycine, and threonine,

had lower concentrations in patients with COPD compared with NCS. We also introduce the possibility that a disruption in histidine-histamine metabolism contributes to COPD pathogenesis.

Metabolic differences likely reflect COPD

Importantly, these metabolic changes are not likely explained by demographic differences in this study, since previous studies reported the metabolic alterations in COPD may be related to sex and BMI.^{4,5} Although we did not directly assess diet information from our study cohort, the Chinese diet is much more consistent and

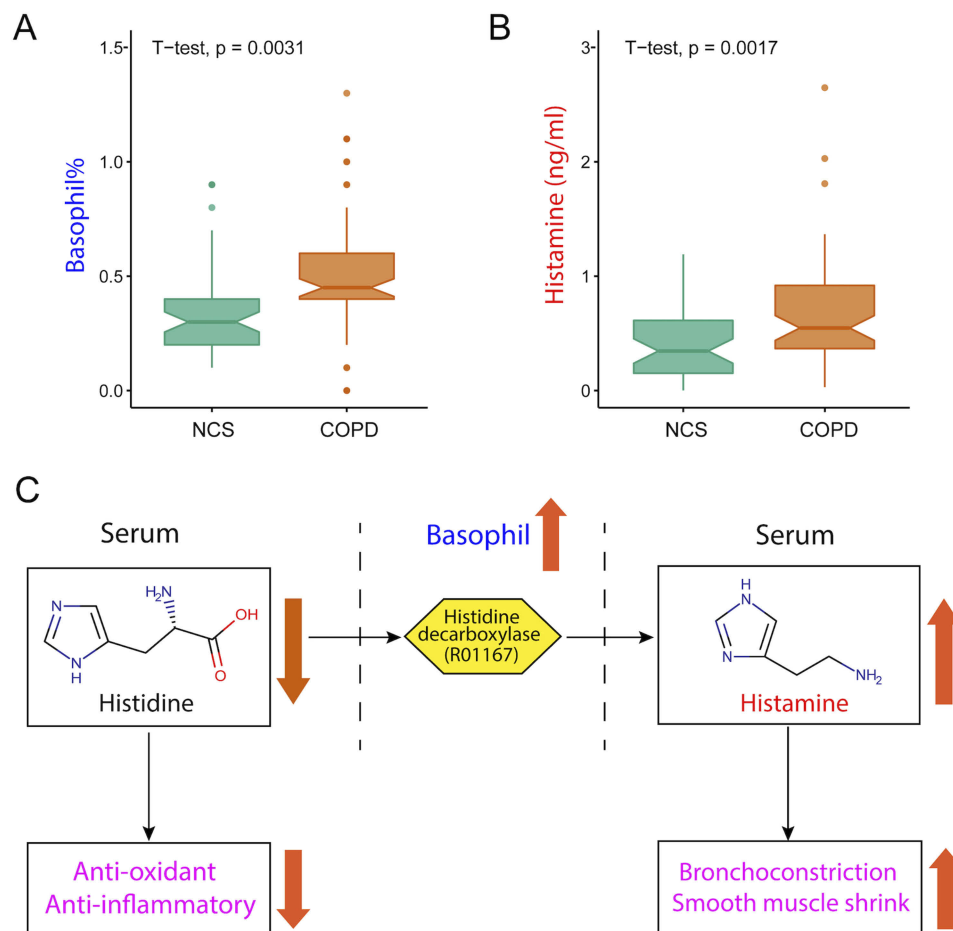


Figure 3 Histidine-histamine metabolism is disrupted in COPD. **(A)** Basophil (%) and **(B)** histamine concentrations are higher in COPD than in non-COPD smokers (NCS). Box and whisker plots (median, 25th and 75th percentiles, min and max). **(C)** Disruption of histidine-histamine metabolism in COPD. Elevated histamine can aggravate bronchoconstriction and shrink airway smooth muscle. Reduced histidine can cause recession of anti-oxidant and anti-inflammatory processes, both of which contribute to the pathogenesis and development of COPD or emphysema. R01167 is the KEGG identifier for the reaction catalysed by histidine decarboxylase.

uniform compared to the Western diet.²⁴ In addition, our study participants had an average BMI ~25 with low variance. Furthermore, smoking status had a minimal impact on the metabolome which implies that the differential metabolites of NCS and COPD were not attributable to current smoking status; this is consistent with previous studies.^{25,26} The absence of a smoking-induced change in the metabolome may have been aided by a 12 hr abstinence prior to the collection of blood for our metabolomics analysis. As such, our findings support the notion that the COPD metabolic derangements we found are likely attributable to the underlying disease.

Comparison with the previous studies

Two earlier studies that included males and females, also investigated the metabolic profiles of COPD using

liquid chromatography-mass spectrometry (LC/MS) and ¹H-NMR metabolomics, respectively.^{7,27} Since the LC/MS study was conducted using a different analytical platform than ours, it is difficult to make a direct comparison with our results.⁷ The earlier ¹H-NMR study of 15 males and 17 females reported a COPD-induced reduction in BCAAs (leucine and isoleucine).²⁷ We made a similar observation but it did not fall below our FDR cut-off of 15%. Additionally, an NMR metabolomics study of exhaled breath condensate (EBC) also distinguished COPD from controls but the metabolic profile of EBC was distinct from that of serum.²⁸ In aggregate, these studies show how different biofluids and the metabolomics data that are acquired from different analytical platforms provide unique information about COPD.

Disorder of energy-related metabolic pathways in COPD

The differentiating metabolites we identified in our study share common metabolic pathways. Creatine, glycine and threonine are part of the glycine, serine, and threonine pathway (Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway: hsa00260). Notably, creatine, a non-proteogenic amino acid, is an important intermediate in the metabolism of tissues with high energy demand such as skeletal muscle.^{29,30} It also acts to maintain ATP levels and serves in an energy shuttle between the sites of ATP synthesis and utilization.³⁰ Given the importance of creatine, glycine and essential amino acids (eg, threonine) for providing energy and preventing skeletal muscle atrophy,^{31,32} it is plausible that their reduced serum concentrations may either contribute to or be a result of exercising tissues dysfunction and increased ventilatory load in COPD patients. Notably, here and as has been previously shown, the extent of emphysema (LAA%) was inversely associated with creatine and histidine concentrations.³³ This likely disruption of energy balance is further corroborated by the ketone body, 3-hydroxybutyrate, in our COPD subjects, which may be a compensatory signal for altered energy metabolism.

Disruption of histidine-histamine metabolism could contribute to a pro-inflammatory COPD phenotype

Histidine is an essential amino acid and is the precursor of histamine.^{34,35} In our COPD cohort, serum histidine was reduced with an associated increase in histamine and basophils (%), cells known to produce histamine.²² Histidine is known to be inversely associated with inflammation and oxidative stress in patients with metabolic syndrome and chronic kidney disease; in fact, histidine supplementation has been shown to suppress inflammatory processes.^{21,36} Moreover, in in-vitro experiments, supplemental histidine lowered the expression of IL-6, TNF- α and nuclear factor kappa-B (NF- κ B) in adipocytes.²¹ We found the same relationship in our cohort as histidine was negatively correlated with serum IL-6 and TNF- α , implying the possibility that lower histidine concentrations may contribute to a pro-inflammatory state in COPD. In aggregate, these findings implicate disruption in histidine-histamine metabolism as contributing to COPD (Figure 3C).

We also observed higher serum levels of histamine, a metabolic product of histidine, in smokers with COPD compared to smokers without COPD. Histamine is

harbored and released from the granules of mast cells and basophils, leading to tissue edema, bronchoconstriction and bronchial smooth muscle contraction via the H₁ receptor.^{37–39} It has also been reported that increased histamine levels are associated with lung function decline and mortality in COPD,^{40–42} it is also widely recognized to play a pronounced role in asthma.⁴³ Given our observed relationship between levels of histidine, histamine and basophil (%) on one hand and pulmonary function, LAA (%) and inflammation markers on the other, we suggest that the disruption of histidine-histamine metabolism could contribute to a pro-inflammatory COPD phenotype.

Limitations

We acknowledge that there are limitations to this study. Our findings are based on a single point in time measurement and we did not assess the influence of medications (eg, steroids) and non-pharmaceutical interventions such as exercise training. Longitudinal assessment of metabolic changes, as well as diet and medication use, will be important for future studies in order to fully assess the stability of the COPD metabolome. Also, given the higher prevalence of COPD in men in China,¹⁰ we limited this initial study to male patients. Given the known sex-related differences in the metabolome,⁵ a more in-depth assessment of such differences in regard to COPD phenotypes is needed. Finally, we acknowledge the preliminary nature of this study and that it will require validation in a larger, independent cohort.

Implications

We identified a broad disruption in energy-related and anti-inflammatory metabolism especially in creatine, glycine, threonine and histidine serum concentrations. This warrants further study and possible consideration that their supplement may improve dysfunction of exercising tissue and ventilatory load, as well as lower inflammation status in COPD patients.

Conclusion

In conclusion, we identified metabolic disturbances in the serum of Chinese male patients that may contribute to the inflammatory phenotype of COPD. In aggregate, these data corroborate previous findings of broad suppression of amino acid concentrations in COPD patients in a male Chinese Han population without BMI bias and they also extend them to introduce a potential mechanistic pathway involving a disruption in histidine-histamine homeostasis that could contribute to inflammatory processes. This

could drive avenues of research including more expansive testing of amino acid supplementation in COPD, which has shown some promise⁴⁴ as well as studies directed at increasing understanding of underlying mechanisms.

Abbreviations

COPD, chronic obstructive pulmonary disease; BMI, body mass index; NMR, nuclear magnetic resonance; FDR, false discovery rate; HRCT, high resolution computed tomography; LAA, low attenuation area; GOLD, global Initiative for chronic obstructive lung disease; NCS, non-COPD smokers; NS, Never smokers; FEV1, forced expiratory volume in the first second; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.

Ethics approval and consent to participate

The study (www.clinicaltrials.gov, NCT03310177) was approved by the Peking University (PUIRB) and the University of Michigan Institutional Review Boards (IRBMed). Written informed consent was acquired from all subjects before enrollment into the study in accordance with the principles of the Declaration of Helsinki.

Data sharing statement

The dataset supporting the conclusions of this article is available in the NIH Metabolomics Workbench repository (<http://www.metabolomicsworkbench.org/>).

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Author contributions

W Diao directed the execution of the study, participated in the assaying of the metabolomics samples, conducted bioinformatic and statistical analyses and interpretation and wrote the manuscript; W Labaki, assisted with data analysis and interpretation and the writing of the manuscript; M Han, T Standiford, B He, N Shen and K Stringer were involved in study design; P Xiang, N Shen, Y Sun, C Guo and M Lu enrolled subjects; L Yeomans, Z Smiley, Y Sun, J Kim and C McHugh performed the metabolomics assays, free haemoglobin assays, NMR spectral analyses and generated the metabolomics data set; C Guo measured IL-6, TNF- α and fibrinogen levels. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

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