Peer Review File

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Reviewer A

I would like to congratulate the authors for the article "Changes in mitochondrial metabolites in lung tissue of Chronic Obstructive Pulmonary Disease".
The study of the metabolic and their impact on human disease is very important and can bring key information for a complete understanding of the disease. The authors studied the similarities and differences of lung metabolites and underlying pathways between COPD patients and CS-exposed mice. This is a very well-written study with an appropriate design.
I have a few comments about the articles:

Comment 1: The authors used lungs from patients with cancer. It is well known that there are significant changes in the body related to the tumor, including the microbiome, cytokines and many metabolic changes that occur in the body due to inflammation that are not limited to the tissue around the tumor. Moreover, if those patients were subjected to chemotherapy treatment, it can also have a significant impact. Most of the time, it is not feasible to obtain lung tissues from patients in "normal" conditions. Therefore, I suggest the authors include in their more information about the treatment used for those patients and a discussion about the implications of using cancer patients.
Re: We thank the reviewer for the comment. The patients in both groups were newly diagnosed lung cancer with stage I or II A and had not received any treatment. We chose patients with lung cancer because lung tissues were available when patients underwent lung surgery. We added the inclusion criteria and exclusion criteria.
Changes in the text: see Page 6-7, line 121-131.

Comment 2: Mice models for COPD are widely used. Understanding their limitation

is important for a proper conclusion. It would be interesting if the authors could discuss more about the implications of their findings in the use of mice models for COPD. Are there any similar studies comparing with other animals used for COPD studies? How do their findings compare to yours?

Re: We thank the reviewer for the suggestion. We added some contents in the Discussion.

A few comparative transcriptomics and metabolomics studies have been performed on murine CS-exposed lungs and human COPD lungs. Murine lung transcriptomic analysis showed that different murine strains overlap with only a portion of the DEGs found in the human COPD lung data. There were few genes overlapping between human COPD and mouse models including VEGFA, HDAC5, P2Y14 and CHN2 [1]. There were 3,723 and 3,106 DEGs respectively in murine lungs after short and long-term cigarette smoke (CS) exposure. A significant overlap of genes in CS-exposed murine lungs, 48% (184 DEGs) from short-term exposure and 44% (168 DEGs) from long-term exposure with those of current-smoker lungs. There were 48 genes that were common to the lungs of both CS-exposed mice and current smokers and severe COPD. These data suggested that murine CS models were strongly representative of molecular processes of human smoking but less of COPD [2]. Recently, comparative transcriptomics analyses of COPD in ferrets, mice and humans were performed to find the uniquely expressed genes. 52 DEGs were common to all three species, 77 DEGs were differentially expressed in humans, 90 DEGs were differentially expressed in mice, and 25 DEGs were uniquely expressed in ferrets and humans, but not mice (3). As far as metabolomics is concerned, the bronchoalveolar lavage fluid (BALF) from CS-exposed mice was positively correlated with human COPD BALF with 2040 metabolites in common, suggesting that mouse models can be used to interrogate human lung metabolome changes[4]. CS-exposed mice had increased glycerolipids and glycerophospholipids in plasma and BALF, which was similar to our study. In our study, we observed that the metabolites like amino acids, carbohydrates and carnitines changed in both COPD patients and mice, but lipids changed more significantly in CS-exposed mice. Therefore, the mouse model of

COPD can partially represent human COPD.

1.Yun JH, Morrow J, Owen CA, Qiu W, Glass K, Lao T, Jiang Z, Perrella MA, Silverman EK, Zhou X, Hersh CP. Transcriptomic Analysis of Lung Tissue from Cigarette Smoke-Induced Emphysema Murine Models and Human Chronic Obstructive Pulmonary Disease Show Shared and Distinct Pathways. Am J Respir Cell Mol Biol. 2017 Jul;57(1):47-58.

2. Obeidat M, Dvorkin-Gheva A, Li X, Bossé Y, Brandsma CA, Nickle DC, Hansbro PM, Faner R, Agusti A, Paré PD, Stampfli MR, Sin DD. The Overlap of Lung Tissue Transcriptome of Smoke Exposed Mice with Human Smoking and COPD. Sci Rep. 2018 Aug 8;8(1):11881.

3.Hussain SS, Edwards YJK, Libby EF, Stanford D, Byzek SA, Sin DD, McDonald ML, Raju SV, Rowe SM. Comparative transcriptomics in human COPD reveals dysregulated genes uniquely expressed in ferrets. Respir Res. 2022 Oct 10;23(1):277.

4.Cruickshank-Quinn C, Powell R, Jacobson S, Kechris K, Bowler RP, Petrache I, Reisdorph N. Metabolomic similarities between bronchoalveolar lavage fluid and plasma in humans and mice. Sci Rep. 2017 Jul 11;7(1):5108.

Changes in the text: see Page 20-21, line 431-435; Page 21, line440-444.

Comment 3: The quality of the figure 3 and 4 is not very good. It is very hard to read it. Please consider upload a figure with higher quality.

Re: We thank the reviewer's comment, we uploaded higher quality figures.

Changes in the text: see Page 35-37.

Reviewer B

In this manuscript, the authors examined the changes in metabolites and metabolic pathways in human and mouse lung tissues of COPD using a targeted metabolomics HM350 analysis in order to explore the potential metabolic pathways involved in the pathogenesis of COPD and the possibility of discovering COPD-associated biomarkers and to examine the similarities and differences of lung metabolites and

underlying pathways between patients and mice. However, there were some significant limitations that dampened the overall enthusiasm for this paper. These included:

Comment 1: From the title, the authors should focus on “mitochondrial metabolites”, but from the whole manuscript, the author did not really do that. The manuscript therefore needs to be reorganized/rewritten.

Re: Thanks for the reviewer's comment. Initially we do want to focus on mitochondrial metabolites. However, there was no such a targeted metabolism analysis. Therefore, we chose HM350 as a targeted metabolism which measures more than 350 different categories of metabolites, including amino acids, fatty acids (including short chain fatty acids), organic acids, bile acids, carbohydrates, carnitine, indole, etc., by using isotope internal standard methods to obtain accurate concentrations of metabolites. We understand that “mitochondrial metabolites” may be inappropriate, so we would like to change the text title as “targeted metabolites”.

Changes in the text: see Page1, line1.

Comment 2: L82-93, the METHODS did not provide inclusion/exclusion criteria.

Re: Thanks for the comment. We added the inclusion/exclusion criteria in methods.

Changes in the text: see Page 6-7, line 121-131.

Comment 3: L175-176, the authors said that clustering of samples within groups was evident and the metabolic differences between groups were significant. However, as seen in Figure 2A, clustering of human samples was not able to separate COPD group from non-smokers.

Re: We compared the differential metabolites of the two groups of human lung tissue samples with t-test, and the results were all significantly different.

The list of metabolites is presented in the following table.

metabolites	P	metabolites	P value

	value		
L-Isoleucine	0.002	L-Malic acid	0.01
L-Leucine	< 0.001	Propionylcarnitine	0.037
L-Norleucine	< 0.001	Homovanillic acid	0.31
2-Hydroxy-2-methylbutyric acid	0.045	Hydroxyphenyllactic acid	0.13
Methylsuccinic acid	0.008	1-Methylhistidine	0.001
Linoleylcarnitine	0.028	Gamma-Aminobutyric acid	0.041
Lithocholic acid	0.032	Glutamyalanine	0.005
Tauro-alpha-muricholic acid	0.046	Glycyl-L-leucine	0.047
L-Thyronine	< 0.001	Glycine	0.009
L-Glutamic acid	0.001	L-Aspartic acid	0.014
m-Aminobenzoic acid	0.002	Ribonic acid	0.048
Hippuric acid	0.043	D-Fructose	< 0.001
4-Hydroxyproline	0.01	D-Xylose	0.042
2-Methylbutyroylcarnitine	0.046	D-Gluconolactone	0.028
Isovelarylcarnitine	0.049	Glyceric acid	0.002
Valerylcarnitine	0.041	Guanidoacetic acid	0.01
Fumaric acid	0.01		

Comment 4: L245-247, the authors provided the reason why the study was based on samples from male COPD patients and male mice models. However, the METHODS (inclusion criteria) did not mention this information.

Re: Thanks for the reviewer's comment. We added the inclusion/exclusion criteria in methods.

Changes in the text: see Page 6-7, line 121-131.

Comment 5: The descriptions of the figures are too brief.

Re:We thank the reviewer’s comment. We modified the description of the figures as suggested.

Changes in the text: see Page 32-38.

Comment 6: In this study, the authors compared the lung metabolites from non-smokers (non-COPD) and COPD patients (smokers). So, how could we confirm whether this difference is due to smokers versus non-smokers, or COPD versus non-COPD?

Re: Thanks for the reviewer's comment. This was indeed a shortcoming of our study, as we did not recruit healthy smokers. Most of the omics studies included COPD patients, smokers and non-smokers. Chen Q used untargeted metabolite profiling of serum samples to compare the differences among healthy smokers, COPD smokers and non-smokers. 62 metabolites were significantly different between healthy smokers and smoker-COPD, of which 17 metabolites were differentially-expressed in smoker-COPD compared to both healthy smokers and non-smokers. 23 metabolites were differentially expressed in COPD-smokers compared with healthy-smokers.This study also demonstrated that cigarette smoke (CS) itself was associated with 107 serum metabolite changes, relative to non-smokers [1]. In a serum lipidome study,CS and COPD were associated with increases in glycero(phospho)lipids and monounsaturated fatty acids, a decrease in polyunsaturated fatty acids and an imbalance in eicosanoids [2]. Both studies indicated smoking had an effect on metabolites. Ideally, there should be a smoker group. We take it as a study limitation which is acknowledged in the study limitations. We will include smokers group in the future.

1.Chen Q, Deeb RS, Ma Y, Staudt MR, Crystal RG, Gross SS. Serum Metabolite Biomarkers Discriminate Healthy Smokers from COPD Smokers. PLoS One. 2015 Dec 16;10(12):e0143937.

2.Titz B, Luettich K, Leroy P, Boue S, Vuillaume G, Vihervaara T, Ekroos K, Martin F,

Peitsch MC, Hoeng J. Alterations in Serum Polyunsaturated Fatty Acids and Eicosanoids in Patients with Mild to Moderate Chronic Obstructive Pulmonary Disease (COPD). Int J Mol Sci. 2016 Sep 20;17(9):1583.

Changes in the text: see Page 21, line 450-452.

Reviewer C

Comment 1: Bronchodilation test was recommended when the authors defined COPD.

Re: Thanks for the reviewer's comment. Our patients all had bronchodilation tests. We added the bronchodilation test to the diagnosis of COPD.

Changes in the text: see Page 6, line 122-126.

Comment 2: There was insufficient evidence to determine whether the mice were COPD mice after only three months of smoking exposure. The reference the authors cited as how to make COPD mice models was improper. In addition, how the model to be considered as the COPD model was not written clearly.

Re: Thanks for the comment. We are sorry for the miss-cited reference, we now changed it with references [1,2]. Indeed, most COPD mouse models were performed at six months, which is time-consuming. A few studies used cigarette smoke (CS) exposure to mouse for 12 weeks to induce COPD model. In one study, the mice were exposed to CS for 60 min/day, 5 days/wk for up to 4, 12, and 20 weeks. The Lm value and alveolar destructive index were significantly increased in CS-exposed mice compared with controls at 12 and 20 weeks [1]. Li Y et al showed that mice were exposed to 10 cigarettes for 60 min per exposure, once daily for 12 weeks, and the presence of emphysema was confirmed by Lm analysis [2]. Shu J et al evaluated the methods of CS-induced mouse model of COPD. The mice were exposed to cigarette smoke (9 cigarettes each hour, 2 hours each time, twice a day and 6 days per week) in either a whole-body exposure system or in a nose-only exposure system for 10 weeks. The bronchial walls of CS-exposed mice thickened, the alveolar walls thinning and fracturing, a large number of macrophages and neutrophils interstitial infiltration, and

even more pulmonary bullae formation [3]. In addition, Heulens N et al and Zhou L et al also used 12-week of CS exposure to mice to establish COPD models[4,5]. In the current study, we exposed mice for twelve weeks (five cigarettes/time, four times/day, five days/week), and we measured lung function in a plethysmograph and Lm in HE-stained sections to verify the success of the COPD mouse model (Figure 1G-1I, see Page 11, line 215-217). Therefore, we think that the 12-week of CS exposure can induce COPD.

1.Sasaki M, Chubachi S, Kameyama N, et al. Evaluation of cigarette smoke-induced emphysema in mice using quantitative micro-computed tomography. Am J Physiol Lung Cell Mol Physiol. 2015 May 15;308(10):L1039-45.

2.Li Y, Yu G, Yuan S, et al. Cigarette Smoke-Induced Pulmonary Inflammation and Autophagy Are Attenuated in Ephx2-Deficient Mice. Inflammation. 2017 Apr;40(2):497-510.

3.Shu J, Li D, Ouyang H, et al. Comparison and evaluation of two different methods to establish the cigarette smoke exposure mouse model of COPD. Sci Rep. 2017 Nov 13;7(1):15454.

4.Heulens N, Korf H, Cielen N, De Smidt E, Maes K, Gysemans C, Verbeken E, Gayan-Ramirez G, Mathieu C, Janssens W. Vitamin D deficiency exacerbates COPD-like characteristics in the lungs of cigarette smoke-exposed mice. Respir Res. 2015 Sep 16;16(1):110.

5.Zhou L, Wu B, Yang J, Wang B, Pan J, Xu D, Du C. Knockdown of circFOXO3 ameliorates cigarette smoke-induced lung injury in mice. Respir Res. 2021 Nov 17;22(1):294.

Comment 3: Smoking differed greatly between COPD and controls, and how can the effect on metabolic outcomes be ignored.

Re: Thanks for the reviewer's comment. This was indeed a shortcoming of our study, as we did not recruit healthy smokers. Most of the omics studies included COPD patients, smokers and non-smokers. Chen Q used untargeted metabolite profiling of serum samples to compare the differences among healthy smokers, COPD smokers

and non-smokers. 62 metabolites were significantly different between healthy smokers and smoker-COPD, of which 17 metabolites were differentially-expressed in smoker-COPD compared to both healthy smokers and non-smokers. 23 metabolites were differentially expressed in COPD-smokers compared with healthy-smokers.This study also demonstrated that cigarette smoke (CS) itself was associated with 107 serum metabolite changes, relative to non-smokers [1]. In a serum lipidome study,CS and COPD were associated with increases in glycero(phospho)lipids and monounsaturated fatty acids, a decrease in polyunsaturated fatty acids and an imbalance in eicosanoids [2]. Both studies indicated smoking had an effect on metabolites. Ideally, there should be a smoker group. We take it as a study limitation which is acknowledged in the study limitations. We will include smokers group in the future.

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2.Titz B, Luettich K, Leroy P, Boue S, Vuillaume G, Vihervaara T, Ekroos K, Martin F, Peitsch MC, Hoeng J. Alterations in Serum Polyunsaturated Fatty Acids and Eicosanoids in Patients with Mild to Moderate Chronic Obstructive Pulmonary Disease (COPD). Int J Mol Sci. 2016 Sep 20;17(9):1583.

Changes in the text: see Page 21, line 450-452.

Comment 4: The inclusion/exclusion on COPD and controls were not strict, resulting in the different effect on outcomes.

Re: We thank the comment. We added the inclusion/exclusion criteria in methods.

Changes in the text: see Page 6-7, line 121-131.

Comment 5: Figure1 legends should began with summative words.

Re 5: Thanks for the comment. We modified the description of Figure 1.

Changes in the text: see Page 32, line 629.

Reviewer D

Comment 1: To unbiasedly characterize the effect of chronic obstructive pulmonary disease on human lung metabolome in the presented research, as least two more factors should be controlled, namely, smoking status (having quitted or not? Biomass or cigarette smoking?) and distance of sample location from cancer lesions.

Re: Thanks for the reviewer's comment. Our COPD patients are currently smoker with more than 20 packs-year, and they were all urban residents with no exposure to other risk factors such as biomass. And we added the inclusion/exclusion criteria in methods.

Changes in the text: see Page 6-7, line 121-133.

Comment 2: To alleviate the influence of smoking on metabolomics in mouse models, it should be considered that a third group of mice were sacrificed and tested after rapid exposure to cigarette smoke. For the reproducibility of mouse model, brand of cigarettes used in this research should be reported.

Re: We thank very much for the reviewer' comment and suggestion . This was indeed a shortcoming of our study, as we did not recruit healthy smokers. Most of the omics studies included COPD patients, smokers and non-smokers. Chen Q used untargeted metabolite profiling of serum samples to compare the differences among healthy smokers, COPD smokers and non-smokers. 62 metabolites were significantly different between healthy smokers and smoker-COPD, of which 17 metabolites were differentially-expressed in smoker-COPD compared to both healthy smokers and non-smokers. 23 metabolites were differentially expressed in COPD-smokers compared with healthy-smokers.This study also demonstrated that cigarette smoke (CS) itself was associated with 107 serum metabolite changes, relative to non-smokers [1]. In a serum lipidome study,CS and COPD were associated with increases in glycero(phospho)lipids and monounsaturated fatty acids, a decrease in polyunsaturated fatty acids and an imbalance in eicosanoids [2]. Both studies indicated smoking had an effect on metabolites. Ideally, there should be a smoker

group. We take it as a study limitation which is acknowledged in the study limitations. We will include smokers group in the future.

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2.Titz B, Luettich K, Leroy P, Boue S, Vuillaume G, Vihervaara T, Ekroos K, Martin F, Peitsch MC, Hoeng J. Alterations in Serum Polyunsaturated Fatty Acids and Eicosanoids in Patients with Mild to Moderate Chronic Obstructive Pulmonary Disease (COPD). Int J Mol Sci. 2016 Sep 20;17(9):1583.

Changes in the text: see Page 21, line 450-452.

Comment 3: Figures should be set at a readable resolution. Additionally, heatmaps of figure 3 make table 2 redundant by reporting differential metabolites.

Reply: We thank the reviewer for the comment. We used higher resolution figures. The heatmaps of figure 3 indeed showed the detailed information of differential metabolites. We deleted the original table 2 and provided two tables in the supplement data.