Responses to comments and revision notes

[The comments of Reviewer #1**]**

The study performs a comprehensive analysis of liver fibrosis, as predictor of significant morbidity and mortality in patients with chronic liver disease, by assessing dynamic changes of different metabolic pathways and serum levels of biomarkers during fibrosis progression using rat models. Metabolites changes were dynamically investigated during the process of liver fibrosis development, based on pathological findings. The novelty of the research consists in identification of two serum biomarkers, muricholic acid (MCA) and cervonoyl ethanolamide (CEA), which proved to be relevant for assessing fibrosis in an animal model and showed superior accuracy in discriminating early vs. advanced fibrosis. Using ROC analyses, these novel biomarkers proved to be effective in framing both early and intermediate cirrhosis stages vs. currently used biomarkers. Therefore, this is an original manuscript that opens new doors in identifying novel and more accurate biomarkers for staging liver fibrosis that may replace in the future liver biopsy, considered as a gold standard but an invasive method for assessing fibrosis.

[Comment 1]

I would recommend that the paper should focus more on describing currently available non-invasive tests for staging fibrosis and to stress on the advantages of these new biomarkers in comparison to the available non-invasive tests (not only the conventional functional liver tests), e.g superiority in identifying early fibrosis or discrimination between early/intermediate stages of fibrosis.

[Response]

We concur with the points and made appropriate changes in “Introduction” section (Page 4, Line 100-106).

[The comments of Reviewer #2**]**

The manuscript entitled “Dynamic changes of key metabolites during liver fibrosis in rats” focused on dynamic changes in metabolic profiles and biomarker concentrations in rat serum during liver fibrosis progression by using ultra-performance liquid chromatography coupled to

quadrupole time-of-flight mass spectrometry. Although the study has been done very well, the proteomic studies on liver fibrosis or cirrhosis are not new. Besides, there are many problems in the manuscript needed to be solved before publication.

[Comment 1]

1. The only new in this study is to find out two serum markers in only one animal model but no ex vivo or in vitro studies in the manuscript to confirm the functional or clinical implications of the two proteins. Besides, only one animal study was so week to support the novel role of these proteins on liver fibrosis and cirrhosis. The authors should add another model to reproduce their experiment to get the same results and to confirm their important role.

[Response]

We appreciate the reviewer's suggestions and comments. The main focus of our present study is metabonomics that which is the science that can better characterize the phenotypes of living organisms, telling us what is "really happening". According to the results of rats experiment，we will intend to collect clinical samples for verification after ethical permission in the follow-up study.

2. The standard evaluation of the animal model should include the fibrosis score which the authors did not mention in the manuscript. The authors did not define which group is fibrosis or cirrhosis.

[Response]

We agree with the reviewer. In this manuscript, the tissue sections were randomly numbered prior to reading and observed by an experienced pathologist. The pathologist graded the liver according to the Ishak's criteria for liver fibrosis （Page 5, Line 138）. Our results showed early fibrosis occurred in the first and fourth weeks, and advanced fibrosis and cirrhosis occurred in the eighth and twelfth weeks.

3. The authors may consult pathologist for the reading of their tissue pathology and hepatologist for the interpretation of clinical implications of the two proteins, who could be included in the coauthor.

[Response]

We thank the reviewer for this suggestion and add the acknowledgment to the

pathologist (Page 15, Line 423-425).

[Comment 2]

The authors did not investigate the functional effects of the two proteins. Therefore, the authors can only cite references to explain the clinical relationship between the two proteins and the manifestation of liver fibrosis or cirrhosis.

[Our Response]

We appreciate the reviewer's point and and made the appropriate changes (Page 13-14). CEA is involved in anti-inflammation and acts as an antagonist of CB2, and β-MCA is related to the processes involved in hepatocyte damage. The ROC results showed both two metabolites had excellent diagnostic value and could be used in clinical diagnosis in the future.

[Comment 3]

3. The discussion is mostly descriptive. There is much hypothesis the authors have to do functional study or check serum levels, such as TNF- , INF- and IL-17 or TBA, to confirm the importance of the two proteins.

[Our Response]

Thank you for the helpful comments. Adding serum levels, such as TNF- , INF- and IL-17 or TBA would be an ideal way to confirm the importance of the two proteins. Serum TBA results have been in the manuscript. Considering the amount of stored rat serum and revised time, we have added liver immunohistochemistry and RT-PCR results of a-smooth muscle actin (α-SMA) and transforming growth factor β1 (TGF-β1) (Page 9, Line 241-246). α-SMA and TGF-β1 also plays a central role in the progression in liver fibrosis. We also rewrote the discussion section (Page 12, Line 318-320).

[Comment 4]

4. Actually, there have been many studies and reviews to report the proteomics of liver fibrosis and cirrhosis and many serum markers were reported. What is the difference between the previous

reports and this study? Is there any relationship between them? Anyway, these studies or reviews should be added in the introduction or discussion section.

[Response]

Early identification of liver fibrosis is very important for the diagnosis and treatment of liver diseases. Although many studies have been reported, there is still no very practical and accurate index to diagnose fibrosis. We carried out a systematic and comprehensive dynamic observation study from liver injury to liver fibrosis by using metabolomics. And, two new potential metabolites have been found. The ROC results showed both two metabolites had excellent diagnostic value and could be used in clinical diagnosis in the future.

[Comment 5]

5. A recently published paper in International Journal of Biological Macromolecules Volume 111, May 2018, Pages 379-392 (Proteomic-genomic adjustments and their confluence for elucidation of pathways and networks during liver fibrosis), is a good reference the authors may be interesting.

[Response]

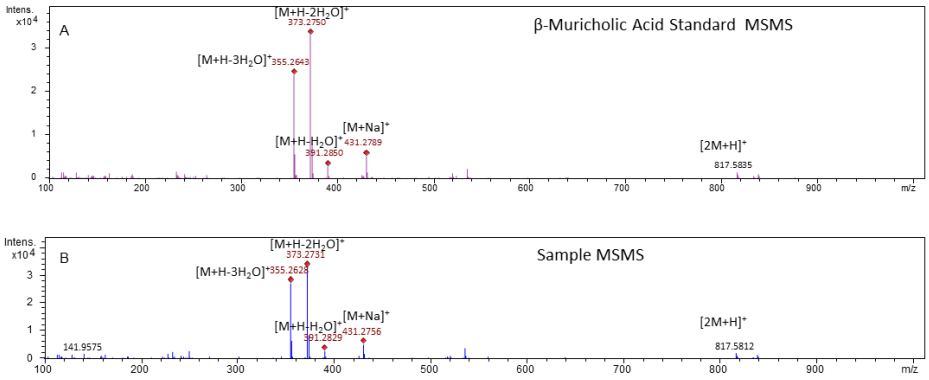
This is a very good article on proteomics. Thank you for your recommendation, but what we did was metabonomics, so we did not cite this article.

[Comment 6]

Minor revisions: 1. There are many subtypes of MCA reported and the most important one is beta-MCA. Can the authors tell reader which subtype of MCA is most important in their study?

[Response]

As mentioned by the reviewer, there are many subtypes of MCA reported. In this study, the subtype we detected is β-MCA. The figures of spectra are as below. Figure A is the standard secondary mass spectrum, and Figure B is the sample secondary mass spectrum. We have mentioned β-MCA clearly in the revision. Special thanks to the reviewer for his great suggestion and comment.



2. Before resubmitting, the authors should send the manuscript for English-editing.

[Response]

We agree with the suggestion and we have invited a professional English expert to revise our article.

[The comments of Reviewer #3**]**

Enjoyed reviewing this manuscript but have a few comments. Authors appeared to have complied with 14 points for peer-review checklist.

[Comment 1]

Introduction: 1. Delete invasive ('invasive' liver biopsy) 2. Limited availability for non-invasive monitorting of liver fibrosis. This is not accurate as clinical trials are now using non-invasive tests such as fibroscan and MRI to monitor hepatic steatosis and fibrosis in lieu of liver biopsies. I think this sentence needs to be rewritten.

[Response]

We fully agree with this suggestion, and we have rewritten the sentences (Page 4, Line 100-106).

[Comment 2]

Results: Authors state cholestatic liver chemistries increased less rapidly than hepatocellular enzymes. However, no explanation for this observation was reported in the discussion and would

be important for readers.

[Response]

Thanks for pointing out our inaccurate statement. We have made corrections on Page 8 Line 226 according to the Reviewer's comments.

[Comment 3]

3.2 Bubble -like morphological changes in liver: not familiar with this term. Did authors mean balloon degeneration of hepatocytes? Investigators used MTC staining performed for collagen staining at predetermined intervals which is acceptable. However, Western blotting is used to detect alpha actin, a sign of stellate cell activation and an events which occurs much earlier before deposition of fibrosis. Could investigators explain why this was not performed?

[Response]

This is a good suggestion. Bubble -like morphological changes in liver means balloon degeneration of hepatocytes. We have added results of liver immunohistochemistry and relative mRNA expression levels of α-SMA and TGF-β1 in revised version (Page 9, Line 241-246).

[Comment 3]

3.5 Profiles most dramatic at early stage of disease yet in discussion authors state most severe metabolic changes were noted at the final stages. Confusing and needs to be clarified.

[Response]

We agree with the reviewer's suggestion and made appropriate changes (Page 10, Line 260-261, Page 12, Line 325-330). In the early stage, the strong change of metabolic spectrum is due to the injury and death of a large number of hepatocytes, which resulting in abnormal liver function indicators. We believe that this is because of the body's stress response to CCl_4. In the late stage, changes in liver metabolic capacity occur after injury of hepatocytes, which induces secondary changes of small molecule metabolites in vivo, as well as strong changes in metabolic spectrum.

[Comment 3]

Discussion: Sentence on ROS and hepatocyte necrosis, cell permeability , steatosis, cirrhosis can

be deleted. Final paragraph needs writing. For example CEA is an agonist against CB2 (ie do you mean antagonist?). Would consider rewriting sentence 'This study identified two novel biomarkers, CEA and MCA involved in x and y, that are able to etc.

[Response]

We agree with the reviewer and rewritten the sentences as required. We have also revised the grammar, layout, etc. of the manuscript（Page 12, Line 335, Page 14, Line 409）.

[The end]