

Dear Associate Editor :

Thank you for your letter and for the reviewers' comments regarding our manuscript entitled "Lipotoxic hepatocyte-derived exosomal miR-1297 promotes hepatic stellate cell activation through the PTEN signaling pathway in MAFLD" (Manuscript NO.: 59907). We have made corrections as suggested by the reviewers and here by submit a revised manuscript. We improved the representation of the data and images. Major corrections in the paper and responses to the reviewers' comments are listed below. We hope that the manuscript will now be accepted for publication in World Journal of Gastroenterology.

Corresponding author: Ming-Yi Xu, E-mail: xumingyi2014@163.com.

Once again, thank you very much for your comments and suggestions.

With all my best regards!

Sincerely yours,

Mingyi Xu

## **Reviewer reports:**

**Reviewer #1: The study reports the vehiculization of mir1297 in Exosomes communicating hepatocytes with HSC. The study is comprehensive and results are presented supporting the hypothesis. PTEN and downstream PI3, AKT have been involved. There are several open questions to be addressed as follows: \**

**1) Is FIBROSCAN an efficient method to assess fatty lever disease and its degree of severity? This issue might be mentioned and a reference (such as Lancet 2020 Newsome PM et al) could be provided.**

Answer: Thank you for kindly reminding us of this point. FIBROSCAN has been recommended to be clinically useful noninvasive tool to assess fatty liver and the severity of NAFLD patients according to the guidance of ASSLD in 2017<sup>[Ref.1]</sup>. We have added the reference in the Method section of revised paper. In page 5, line 12-15: “FibroScan (Echosens, Paris, France) analysis was applied to assess the controlled attenuation parameter (CAP) and Liver Stiffness Measurement (LSM) of these patients according to the guidance of ASSLD <sup>[10]</sup>

[Ref.1] Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology*. 2018;67(1):328-357. doi:10.1002/hep.29367

**2) Data analysis of micro RNaseq experiments must be described.**

Answer: Thanks for this opinion. We have added the detailed analysis of the micorRNA seq in the Method section of revised paper. In page 6, line 18-23: “Low-quality reads and adapters were filtered out from the raw reads to obtain clean reads. The clean reads were aligned against the miRBase database and the expression of miRNAs in each sample was determined. Differentially-expressed miRNAs were screened using edgeR, under the following criteria:  $|\log_2(\text{fold-change})| \geq 1$  and  $p < 0.05$ . Heatmaps of gene expression were generated with MeV software (MeV Ltd., Stockport, UK).”

**3) Figures 3 and 4 are missing.**

Answer: Apologies for the confusion. We have updated the correct version of Figure 3 and Figure 4 in the revised paper.

**4) Are there differences between cells-derived and serum Exosomes?**

Answer: Thanks for the kind opinion. Exosomes collected from the cell culture medium were usually used for *in vitro* study, while exosomes isolated from serum from mice or human were usually used for *in vivo* study, which was generally accepted in exosomes related research.

In previous study, Liu et al measured the expression levels of miR-192-5p in exosomes from both NAFLD patients and PA (Palmitic Acid)-treated THP-1 cells to better clarify the expression in the two conditions as shown in Fig. A and Fig. B <sup>[Ref.2]</sup>,

which was similar to our research design. So by referencing the similar work, we also used cell-derived and serum-derived exosomes in our work.

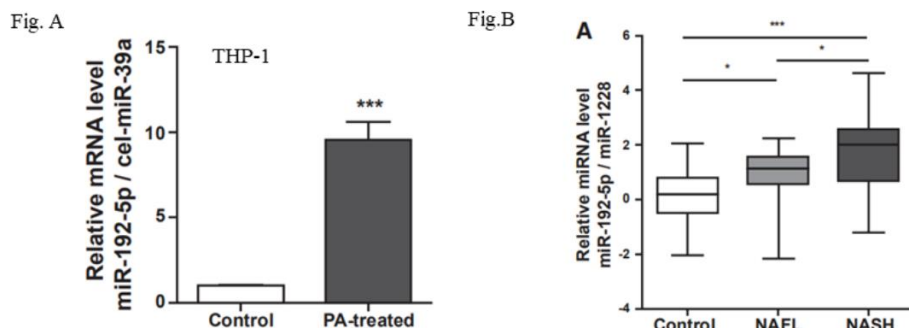


Fig.A: The relative expression levels of miR-192-5p in exosomes from THP-1 cells after treated with Bovine serum albumin (control) or Palmitic Acid [Ref.2].

Fig.B: The relative expression levels of serums exosomal miR-192-5p from NAFLD patients in different stages [Ref.2].

[Ref.2] Liu XL, Pan Q, Cao HX, et al. Lipotoxic Hepatocyte-Derived Exosomal MicroRNA 192-5p Activates Macrophages Through Rictor/Akt/Forkhead Box Transcription Factor O1 Signaling in Nonalcoholic Fatty Liver Disease. *Hepatology*. 2020;72(2):454-469. doi:10.1002/hep.31050

**5) 1400 fold over expression of mir1297 in LX2 cells is far from physiological conditions. This might lead to artifactual effects and misleading interpretations. Please, discuss.**

Answer: Thanks a lot for kindly reminding us of this point. The negative control mimics (mi-NC) and miR-1297 mimics (mi-miR) in our research were purchased from GenePharma Co., Ltd. (Shanghai, China), which have been widely applied in researches in a variety fields. As the manufacturer stated, the over-expression fold change of microRNA mimics produced by GenePharma ranged from dozens of times to thousands of times, depending on the cell type and the specific miRNA original abundance. As shown in Fig. C, the miR-145 mimics transfections could make a 1500 times over-expression in LX2 cells [Ref.3]. By reviewing our previous study, we could find the expression of miR-194 reached an 800 times fold change in LX2 cells as shown in Fig.D [Ref.4]. Based on the above factors, we used this method in our research.

Fig. C

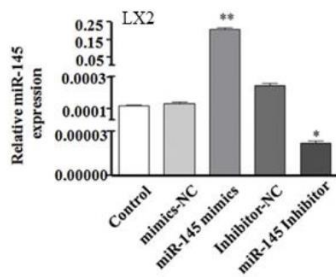


Fig.D

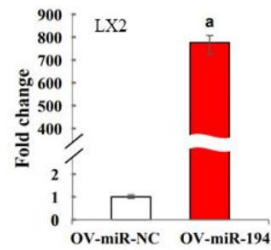


Fig.C: The relative expression levels of miR-145 in LX2 cells after miR-145 mimics or miR-145 inhibitors transfection [Ref.2].

Fig.D: The relative expression levels of miR-194 in LX2 cells after the miR-194 mimics transfection [Ref.3].

[Ref.3] Zhou DD, Wang X, Wang Y, et al. MicroRNA-145 inhibits hepatic stellate cell activation and proliferation by targeting ZEB2 through Wnt/ $\beta$ -catenin pathway. *Mol Immunol.* 2016;75:151-160. doi:10.1016/j.molimm.2016.05.018

[Ref.4] Wu JC, Chen R, Luo X, Li ZH, Luo SZ, Xu MY. MicroRNA-194 inactivates hepatic stellate cells and alleviates liver fibrosis by inhibiting AKT2. *World J Gastroenterol.* 2019;25(31):4468-4480. doi:10.3748/wjg.v25.i31.4468

## 6) Please describe PTEN mutant.

Answer: Thank you for kindly reminding us of this point. We have added the detailed information of PTEN mutant in the Method section of revised paper. In page 6, line 9-15: “The 3'-UTR sequence of PTEN predicted to interact with miR-1297, together with a corresponding mutated sequence within the predicted target sites, were synthesized and inserted into the luciferase reporter (Promega, Madison, WI, USA) to generate PTEN wild type (WT) or PTEN mutant (MUT) (primers was shown in Suppl.Table2). The pGL3-Basic luciferase vector containing PTEN WT or PTEN MUT were co-transfected with miR-1297 mimics or negative control mimics by using Lipofectamine2000 (Invitrogen).”.

**Reviewer #2: the manuscript by Xin Luo et al, is a good work, well presented and describes its methodological development and results very well. Congratulations**

Answer: Thank you for your kind encouragement of our work.

**Reviewer #3: General comments** This study analyzed the role of lipotoxic Exosomal miR-1297 in the pathogenesis of MAFLD. As author stated, MAFLD is becoming more prevalent, and there are many clinical trials to evaluate the efficacy of medications to prevent progression of MAFLD to cirrhosis. As such, elucidating the pathogenesis of MAFLD has clinical relevance for future drug development. Please note, I am not a basic scientist, so I cannot comments on the technical aspect of this study, however this is a well written manuscript with clinical importance, so this should be considered for possible publication after

**addressing minor comments as below. Specific comments For methodology section.**

**1) It stated that fibroscan was used to classify mild vs sever fatty liver. Biopsy is usually regarded as the gold standard. Was there any biopsy result?**

Answer: Thank you for kindly reminding us of this point. FIBROSCAN has been recommended to be clinically useful noninvasive tool to assess the severity of NAFLD patients according to the guidance of ASSLD in 2017<sup>[Ref.1]</sup>. Since our cohort was diagnosed according the non-invasive method, so there were no liver biopsy results could be provided. We have added the reference in the Method section of revised paper. In page 5, line 12-15: “FibroScan (Echosens, Paris, France) analysis was applied to assess the controlled attenuation parameter (CAP) and Liver Stiffness Measurement (LSM) of these patients according to the guidance of ASSLD <sup>[10]</sup>

[Ref.1] Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology*. 2018;67(1):328-357. doi:10.1002/hep.29367

**Reviewer #4: Authors used primary hepatocyte (HC) line (LO2) cultures to isolate Exosomes by treating HC with palmitic acid, identified miR-1297 top upregulated microRNA among DE-miRs, showed activation of hepatic stellate cell (HSC) line (LX2) by mi-1297 and Exosomes isolated from serum samples of patients with MAFLD, and associated HSC activation with PTEN/PI3K/AKT signalling pathway. I would like to congratulate all authors for conducting such complex study with a small team and also clearly expressing the data within the manuscript. My comments:**

**1) MAFLD is proposed as new terminology for NAFLD, but the consensus has not been developed to use it. Therefore, I suggest to use NAFLD or at least make a referral to this terminology change.**

Answer: Thanks a lot for kindly reminding us of this point. We have added the contents about the explanation on MAFLD being proposed as new terminology for NAFLD and the relevant conference in the revised paper. In page 3, line 28-29 and page4, line1-3: “Metabolic associated fatty liver disease (MAFLD), which was formerly terminated as Non-alcoholic Fatty Liver Disease (NAFLD), is highly prevalent worldwide and affects approximately 25% of the global adult population <sup>[1]</sup>. Recently, a consensus of international experts proposed the terminology change from NAFLD to MAFLD in order to better demonstrated the etiological features of this disease <sup>[2]</sup>”

**2.1) The histologic (NAS and fibrosis) spectrum of patients must be given.**

Answer: Thank you for kindly reminding us of this point. In our study, FIBROSCAN

was used for the diagnosis of NAFLD patients. FIBROSCAN has been recommended to be clinically useful noninvasive tool to assess severity of NAFLD patients according to the guidance of ASSLD in 2017<sup>[Ref.1]</sup>. Since our cohort was diagnosed according the non-invasive method, so there were no liver biopsy results could be provided. We have added the reference in the Method section of revised paper. In page 5, line 12-15: “FibroScan (Echosens, Paris, France) analysis was applied to assess the controlled attenuation parameter (CAP) and Liver Stiffness Measurement (LSM) of these patients according to the guidance of ASSLD <sup>[10]</sup>

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## **2.2) Why were so much (20) serum samples used?**

Answer: Thanks for the comment. At the beginning of our human samples collection, we referred to some similar study about NAFLD to determine the proper amount of human patients. In a research on exosomal miRNAs profiling of children's NAFLD, there were 20 NAFLD patients enrolled <sup>[Ref.2]</sup>. Considering the limitation of experiment budget and time, we finally design a small cohort of 20 NAFLD patients (10 mild fatty liver and 10 severe fatty liver) in our research.

[Ref.2] Zhou X, Huang K, Jia J, et al. Exosomal miRNAs Profile in Children's Nonalcoholic Fatty Liver Disease and the Correlation with Transaminase and Uric Acid. *Ann Nutr Metab*. 2020;76(1):44-53. doi:10.1159/000506665

## **2.3) Why analysis were done according to level of hepatosteatosis, instead of fibrosis, since mi-1297 was found to be related to fibrosis pathways?**

Answer: Apologies for causing such a confusion. As the previous study demonstrated that the lipotoxic hepatocyte exosomes (HC-Exo) could deliver the miRNA to the receipt cell HSC, contributing to HSC activation <sup>[Ref.3]</sup>, but the exact mechanism under the HC-Exo and HSC crosstalk remained unclear. So for *in vitro* study, we treated PHC with palmitic acids or BSA to generate the lipotoxic group or control group, and found miR-1297 was greatly up-regulated in lipotoxic HC-Exo. Therefore when it came to *in vitro* study, we conducted qPCR verification according to the hepatic steatosis of NAFLD patients to parallel with design of *in vitro* experiment.

[Ref.3] Lee YS, Kim SY, Ko E, et al. Exosomes derived from palmitic acid-treated hepatocytes induce fibrotic activation of hepatic stellate cells. *Sci Rep*. 2017;7(1):3710. Published 2017 Jun 16. doi:10.1038/s41598-017-03389-2

## **2.4) Was there any correlation between level of fibrosis in the liver and**

**experimental finding?**

Answer: Thanks for this question. As we mentioned in Question2.3, we designed our experiment according to hepatic steatosis of NAFLD patients. The cohort of research consisted of 20 NAFLD patients including 10 mild fatty liver and 10 severe fatty liver. They differ from the hepatic steatosis but the severity of liver fibrosis were similar. It was regretful that we neglected this point and caused a confusion. We have added this criteria of patients selection in the Methods section of revised paper. In page 5, line 9-10: “Among the 20 MAFLD patients with similar liver fibrosis severity, 10 patients were diagnosed as mild fatty liver while the other 10 patients were diagnosed as severe fatty liver depending on their hepatic steatosis.”

So the fibrosis severity and our experimental findings did not correlate.

**2) Fig2: Labels on X and Y axes cannot be read.**

Answer: Apologies for the confusion. We have updated the new version of Figure 2 with a higher resolution in the revised paper.

**3) Fig3-4: It is not available.**

Answer: Thanks for remind us of the confusion. We have updated the correct version of Figure3 and Figure 4 in revised paper.

**4) Suppl.Table1: the abbreviation LSM should be explained, and CAP values must be added to table.**

Answer: Thanks for remind us of the mistake. The abbreviation LSM was short for “Liver Stiffness Measurement”, but the data in the LSM line were actually the CAP value. The caption was a confusion by mistake. We have corrected the error in the revised paper and both the CAP value and LSM value have been given in Suppl.Table1.

**Reviewer #5: Congrats a quality work**

**This study is novel and the experiments are convincing.**

Answer: Thank you for your kind encouragement of our work.

Dear Editor :

Thank you for your letter and for the reviewers' comments regarding our manuscript entitled "Lipotoxic hepatocyte-derived exosomal miR-1297 promotes hepatic stellate cell activation through the PTEN signaling pathway in MAFLD" (Manuscript NO.: 59907) for the first round revision. We have made corrections as suggested by the reviewers and here by submit a revised manuscript. We improved the representation of the data and images. Major corrections in the paper and responses to the reviewers' comments are listed below. We hope that the manuscript will now be accepted for publication in World Journal of Gastroenterology.

Corresponding author: Ming-Yi Xu, E-mail: xumingyi2014@163.com.

Once again, thank you very much for your comments and suggestions.

With all my best regards!

Sincerely yours,

Mingyi Xu



## **Reviewer reports:**

**Reviewer #1: The authors have addressed some of the issues raised by this reviewer. However, several aspects require further consideration:**

**1. Similarities and differences of cellular and serum exosomes should be further discussed and comments should be included in the manuscript.**

Answer: Thanks for the comment. We have added the further comments on the differences of cellular and serum exosomes in Discussion Section when talking about the qPCR verification in both cellular and serum exosome samples. Changes could be seen in the revised manuscript in page 14, line 10-20: “Then miR-1297 was found to be the top up-regulated microRNA in both lipotoxic PHC-derived exosomes and severe fatty liver patients’ serum exosomes compared to their control groups via qPCR verification. Cellular exosomes were isolated from cell culture mediums and could represent an MAFLD cell model progression. We found miR-1297 was significantly up-regulated in lipotoxic PHC-derived exosomes, however sometimes the experimental results did not parallel with the actual disease progression. So we measured the expression of miR-1297 in serum exosome samples of MAFLD patients. Exosomes derived from serum usually originated from different organs and could reveal a rather complicated but more real condition during the disease progression. In serum exosomes samples we also found miR-1297 was markedly over-expressed in severe MAFLD patients. Both results revealed that miR-1297 was obviously up-regulated during the MAFLD progression, so miR-1297 was finally chosen for the follow-up study.”

**2. Even though the authors provide previous studies reporting very large over expression of particular genes, it doesn't really respond the question of whether this unphysiological effect might lead to artifactual biological responses leading to misleading interpretations.**

Answer: Apologies for the confusion. Normally microRNA mimics transient transfection could reach a very large over-expression ranging from dozens to thousands, but the previous study had affirmed that this large over-expression will not lead to an artificial effect and misleading interpretation. In a previous study published in *Gastroenterology* [Ref.1], in order to illustrate the potential artificial biological effect of exogenous miRNA mimics transfection, both transient and stable miR-139 over-expression HCC cell model were established to observe if the two kind of over-expressions would make a huge difference to cell function. As shown in Fig. A and Fig. B, the stable miR-139 SMMC-7721 cells (a HCC line) make an almost 50 times up-regulation while the transient transfection reached a 3000 times over-expression. Transwell essays was conducted to evaluate their invasion capacity. As shown in Fig. C and Fig. D, both stable transfection and transfection of miR-139 markedly suppressed the invasion of SMMC-7721 cells and showed a similar decline degree, which proved that the large over-expression of exogenous miRNA mimics

transfection and physiological over-expression showed a similar cell function and would not lead to an artificial biological response and misleading interpretation.

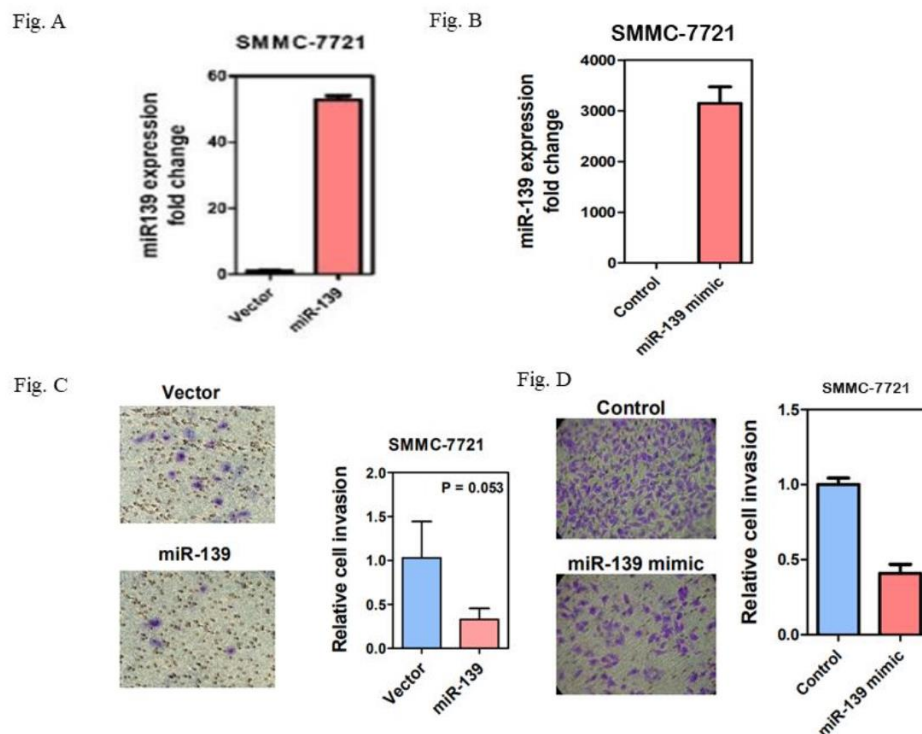


Fig. A: The relative expression levels of miR-139 in stable miR-139 over-expressed SMMC-7721 cells

Fig. B: The relative expression levels of miR-139 in SMMC-7721 cells with transient miR-139 mimics transfection.

Fig. C: The stable miR-139 over-expressed SMMC-7721 cells showed a significant reduction of cell invasion compared with the control cells.

Fig. D: SMMC-7721 cells with miR-139 mimics overexpressing showed a significant reduction of cell invasion compared with the control group.

[Ref.1] Wong CC, Wong CM, Tung EK, et al. The microRNA miR-139 suppresses metastasis and progression of hepatocellular carcinoma by down-regulating Rho-kinase 2. *Gastroenterology*. 2011;140(1):322-331. doi:10.1053/j.gastro.2010.10.006