Point by Point Response to Reviewer's Comments

Reviewer #1 (Remarks to the Author):

1. Saeed et al. examined the role of necroptosis in NASH using RIP3KO-mice with NC/HF-diet. Study seems to do well. 1. Authors should show the evaluation of apoptosis.

☞Thanks for your good point. We fully agree with reviewer that evaluation of apoptosis could have provided more useful information; however, it was not primarily the aim of our study. We have already mentioned about this limitation of our study in discussion section on page 17 as follows "Moreover, we also did not evaluate the previously highlighted contribution of increased hepatic and adipose tissues apoptosis associated with RIP3 deletion in NAFLD. RIP3 ablation in adipose tissue leads to the metabolic phenotype in RIP3KO mice. Moreover, RIP3 has a role in maintaining white adipose tissue homeostasis and systemic RIP3 ablation lead to insulin resistance and glucose intolerance. RIP3 overexpression is thought to balance caspase-8 mediated increased apoptosis. Following, RIP3 deletion, a switch towards increased apoptosis in both liver and adipose tissues was observed and increased adipocytes apoptosis was thought to mediate systemic effects [1]."

References

1. Gautheron J, Vucur M, Schneider AT, Severi I, Roderburg C, Roy S, Bartneck M, Schrammen P, Diaz MB, Ehling J, Gremse F, Heymann F, Koppe C, Lammers T, Kiessling F, Van Best N, Pabst O, Courtois G, Linkermann A, Krautwald S, Neumann UP, Tacke F, Trautwein C, Green DR, Longerich T, Frey N, Luedde M, Bluher M, Herzig S, Heikenwalder M, Luedde T. The necroptosis-inducing kinase RIPK3 dampens adipose tissue inflammation and glucose intolerance. *Nature communications* 2016; **7**: 11869 [PMID: 27323669 PMCID: PMC4919522 DOI: 10.1038/ncomms11869]
2. Roychowdhury S, McCullough RL, Sanz-Garcia C, Saikia P, Alkhouri N, Matloob A, Pollard KA, McMullen MR, Croniger CM, Nagy LE. Receptor interacting protein 3 protects mice from high-

fat diet-induced liver injury. *Hepatology (Baltimore, Md)* 2016; **64**(5): 1518-1533 [PMID: 27301788 PMCID: PMC5074889 DOI: 10.1002/hep.28676]

2. "Histological assessment of liver biopsy samples" "1% P/S"? "DPBS"?....

☞Thanks for your good point the terms 1% P/S, DPBS, and DMEM have been elaborated, mentioned and highlighted as they appeared first in the manuscript, Page 10, Subheading, HepG2 cells culture and maintenance.

3. Authors should ask native English speaker to edit your manuscript again.

☞As reviewer pointed, English has been extensively revised and improved by a native English speaker. The mentioned errors have been corrected as suggested.

Reviewer #2 (Remarks to the Author):

1. Please strengthen the aim of your study in the abstract.

☞Thanks for your good point. The aim of study has been revised according to journal's guidelines. Moreover, it is strengthened and re-written in abstract as follows: "To validate the effects of RIP3 deletion in NAFLD and to clarify the mechanism of action."

2. Please improve the introduction section, it is poor and misses in important and fundamental details. It must be improved and updated related to the current literature. The authors should go deeper into different and relevant aspects of NAFLD. I recommend checking the following interesting and fundamental papers, or similar, and comment them in relation to the study topic: Early effects of high-fat diet, extra-virgin olive oil and vitamin D in a sedentary rat model of non-alcoholic fatty liver disease. Histol Histopathol. 2018, 33, 1201-1213 doi: 10.14670/HH-18-008. PMID: 29855033. Echocardiography and NAFLD (non-alcoholic fatty liver disease). Int J Cardiol. 2016 Oct 15;221:275-9. doi: 10.1016/j.ijcard.2016.06.180. PMID: 27404689. Fatty liver disease and lifestyle in youngsters: diet, food intake frequency, exercise, sleep shortage and fashion. Liver Int. 2016 Mar;36(3):427-33. doi: 10.1111/liv.12957. PMID: 26346413. 4Ps medicine of the fatty liver: the research model of predictive, preventive, personalized and participatory medicine-recommendations for facing obesity, fatty liver and fibrosis epidemics. EPMA J. 2014 Dec 7;5(1):21. doi: 10.1186/1878-5085-5-21. PMID: 25937854;

☞Thanks for your good point. The introduction section of the study has been extensively revised and re-written. As the reviewer pointed, the relevant different aspects of NAFLD have been updated in the introduction section according to the mentioned literature and similar references on Page 7 as follows: NAFLD, the hepatic component of metabolic syndrome, is a multifactorial wide spectrum disease ranging from simple steatosis to steatohepatitis and further progressing to fibrosis and the hepatocellular carcinoma. In NAFLD increased lipids accumulation in hepatocytes leads to steatosis, inflammation

and fibrosis. NAFLD could also be hinting towards decreasing heart function[1]. In youngers NAFLD is also associated with decreased sleep, decreased quality and frequency of food intake, and the sedentary life-style [2]. The lifestyle modifications directed towards reduced steatosis in NAFLD would not only improve NAFLD but also the cardiac function [1]. Although the prevalence of NAFLD is increasing; however, there are still numerous diagnostic and treatment issues associated with NAFLD. For instance, although, liver biopsy remains the gold standard method for NAFLD diagnosis; however, currently no diagnostic method can correctly distinguish between simple steatosis and steatohepatitis. Moreover, there is still a lack of satisfactory treatment strategy for NAFLD [3].

In NAFLD, the 'first hit' comprises of accumulation of fatty acids in hepatocytes facilitated by increased fatty acids synthesis and increased insulin resistance. Later, the multiple 'parallel hits' mainly comprising of endoplasmic reticulum stress, mitochondrial dysfunction, oxidative stress, and inflammatory cytokines further facilitate the hepatocytes dysfunction and death [4].

3. Please strengthen the aim of your study at the end of the introduction section.

☞As reviewer pointed, we have strengthened the aim of the study in introduction section and it has been re-written as follows on Page 8, "Therefore, by using HF diet induced NAFLD in RIP3KO mice, we aimed to validate and evaluate the precise underlying mechanism of steatosis and inflammation in hepatocytes and inflammatory cells."

4. Please strengthen and improve the conclusion, adding the clinical relevance of your work and some important suggestions for the scientific community.

☞As reviewer pointed, the conclusion section has been improved. Moreover, the clinical relevance and important future suggestions have been mentioned as follows on page 18, "The future research should consider the diverse and unwanted systemic consequences of RIP3 deletion in NAFLD. The role of RIP3 could be a double-edged sword in NAFLD. Although RIP3 has a crucial role in necroptosis, RIP3 showed diverse effects in metabolic disease. Therefore, careful attention and more extensive studies are

needed to further elaborate the interactions between RIP3 and NAFLD associated signaling pathways."

5. Please refresh and update the reference list section.

☞As reviewer asked the references have been refreshed, updated and highlighted.

References

1. Trovato, F. M. et al. Echocardiography and NAFLD (non-alcoholic fatty liver disease). International journal of cardiology 221, 275-279, doi:10.1016/j.ijcard.2016.06.180 (2016).

2. Trovato, F. M. et al. Fatty liver disease and lifestyle in youngsters: diet, food intake frequency, exercise, sleep shortage and fashion. Liver international : official journal of the International Association for the Study of the Liver 36, 427-433, doi:10.1111/liv.12957 (2016).

3. Oh, H., Jun, D. W., Saeed, W. K. & Nguyen, M. H. Non-alcoholic fatty liver diseases: update on the challenge of diagnosis and treatment. Clinical and molecular hepatology 22, 327-335, doi:10.3350/cmh.2016.0049 (2016).

4. Bessone, F., Razori, M. V. & Roma, M. G. Molecular pathways of nonalcoholic fatty liver disease development and progression. Cellular and molecular life sciences : CMLS, doi:10.1007/s00018-018-2947-0 (2018).