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17th July 2020
Re : World Journal of Hepatology Manuscript NO: 57567 response to review

Dear Sirs,
I am writing on behalf of the authors to detail our responses to the review of our manuscript "Inhibition of VAP-1 modifies hepatic steatosis in vitro and in vivo". We are grateful to the reviewers and the editorial team for their comments and respond to each comment (in bold) in a point by point manner below (plain text). Reviewer 1 : we are pleased that the reviewer stated our manuscript was interesting. They requested clarification on our staining in Figure 1 noting that **"Better quality figures at lower magnification are necessary to reveal more features, so that they can be clearly evaluated. Nuclear staining in H&E is not clear. The staining for Picrosirius Red is not correct, cytoplasm should be stained yellow and collagen red. The collagen staining in NASH sample is not convincing. Please include scale bar"**

The reviewer is correct in that we had mislabelled our fibrosis staining as picrosirius red (which would indeed be yellow and red) when we had in fact utilised a Van Gieson's staining protocol for our human specimens to yield blue nuclei and red collagen staining. To address this, we have corrected the methods information and figure legend accordingly. We have also replaced the images with clearer examples at lower power and have included scale bars as requested.

Figure 4 and Supplemental Figure 2. Except for a few genes, the fold change in most genes appeared to be rather marginal. Please provide the fold change either in a separate table or in the figure. Statistical analysis should be provided.
Here we have compared two different situations. For Figure 4 the data was collected from human liver slices exposed to methylamine for only 4.5 hours. Thus it is perhaps not surprising that we see only small magnitude changes in expression of genes involved in the early stages of nutrient handling such as FABP2, VLDLR, caveolin and Insulin Receptor subunits. We have added a supplemental data table (Supplemental table 2) showing the fold changes/statistical analyses and have amended the discussion to highlight the context. Similarly, in supplemental Figure 2, this data is for steatotic, NASH and ALD explanted liver tissue from four human donors in each category. The cellular composition of livers and relative proportions of hepatocytes change dramatically in cirrhosis. Thus genes in hepatocytes which are responsive to metabolic signalling

will be expressed in a smaller percentage of the liver as a whole, even as the expression of the individual genes increases. The raw data is now shown in the new Supplemental Table 3

As hepatic steatosis is primarily excessive lipid accumulation in hepatocytes it will be important to measure triglycerides level in the tissues instead of triglyceride secretion (Figure 3A).

We agree, the data generated in panel 3B is generated from precision cut human liver slices where we have stained and extracted neutral lipid from the tissue sample and quantified it with a spectrophotometric method. This was the most robust and reproducible methodology available to us using the small amounts of human sample that are available to us in PCLS from each donor after performing our functional/inhibitor assays. The data confirm that exposure of slices to methylamine plus VAP-1 significantly increased hepatic lipid content by 25-30% and that addition of VAP-1 enzyme inhibitor reduced this back to control levels.

It will also be important to know if there is a reduction in fatty acid beta-oxidation which is related to PPARA expression (Figure 3A and B).

As expected, an excess of lipid fuel in the context of exposing hepatocyte cells to oleic acid leads to an increase in PPARA transcription (Figure 2) as reported by others[1]. This leads to enhanced beta oxidation which can drive mitochondrial damage and cellular stress after sustained exposure and typically utilizes lipid fuels thereby reducing hepatocyte accumulation. However we have used a dose of palmitic acid significantly below that required to cause apoptosis[2] which is in keeping with our relatively modest fold induction of PPARa. This suggests we might see a small effect on beta oxidation but it is unlikely to be significantly reduced. When VAP-1 was activated by exposure of tissue slices to methylamine in the short term we also saw a small compensatory induction of PPARa. Figure 3 shows that this also led to increased lipid accumulation in tissue slices and reduced export to the supernatant. Previous studies of adipocyte function have shown that as adipocytes differentiate and store more lipid they upregulate VAP-1/SSAO. This drives conversion of carbohydrate and lipid precursors into fatty acid synthesis for storage[3]. Thus it is unlikely that PPARa induction in our setting is linked to reduced beta oxidation, and in light of post covid restrictions upon our human tissue access performing Seahorse metabolic analysis or equivalent to clearly assess mitochondrial function is beyond the remit of this study. This is something that we will investigate further in future studies.

Bromoethylamine (VAP-1 inhibitor) reduced accumulation to control levels, whilst inhibitors of other amine oxidases (MAOA and MAOB and LOX) did not reduce the uptake seen in the presence of VAP-1 plus substrate." This is not consistent with the data shown. e.g LOX inhibitor, less MOA A/B inhibitors, similarly reduced lipid accumulation. Please explain this outcome. A statistical analysis should be done comparing values with VAP-1 and LOX inhibitors, and with VAP-1 and MOA A/B. inhibitors.

Here the reviewer is referring to our data in Figure 3B where we have exposed human liver tissue slices to a combination of VAP-1 and methylamine and have also added in individual enzyme inhibitors. We have done the statistical analysis for all samples and those which came out as significantly different were indicated by the stars on panel 3B. Hence VAP-1+ methylamine induced a significant increase in lipid accumulation compared to control, this was significantly reduced when we added bromoethylamine (ie column 6 vs column 4). We also saw a significant reduction in the VAP-1+ methylamine response when we added LOX inhibitor (column 7 vs column 4). However the MAO A/B inhibitor did not significantly reduce lipid accumulation in the presence of VAP-1+methylamine. Thus the reviewer is correct and we have amended the sentences describing the LOX data in the results and discussion sections.

Figure 3C. As VAP-1 has been implicated to play a role in immune cell trafficking, which can affect lipid accumulation. It will be important to show H&E stain of the WT and KO livers, and changes in lipid transporter and key metabolic response gene expression, before HFD and after 12 weeks of diet. What is the protein expression of VAP-1 in WT liver before and after 12 weeks of HFD? We agree with the reviewer given the key role of inflammatory macrophages in particular in driving hepatic steatosis. We and others have previously published a significant amount of phenotypic data for metabolic liver disease models (HFD and MCD) in the WT and VAP-1-deficient context. In particular we have extensively characterised both the expression of VAP-1 in response to diet, and the hepatic and peripheral inflammatory response (see data and supplemental files in 10.1172/JCI73722). We have shown that WT mice have significantly higher hepatic inflammatory scores than VAP-1 deficient mice on HFD, and that dietary modification significantly increases hepatic VAP-1 mRNA expression. To avoid duplication of this data in our current study we have clarified the discussion section of the current manuscript to reiterate these findings and signposted the original VAP-1 inflammation manuscript more explicitly.

The reviewer states that **"The changes in lipid transporter and key metabolic response gene expression, is at best, associated/correlated with the pathology. They do not reveal the mechanism. In the first instance, it is unclear if VAP-1 is involved in their expression. It will be important to extend the finding from Figure 4 to include treatment with VAP-1 inhibitor. How does VAP-1 alter the expression of these genes? Please discuss about the limitations of this study.**
We agree that the human data allows us to correlate changes in gene expression with diseases that we know lead to increased circulating and hepatic VAP-1 expression. We have then used our in vitro and murine studies to begin to dissect mechanisms by which elevated VAP-1 activity could alter lipid homeostasis. We agree it would be key to test the effects of VAP-1 inhibition on gene expression in the human ex vivo system as this is a limitation of the current data. We are unable to perform these experiments currently due to restrictions on human sample access relating to covid. However, we have discussed the limitations in the revised manuscript. We have also included a new schematic diagram to clarify the mechanisms by which VAP-1 may influence the pathogenesis of NAFLD (Figure 5)

Minor Figure 2A. Please include scale bar. Page 11, tryglyceride should be triglycerides
Thank you, we have corrected these errors/omissions.

Reviewer 2 : we are pleased that the reviewer stated our manuscript was very interesting with translational impact.

Editorial Office Comments : the scientific editor requested that we clarify the underlying mechanism in light of reviewers comments. They requested we improve the ARRIVE guidelines document and detail 'Author contributions'. They also requested we provide figures in PowerPoint format and update the reference list to include PMID and DOI for all references. Addition of an 'Article Highlights' section at the end of the main text was also requested.

We thank the team for the thorough review of the manuscript and associated documents and have amended the revised paperwork in light of these comments. Please do not hesitate to contact me if you require additional information

Yours sincerely

Dr Patricia Lalor
Reader in Experimental Hepatology

1 Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S, Lonardo A, Carulli N, Loria P. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. J Gastroenterol Hepatol. 2009: 830 [PMID: 19207680 10.1111/j.1440-1746.2008.05733.x: 10.1111/j.1440-1746.2008.05733.x]

2 Mei S, Ni HM, Manley S, Bockus A, Kassel KM, Luyendyk JP, Copple BL, Ding WX. Differential roles of unsaturated and saturated fatty acids on autophagy and apoptosis in hepatocytes. J Pharmacol Exp Ther. 2011: 487 [PMID: 21856859 10.1124/jpet.111.184341: 10.1124/jpet.111.184341]

3 Yang H, Ralle M, Wolfgang MJ, Dhawan N, Burkhead JL, Rodriguez S, Kaplan JH, Wong GW, Haughey N, Lutsenko S. Copper-dependent amino oxidase 3 governs selection of metabolic fuels in adipocytes. PLoS Biol. 2018: e2006519 [PMID: 30199530 10.1371/journal.pbio.2006519: 10.1371/journal.pbio.2006519]