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Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 11106-review.doc).

Title: NASH a precursor for HCC development

Author: Chunmeng Jiang, Chunwen Pu, Yahui Hou, Zhe Chen, Mohammed Alanazy, Lionel Hebbard

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 11106

We thank the reviewers for their time.

Reviewer 1

Question 1. This is an interesting review on the linkage between inflammation in NAFLD and HCC development. It is reasonably well written but needs some polishing (typo's, minor English corrections, etc.).

Response: these have been corrected.

Question 2. Specific comments: -the claim that NASH is now the most important cause of HCC is not substantiated by data; this statement should be removed -it is true that HCC can arise without cirrhosis and that inflammation is the driver; however, it hardly ever happens without advanced fibrosis, which is an indicator of long-standing inflammation; this should be mentioned

Response: We have toned down these comments. On pages 4 (last three lines) and 5 (first line) "Importantly, work has illustrated in comparison to diabetes and HCV that NAFLD/NASH is now a growing underlying etiological risk for HCC (15). Moreover, research has shown that HCC is now occuring more frequently from non-cirrhotic NASH (16, 17)(and reviewed in (18)).

With regards to the statement that HCC can occur in the absence of fibrosis I wish to bring the following published reports to the reviewers attention.

Firstly, we agree that cirrhosis precedes HCC in most cases. However, recent data has shown that the number of cases in which HCC develops in the absence of cirrhosis is understated. The evidence is as follows: (i) A US review of 804 patients with HCC illustrated a lack of cirrhosis in 42.6% of the cases (Nzeako et al., Am J Clin Path 1996;105:65-75); and (ii) In HCV linked HCC a systematic review of 19 studies has shown that 6.7 to 50.1% of all cases, were reported to occur in the absence of cirrhosis (Madhoun et al., Am J Med Sci 2010;339:169-73). Moreover, a growing weight of evidence has been published showing a significant association between NAFLD and non-cirrhotic HCC (Guzmann et al., Arch Pathol Lab Med 2008;132:1761-6; Paradis et al., Hepatology 2009;49:851-9; Chagas et al Braz J Med Biol Res 2009;42:958-62; Ertle et al., In J Cancer 2010;128:2436-43). In a US based review of health care claims, it was identified that 4406 HCC cases were associated with NAFLD as the major risk factor (59%), followed by diabetes (36%) and

chronic HCV infection (22%). Of the NAFLD-associated cases only 46% were reported to have cirrhosis, while 78% of the HCC HCV cases had cirrhosis (Sanyal et al., Curr Med Res Opin 2010;26(9):2183-91). Furthermore, analysis of the US SEER Medicare database found a total of 17,895 HCC cases, of which 2863 were due to biopsy proven NAFLD. Importantly, 1031 (36%) of these cases were diagnosed in non-cirrhotic livers (Rahman et al., Hepatology 2012;56(4(Suppl)):241A). These data suggest that HCC in the absence of cirrhosis is much more prevalent than previously thought, and that cirrhosis is not strongly linked to HCC development in the context of obesity.

Question 3. Page 6: the explanation of removal of IKKb from hepatocytes vs. hepatocytes and immune cells should be improved: it is hard to understand why they lead to different phenotypes.

This section has been rewritten:

In mice, the ablation of IKKβ from hepatocytes to foster NF-κB inactivation, and subsequent treatment with the mutagen diethylnitrosamine (DEN), resulted in a greater incidence of HCC (26). To explain this phenotype it was found that on exposure to excess TNFα, prolonged c-Jun N-terminal kinase (JNK) activation was stimulated, and resulted in increased hepatocyte apoptosis with compensatory hepatocyte proliferation. These events facilitated the accumulation of genetic errors to enhance HCC growth. Accordingly, increased JNK activity can also promote insulin resistance as $JNK^{-/-}$ mice have improved insulin sensitivity and less hepatic inflammation and fibrosis (27, 28). Alternatively, the removal of IKKβ from hepatocytes and Kupffer cells reduced the number and size of HCCs, after DEN treatment (26). Mechanistically, it was found that the proliferation of hepatocytes after exposure to DEN, were dependent on the IKKβ induced production of the hepatomitogens TNFα, IL-6 and HGF from Kupffer cells.

Further studies showed that the deletion of NEMO from hepatocytes, which modulates the phosphorylation and degradation of IκBs in the TNFα pathway, spontaneously promoted HCC development. It was found that NEMO absence completely blocked NF-κB activation and sensitized the NEMO-null hepatocytes to lipopolysaccharide (LPS) treatment, suggesting the involvement of the innate immune response and microbiome in HCC development (29).

To convey the importance of obesity in driving steatosis and inflammation, it has been shown that TNF-α and IL-6 are important in obesity driven HCC. Here liver cancer was promoted by DEN treatment of IL-6 or TNFR1 null mice and subsequent high-fat feeding. It was observed that compared to wild-type controls that IL-6 or TNFR1 null mice had significantly reduced steatosis, HCC number and size (30). Collectively, these studies show the key role of the TNFα pathway in hepatic inflammation, and suggest that imbalances in this pathway are important in the transition from NASH to HCC.

Question 4. Adiponectin: here, the authors should look in more detail on the effect of adiponectin in hepatocytes vs. inflammatory cells in relation to NF-kB activation

Response: the dominant anti-inflammatory effect of adiponectin is on the hepatic inflammatory cells. We have written this paragraph from page 9-10 and included new references.

Given the different adiponectin forms and receptors, adiponectin has a multitude of activities. In the liver adiponectin activates AMPK to reduce hepatic gluconeogenesis, stimulate fatty acid oxidation, and limit hepatic *de novo* lipogenesis through inhibition of SREBP-1c, a dominant regulator of triglyceride and fatty acid synthesis (48, 49). Adiponectin can also activate PPARα to promote fatty acid oxidation. Importantly, in the context of liver diseases adiponectin can limit inflammation by inhibiting the nuclear factor (NF-kB) activation to suppress TNFα release (50, 51). Adiponectin can also further suppress macrophage function and the proliferation and migration of vascular smooth muscle cells (52).

Reviewer 2

Dear Author This is very interesting paper. The pathogenesis of NASH remains unclear. Several observations have suggested that small intestinal bacterial overgrowth (SIBO) may play a role in NASH. I think gut bacteria contribute to the pathogenesis of NAFLD by increasing gut luminal ethanol production, metabolizing dietary choline and through production of LPS, which may activate proinflammatory cytokines in luminal epithelial cells, liver macrophages, or both. DCA,which is known to induce DNA damage, enhance HCC growth and progression.

I ask question.

Question 1. Please explain the etiology of SIBO in NASH.

Response: I have not been asked to incorporate this point in the text, thus I will give just a brief comment.

The causes of SIBO are diverse and complex. The digestion of alcohol will impair the intestinal epithelial barrier and promote colonic dysbiosis. Alterations in diet towards high sugar consumption could also promote SIBO. Importantly, fructose has been shown to significantly elevate triacylglycerol and the hepatic expression of TLR4 1-4 and 6-8 (Wagnerberger et al., Br J Nutr 2012;107:1727-38). The relationship between the gut flora and obesity in mice is strong, whilst germ free mice resist the development of obesity when fed a high-fat, high sugar diet (Backhed et al., PNAS 2007; 101:979-84). In patients the data is controversial. In one study, a higher prevalence of SIBO (50%) was found in NASH patients (Wigg et al., Gut 2001;48:206-11). However, in another SIBO was not associated with NASH (Sabate et al., Obes Surg 2008;18:371-377). Thus, despite the findings in small rodents, additional studies are needed to support a role of SIBO in NASH patients. See the recent review by Imajo et al., (Semin Immunopathol 2014; 36:115-32) for further information.

Question 2. Please tell me what kinds of bile acids could be a deciding step in the progression from NASH to HCC.

Response: I have not been asked to incorporate this point in the text, and it is very speculative, thus I will give just a brief comment.

As explained in the manuscript, the bile acid with a well described experimental role in HCC progression is the secondary bile acid DCA. Here, high levels of DCA in conjunction with a mutagen were able to promote HCC growth (Yoshimoto et al.). They found that that DCA can promote the activation of a senescence–associated secretory phenotype in hepatic stellate cells, reflected by the secretion of IL-1β.

Alternatively, as bile acids suppress inflammation, in particular FXR ligands, it is plausible that reduced levels of bile acids could promote dysbiosis, leading to increased hepatic inflammation and ultimately HCC. Mouse models have shown that FXR null mice are more susceptible to HCC, suggesting that reduced levels of the FXR binding bile acids maybe a promoter of HCC.

Additionally, TGR5 ligands can also inhibit inflammation, but a direct role for this receptor has not yet been published in mouse models.

Question 3. I think the use of FXR agonist help control SIBO. Please explain much more detail how to use FXR agonist to limit hepatic inflammation and NASH progression.

Response: I have rewritten this section and included two more references. The rewritten passage is listed below.

FXR regulates bile acid synthesis by inhibiting the transcription of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the conversion of cholesterol to bile acids. FXR can also repress the sterol-regulatory element binding protein-1c (SREBP-1c) transcription to reduce triglyceride synthesis, and promote the β-oxidation of fatty acids through augmented peroxisome proliferator-activated receptor α (PPARα) signalling (82). In agreement FXR null mice have elevated serum triglycerides and cholesterol and are prone to develop steatohepatitis (83). Moreover, FXR agonists can antagonize NF-κB activity and limit hepatic inflammation *in* vivo (84). In this light, studies have illustrated the potential of FXR agonists to treat NASH. In the mouse MCD model, treatment with WAY-362450, reduced liver injury and inflammation (85). In a recent phase II clinical trial a study was undertaken to evaluate the effects of Obeticholic acid (OCA; INT-747, 6α-ethyl-chenodeoxycholic acid) on insulin sensitivity in patients with nonalcoholic fatty liver disease and type 2 diabetes mellitus. It was found that within 6 weeks OCA increased insulin sensitivity and reduced the markers of liver inflammation treatment and fibrosis (86). Given FXR's important role in liver function, it has also been shown that FXR null mice can spontaneously develop liver tumors as they age, and treatment with CA further potentiated DEN-initiated liver cancer.

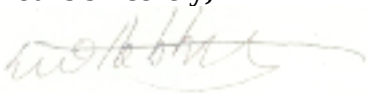
Reviewer 3

Response: Many thanks for your suggestions. As a native English speaker I have taken on the following of your corrections.

Page 3 last line: removed "they"
Page 4: corrected all suggested changes
Page 5 2nd line: Inserted "Taken" before together.
Page 6 line 6: corrected to "leading to its degradation and the subsequent movement of NF-κB into the cell nucleus"
Page 7 8th last line: replaced expression with "protein levels"
Page 8: space removed
Page 9: changed multitude to "plethora"
Page 10: changed sum to "summary"
Page 10: comma removed
Page 10: sentence rewritten "Additionally, evidence also suggested that the accumulation of hepatic cellular fat could promote the release of reactive oxygen species to interfere with cellular functions such as cellular respiration to cause the release of toxic lipids species, to result in hepatocyte dysfunction and apoptosis (as reviewed by us (65))."
Page 11: sentence "This we will now consider" removed
Page 11: Probiotics spelling corrected.
Page 12: by replaced with "for"
Page 13: inserted evidence between " above" and "suggests"

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology.*

Yours sincerely,



Dr Lionel Hebbard.