ANSWERING REVIEWERS

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World Journal of Gastroenterology

Jin-Lei Wang

Director, Editorial Office

Yuan Qi

Science Editor, Editorial Office

Dear Dr. Jin-Lei Wang and Yuan Qi,

Thank you very much for giving us the opportunity to resubmit our manuscript.

We are enclosing the full version of our revised manuscript in Word format (file name: ESPS Manuscript No. 6063-review.docx), entitled: "**Hierarchical and selective roles of galectins in hepatocarcinogenesis, liver fibrosis and inflammation**" co-authored by *María L. Bacigalupo, Malena Manzi, Gabriel A. Rabinovich and María F. Troncoso* to be considered for publication in World Journal of Gastroenterology.

We would like to thank the reviewers for their thorough revision of our manuscript, for their detailed advice and for bringing to our attention important points to be considered in our analysis that will undoubtedly improve our manuscript.

We made our best to appropriately address all the reviewers' concerns and resubmit the modified manuscript. We are sending the revised manuscript with changes underlined.

A point-by-point to the reviewers' and editor's suggestions is included in the following pages.

Briefly, in the modified manuscript we revised the gene and protein nomenclature, we clarified some paragraphs in the "Galectins" section and we prepared three tables to abbreviate our manuscript.

Hoping that you will find this manuscript suitable for publication in World Journal of Gastroenterology, we thank you very much for your consideration.

Kind regards,

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Comments from Reviewer Nº 02444965:

Comment: "In the present review authors summarized current knowledge on the involvement of galectins on liver diseases. The review is very detailed, well-organized and presented critically. Its publication is appropriate as it puts together a good resource for current knowledge in the implication of the galectins, especially Galectins-1, -3, -4, -8 and -9, in the pathogenesis of liver cancer.

The review is clearly written and I have only a minor comment on gene nomenclature. As indicated by HNGC Guidelines, human gene names should be written in capitalized italic characters and mouse genes in italics with capitalized first character and human proteins should be written in capitalized characters meanwhile only the first letter is capitalized in mouse proteins. I have found that the nomenclature used is confusing. As an example:

- Gal1 gene (*Lgals1)*, Page 8, 2nd paragraph. According to HNGC Guidelines it should be a mouse gene, but the 2 paragraphs following this shows results with human HCC. If referring to the human gene it should be substituted with *LGALS1.*

Similarly to this, nomenclature should be revised all along the manuscript. In a non-exhaustive search I have found the following:

- *Lgals1* gene, several paragraphs in page 9.
- *Lgals3* gene, several paragraphs in page 16.
- *Lgals3* promoter, several paragraphs in page 16.
- Gal4 gene (*Lgals4*), page 23.
- Gal8 gene (*Lgals8*), page 24.

I did not check deeply for protein nomenclature errors, but authors should do an exhaustive revision on the manuscript looking for them."

We checked thoroughly the manuscript for gene and protein nomenclature mistakes:

- Human and mouse gene names were revised. Changes are underlined in pages 6, 7, 12, 17, 18 of the revised version.

- As along our manuscript, we generalize or discuss the varied roles of human galectins, but we also describe results obtained from animal experimental models, we decided not to abbreviate the term "galectin" when referring to the protein, to avoid misunderstanding. Changes are underlined throughout the revised manuscript.

Comments from Reviewer N° 02444872:

Comment: "Cancer of liver is a worldwide disease as cancer itself. Cancer is a very heterogeneous disease involving many risk factors and multiple biochemical and signaling pathways. A lots of research efforts have been invested globally so far and still needs so on. Present review article focusses on the potential role and pathways of galectins proteins in the pathogenesis of hepatocarcinoma towards an attempt to target galectins in course of hepatocarcinoma treatment. In my opinion, authors have done a very good work in gathering and presenting critical recent findings related to up regulation of galectins-1, -3 and -4 type-, and down regulation of –lectins galectins-8 and -9 type- in pathogenesis of liver cancer. This article significantly add to the understanding of galectins involvement in development and progression of liver cancer.
Introduction section needs following two clarification."

Comment: "What are the major cell types that synthesize galectins and secrete, though, through a poorly understood pathways?"

We answered this question describing the cell types or tissues that synthesize galectins and, giving some examples of cell types that secrete them.

In the "**GALECTINS**" section, page 4, line 20, the following paragraph was added: "Some galectins (*e.g.* galectin-1, galectin-3 and galectin-9) are widely expressed among different tissues including, immune cells, endothelial and epithelial cells, and sensory neurons (reviewed by [27-29]); whereas other family members have a more restricted tissue localization and compartmentalization (*e.g.* galectin-7 is preferentially found in the skin,

galectin-12 is abundantly expressed in adipose tissue, galectin-5 is restricted to rat reticulocytes, and galectin-10 is strongly represented in human but not mouse eosinophils)[27]."

Also, in the "**GALECTINS**" section, page 4, line 28, we introduced the following paragraph: "For instance, non-clasical secretion of galectin-1 has been observed in skeletal muscle during *in vivo* development and in cultured myoblasts during differentiation[33]. Besides, secretion of galectin-3 from macrophages and renal and polarized intestinal epithelial cells has been detected[34, 35]. There is also evidence for secretion of galectin-9 in activated Jurkat T cells[36] and CD4 T cells expressing galectin-9 on the cell surface upon T cell receptor stimulation[37]."

Thus, we incorporated the corresponding references.

Comment: "Are these proteins endocytosed in target cells, the hepatocytes, after binding with cell surface glycoconjugates and then transmit their signals?"

To answer this concern, we incorporated to the discussion another article (Reference 41 in the revised version) which describes the endocytosis of galectin-1 by Jurkat T cells and, a finding of our group that demonstrated that this galectin is internalized by hepatocellular carcinoma cells (Reference 85 in the revised version).

In the "**GALECTINS**" section, page 4, line 35, we introduced the following paragraph: "Remarkably, it has also been demonstrated that galectin-1 can be internalized by Jurkat T cells in a carbohydrate-dependent mechanism, following dual pathways involving clathrin-coated vesicles and raft-dependent endocytosis[41]."

Accordingly, we incorporated the corresponding reference.

Besides, in the "***Galectin-1 in HCC and in inflammation-associated liver injury***" section, page 8, line 6, the following sentence was added: "and remarkably, we also found that exogenously added recombinant galectin-1 was internalized by HepG2 cells[85]".

Comment: "What extent galectins are associated with carcinomas other than hepatomas? A brief paragraph needed here summarizing all galectins although specific discussion provided in respective segments."

To address this concern, in the "**GALECTINS**" section, page 5, line 9, the following underlined sentence was added: "Galectins are often aberrantly expressed in ~~tumor cells~~ many different tumor types including astrocytoma, melanoma and prostate, thyroid, colon, head and neck, bladder, kidney, stomach, lung, bladder, uterine, breast and ovary carcinomas[27, 47, 48]." Moreover, mounting evidence indicates that these proteins play fundamental roles in cancer biology including tumor transformation, tumor growth, angiogenesis, migration, metastasis and tumor-immune escape[49-52]. Given these pleiotropic activities in the tumor microenvironment, galectins are being increasingly recognized as molecular targets for innovative cancer therapy[26, 52-56].

Comment: "The author could better have shortened the paper had the prepared a table making comparisons on the role of various galectins in the development of hepatomas and other critical findings with references."

As the reviewer suggested, we prepared tables that allow comparison of the role of the different members of the galectin family in the pathogenesis of hepatocellular carcinomas (Table 1), in inflammation-associated liver injury (Table 2) and fibrosis-related liver pathologies (Table 3).

Table 1 was prepared by summarizing the results from relevant articles that highlight the involvement of galectins in hepatocellular carcinoma, with the corresponding references.

The following paragraphs were modified. Some sentences were deleted from the manuscript text (crossed out, underlined); other sentences were added (underlined). The

information was abbreviated and incorporated in Table 2 with the corresponding references:

- Page 9, line 3: "Interestingly, it has been demonstrated that ~~Gal1~~galectin-1 exerts a protective role on Con A-induced autoimmune hepatitis in mice (Table 2)[89]. ~~Gal1 pretreatment prevented both liver injury and T-helper cell liver infiltration induced by Con A. *In vivo* and *in vitro* experiments indicated that the protective effects of Gal1 involved apoptosis of Con A-activated T cells due to carbohydrate-dependent lectin-receptor interactions. In addition, Gal1 almost completely suppressed plasma levels of TNF and IFN-γ induced by Con A[89].~~"

- Page 9, line 14: "~~In these mice, Gal1 was manly localized at the cytoplasm of non-hepatocyte cells including immune cells and cholangiocytes and rarely, at the cytoplasm of hepatocytes.~~ To highlight the relevance of the endogenous protein ~~Gal1~~, ~~Gal1~~galectin-1-knockout (KO)/B6 mice were used in the context of Con A-induced autoimmune hepatitis. ~~While Gal1 transcript was up-regulated following Con A injection in both strains, only B6 Gal1-KO mice presented an increased sensitivity to Con A-induced hepatitis.~~ The results demonstrated that endogenous ~~Gal1~~galectin-1 selectively protects against Con A-induced liver injury in B6 mice (Table 2)[91]."

- Page 15, line 19: "~~Nomoto *et al.* 2006 reported that Gal-3 null mutant mice develop NAFLD/NASH spontaneously with aging, which eventually results in dysplastic and neoplastic liver nodules~~. On one hand, it has been demonstrated that in choline-deficient L-amino-acid (CDAA) diet-induced NAFLD/NASH hepatic injury was more severe in ~~Gal3~~galectin-3 KO mice, as compared to wild type mice[142]. ~~Also using gene microarrays, the authors showed that Gal3 deficiency caused alterations in the expression of various genes associated with carcinogenesis and lipid metabolism. As a possible mechanism responsible of promoting hepatocellular damage under Gal3 deficiency the authors proposed an increase in lipopolysaccharide-mediated signaling, an effect that is involved in the pathogenesis of NASH/NAFLD and is negatively regulated by Gal3[143].~~"

- Page 15, line 31: "~~NASH attenuation was associated with inhibition of HSC-driven fibrosis, reduction of inflammatory-cell infiltration, pro-inflammatory pattern of cytokines and transcription factors and endoplasmic reticulum stress and hepatocyte apoptosis. Both Th1/M1 and Th2/M2 inflammatory responses, which drive hepatocyte damage and fibrosis, respectively, were also down-regulated in Gal3-deficient animals.~~"

- Page 16, line 10: "~~In normal liver samples, staining for RAGE was observed in hepatocytes and bile ducts, whereas Gal3 expression was negative in hepatocyes but positive in bile ducts. Both receptors were negative in Kupffer cells. However, in samples from patients with hepatic dysfunction, the authors found marked amounts of RAGE in hepatocytes and bile ducts independently of diagnosis. Moreover, strong staining for Gal3 in hepatocytes was observed only during the course of cirrhosis where this galectin was highly expressed in Kupffer cells. Thus,~~ They observed that when liver function is impaired and AGE levels rise, over-expression of ~~Gal3~~galectin-3 appears to contribute to tissue protection (Table 2)[145]. ~~In line with these findings, in the murine NASH model induced by an atherogenic diet, Iacobini *et al.* demonstrated that Gal3 is a major scavenger receptor involved in ALE/AGE uptake by the liver. Also, marked reductions of ALE/AGE accumulation within the liver and, increased serum levels of these compounds were observed, pointing to a decreased ALE/AGE clearance.~~"

- Page 16, line 30: "~~Also, in Gal3 deficient mice the number of pro-inflammatory M1-type liver macrophages was significantly decreased following APAP administration. Moreover, expression of the classical macrophage activation markers iNOS, TNF, and IL-12, and pro-inflammatory chemokines and chemokine receptors were markedly reduced~~[148]."

- Page 17, line 5: "~~T lymphocytes (both $CD4^+$ and $CD8^+$), B lymphocytes, dendritic cells, NK and NKT cells~~[149]. Moreover, pretreatment of wild type mice with a selective inhibitor of ~~Gal3~~galectin-3 (TD139) attenuated Con A–induced liver injury and reduced the number of $CD4^+$ and $CD8^+$ T cells (Table 2)[149]. ~~Also, this inhibitor favored the influx of IL-10-producing $CD4^+$ T cells in the liver and the alternative activation of macrophages, decreased serum levels of IFN-γ, IL-17 and IL-4, while increased the amounts of IL-10 in~~

~~Con A-treated animals. In addition, deletion of Gal3 enhanced the apoptosis of mononuclear cells[149].~~"

- Page 21, line 29: "In this context, blockade of the TIM-3/galectin-9 pathway exacerbated local inflammation and liver damage (Table 2)[190]. ~~using an anti-TIM-3 antibody, an increased hepatocellular damage, local neutrophil infiltration, T cell and macrophage accumulation and liver cell apoptosis was observed. Moreover, blockade of the TIM-3/Gal9 pathway using an anti-TIM-3 or anti-Gal9 mAb, led to increased IFN-γ production by ConA-stimulated spleen T cells and augmented TNF and IL-6 production by ConA-stimulated macrophages/T cells[190].~~"

- Page 22, line 5: "On the contrary, biochemical and histopathological data indicated that a single injection of ~~Gal9~~galectin-9 was sufficient to protect mice against Con A-induced induced hepatitis (Table 2)[191]. ~~The authors demonstrated that the protective effects of Gal9 involved selective elimination of activated CD4$^+$ effector T cells as well as prevention of the synthesis and/or release of proinflammatory cytokines[191].~~"

- Page 22, line 22: "It has been demonstrated that galectin-9 limits the inflammatory response in a mouse model of diet-induced nonalcoholic fatty liver disease (NAFLD) (Table 2)[195]. ~~In a mouse model of diet-induced nonalcoholic fatty liver disease (NAFLD), Tang *et al.* demonstrated that Gal9 induces apoptosis of NKT cells, thus limiting the inflammatory response, although it induces the production of IL-15 by Kupffer cells[195]. IL-15, in turn, induces the proliferation of NKT cells.~~" –

The following paragraphs were modified. Some sentences were deleted from the manuscript text (crossed out, underlined); other sentences were added (underlined). The information was abbreviated and incorporated in Table 3 with the corresponding references:

-Page 10, line 1: "~~. They also observed up-regulation of Gal1 in fibrotic endothelial and Kupffer cells, although at a lower extent than in stellate cells, whereas no expression of this lectin was detected in hepatocytes~~[93]. When the biological role of ~~Gal1~~galectin-1 was investigated in HSCs, it was found that this lectin stimulated the proliferation rate and migratory activity of cultured HSCs through carbohydrate-dependent mechanisms (Table 3)~~via an ERK1/2 signaling pathway, although protein kinases C (PKC) and A (PKA) were not implicated~~[94]. ~~Treatment with thiodigalactoside, a potent galectin inhibitor, significantly decreased the ability of Gal1 to activate phosphorylation of ERK1/2, suggesting that this effect proceeded through carbohydrate-dependent mechanisms. In addition, these authors confirmed that Gal1 enhanced the migratory activity in HSCs."~~

-Page 10, line 17: "~~In contrast, endothelial cells of the portal vein and hepatic arterial branches and nerve bundles in the portal tracts were positive for Gal1. Moreover, staining for this galectin in connective tissue in portal tracts and parenchyma was weakly positive.~~ Remarkably, 73 % of the intrahepatic cholangiocarcinoma (ICC) samples analyzed were positive for ~~Gal1~~galectin-1[96]. Expression of this lectin significantly correlated with histologic dedifferentiation of ICC, vascular invasion, and lymph node metastasis of ICC. ~~Besides, Gal1 was also strongly expressed in the cancerous stroma of ICC. The cholangiocarcinoma cell line, CCKS1, was also found to express Gal1 in intracellular and extracellular compartments~~[96]. These results suggest that ~~Gal1~~galectin-1 over-expression in ICC cells is associated with neoplastic progression and tumor cell proliferation (Table 3)."

-Page 13, line 32: "*In vitro* experiments and different experimental models of liver injury and fibrosis demonstrated that galectin-3 stimulated the proliferation rate of cultured activated HSCs and is also involved in myofibroblast activation, identifying galectin-3 as a potential therapeutic target in the treatment of liver fibrosis (Table 3)[94, 132-134]. ~~Gal3 stimulated the proliferation rate of cultured HSCs via MEK1/2-ERK1/2 signaling pathway, involving PKC and PKA pathways. The effect of this lectin on proliferation was dependent on its carbohydrate-binding properties. Interestingly, Henderson *et al.* showed that TGF-β, a major pro-fibrogenic cytokine, requires intracellular Gal3 to stimulate~~

~~myofibroblast activation and pro-collagen production independent of Smad-2 and Smad-3[132]. Moreover, siRNA-mediated silencing of Gal3 *in vivo* using knockdown technologies reduced myofibroblast activation in a rat model of reversible carbon tetrachloride (CCl_4) induced liver fibrosis. Using the same animal model, Yamazaki *et al.* demonstrated that Gal3 expression is aberrantly induced in the cytoplasm of periportal hepatocytes of adult rat liver[133]. Accordingly, Jiang *et al.* observed that Gal3 was up-regulated in HSC isolated from rats with bile duct ligation (BDL), another experimental model of liver injury and fibrosis[134]. Induction of Gal3, mediated by NF-κB, was required for elimination of apoptotic cells by HSCs. This process was integrin-dependent and involved secretion of this chimera-type lectin. Thus both autocrine and paracrine mechanisms of Gal3 stimulation may underlie HSC activation during liver fibrosis. These results identify Gal3 as a potential therapeutic target in the treatment of liver fibrosis."~~

-Page 14, line 16: "In patients with liver cirrhosis galectin-3 is not extracted by the liver[136], and also, its expression is induced in hepatocytes of cirrhotic liver[124, 136]. ~~In liver healthy controls, concentrations of Gal3 were found to be significantly higher in portal compared to hepatic vein suggesting that the liver can remove this protein from the circulation[137]. On the contrary, in patients with liver cirrhosis Gal3 levels were found to be similar or even higher in the hepatic vein compared to portal vein blood, indicating that Gal3 is not extracted by the cirrhotic liver. Also, in healthy liver, Gal3 was not found to be expressed in hepatocytes, but its expression was induced in hepatocytes of cirrhotic liver[124, 136].~~ Furthermore, ~~Gal3~~galectin-3 was negatively associated with liver function in patients with alcoholic liver cirrhosis, an effect which might be partly explained by the impaired hepatic removal and/or by higher hepatic synthesis of ~~Gal3~~ galectin-3 (Table 3)[136]."

-Page 14, line 27: "Shimonishi *et al.* examined ~~Gal3~~galectin-3 expression pattern in intrahepatic cholangiocarcinoma (ICC), and found that 93 % of the ICC samples analyzed were positive for ~~Gal3~~ this lectin[96]. ~~Gal3 was constitutively but weakly expressed in normal intrahepatic bile ducts and hyperplastic intrahepatic bile ducts, while its expression was strong in biliary dysplasia. Furthermore, 93 % of the ICC samples analyzed were positive for Gal3.~~ The expression was more intense in well-differentiated ICC, and

was significantly decreased in dedifferentiated areas or poorly differentiated ICCs, indicating ~~The authors concluded~~ that ~~Gal3~~galectin-3 expression is rather related to the preneoplastic and early neoplastic stages of ICC, and tends to disappear at later stages of ICC (Table 3)[96, 137]."

-Page 15, line 2: "~~Besides, silencing of Gal3 expression in two human cholangiocarcinoma cell lines using siRNA strategies significantly increased cell migration and invasion without altering cellular proliferation[137]. In subsequent studies, the authors demonstrated that.~~ Also, it has been demonstrated that ~~Gal3~~galectin-3 played a role in apoptosis and response to chemotherapy in cholangiocarcinoma cell lines (Table 3)[138]. ~~Gal3 expression levels correlated positively with its anti-apoptotic activity, and to resistance to chemotherapeutic agents as substantiated by findings showing that reduction of Gal3 enhances the sensitivity of antitumor drugs[138].~~"

By doing this, our manuscript has been shortened.

Additionally, we altered the numbering of references.

Editor's suggestions:

Comment: "Could you give me the word version document about your article ? so that I can edit them easily."

We have up-loaded our revised manuscript as a Word version document.

Comment: "For manuscripts submitted by non-native speakers of English, please provided language certificate by professional English language editing companies mentioned in`The Revision Policies of BPG for Topic Highlights'."

We have read the section about **Manuscripts submitted by non-native speakers of English** included in the **BPG's Revision Policies for Topic Highlight**. Nevertheless, according to what is declared in the **Note** paragraph, we prefer not to make use of a professional English language editing company. We are confident that our manuscript will reach Grade A in the language evaluation.

The corresponding author signs as guarantee.

María Fernanda Troncoso