Jin-Lei Wang
Director, Editorial Office
World Journal of Hepatology

Dear Dr Wang

We would like to thank you and the reviewers for your help in considerably improving our manuscript "Serum biomarkers and risk of hepatocellular carcinoma recurrence after liver transplantation", manuscript number 42566.

We have revised the manuscript according to your comments and the reviewer`s comments and we hope this version will be found suitable for publication in *World Journal of Hepatology*.

In the updated version of the manuscript, changes have been made according the CrossCheck report, as you suggested. We have carefully revised all the reviewer`s comments, and point by point detailed resposes to reviewers are detailed below. We have also updated the manuscript according to the Guidelines and Requirements for Manuscript Revision and have used language editing services. Finally, in addition of the updated version of the manuscript, we have submitted an audio core tip, the Copyright License Agreement and the Non-Native Speakers of English Certificate.

Sincerely

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RESPONSES TO THE REVIEWERS´ COMMENTS

Reviewer 02530754

Thank you very much for your valuable comments that have improved the manuscript quality. The manuscript has been updated according your comments and below you can find the detailed changes in response to your comments.

The present manuscript by María José Citores et al. is a comprehensive review on the current state and potential utility of serum biomarkers of HCC in candidates for liver transplantation. The topic is of high interest given the inaccuracy of tumor burden, as assessed by dynamic imaging techniques, to predict tumor recurrence after liver transplantation. Not invasive surrogate markers of tumor biology, either alone or in combination with existing radiological criteria, may provide a more rational approach to select candidates for liver transplantation and to prioritize them within the waiting list. The authors are kindly invited to consider the following comments:

- In the introduction it can be read: "preoperative biopsy… is not currently recommended for HCC evaluation because of the risk of needle tract tumor seeding". While such risk exists, tumor biopsy is still needed in patients with atypical radiological features. I agree that liver biopsy is not systematically needed, but it is still recommended in doubtful cases (and this trend will probably increase in the next years). Consider rephrasing.

- Regarding the above referred paragraph: Microvascular invasion cannot be assessed or predicted by using a needle biopsy. The whole liver specimen (either resected or explanted) is needed for such evaluation. An unequivocal statement is needed.

Considering both recommendations above, the paragraph regarding liver biopsy has been changed as follows:

"…However, preoperative biopsy often underestimates poorly differentiated tumors and does not accurately predict microvascular invasion, when

compared with the final specimen examination after liver resection or LT[11-12]. Due to these limitations and because of the risk of needle tract tumor seeding, preoperative biopsy is not currently recommended for routine HCC evaluation; although, it is still needed in patients with atypical radiological features and in doubtful cases".

- Regarding systemic inflammatory markers, several meta-analyses of observational studies are quoted. It is fair to state in the manuscript that these studies are based in very low quality evidence and that they are limited by a high risk of publication bias. Indeed, those studies evaluating inflammatory markers with positive results are more likely to be published as compared with studies describing negative findings. In addition, abstracts presented in congresses but not published in full, which are more likely to report negative findings, are systematically not considered. In addition the referred meta-analyses do not provide a valid assessment of risk of publication bias (funnel plots or similar). The authors should therefore refer to these meta-analyses and derived results with great caution.

We agree with these comments, and especially with regard to PLR. We have added some comments as follows:

- Page 12, line 6: "However, this association must be taken in consideration with great caution since a moderate level of heterogeneity was found among the studies included".
- In section "Limitations of pretransplant serum biomarkers", last pratagraph: …"Albeit the serum markers reviewed here are potential markers to be included in patients selection for LT, their utility is limited and they cannot be universally applied in all patients. Although AFP is considered the most useful pretransplant marker of HCC recurrence after LT, its utility is restricted by the existence of non-AFP secreting HCC. More restricted is the utility of systemic inflammatory markers because of different reasons. More restricted is the utility of systemic inflammatory markers, for different reasons. Although some metaanalyses have

suggested NLR[83] and PLR[84] as useful pretransplant biomarkers for HCC recurrence, they are based on very few retrospective studies (four and five studies respectively), with most having a small sample size….

- Aligning with the comment above, the main barrier for implementation of inflammatory markers to select candidates for LT or to prioritize them within the waiting list is their lack of specificity. As the authors acknowledge thereafter, these markers may be increased in other situations such as infections, which are frequent in patients with end stage liver disease. This limitation should be further highlighted in the manuscript.

And after the sentences included above, we have highlighted this limitation as follows:

"…However the most important limitation may be that these inflammatory serum biomarkers can be affected by other conditions, such an acute infection, hematologic disorders, hypersplenism, gastrointestinal tract bleeding or systemic inflammatory diseases, which are frequent in patients with end-stage liver diseases".

- In page 7, last line, the authors quoted a statement from the EASL-EORTC guidelines where AFP was considered a suboptimal marker but as far as I know this pertains to HCC screening only, whereas the authors referred to the whole HCC routine clinical practice. Please revise.

Thank you for your comment. You are right that the EASL-EORTC recommendation refers to HCC screening and surveillance and not to the whole clinical practice. So, the sentence has been corrected, and we indicate "*routine screening of early HCC"* instead of "*routine clinical practice*"

- AFP-L3 has been invoked as more specific and may be particularly useful in patients with increased tumor burden but with normal or mildly increased conventional AFP. Please comment.

The main scope of this review was to review the most widely analyzed serum biomarkers with potential utility at present to improve the performance of Milan criteria for predicting HC C recurrence after LT. Following your recommendations, we also think it is worth mentioning some of them such as AFP-L3% or cell free DNA and miRNAs , among others, as you also mentioned below. We have added a new section entitled "Other potential serum biomarkers", before the conclusion section, including some of these new biomarkers as follows:

"OTHER POTENTIAL SERUM BIOMARKERS

In addition to the serum biomarkers reviewed here, some other markers have been proposed as potential risk factors for HCC recurrence after LT.

AFP-L3%, which represents a serum AFP fraction reactive with lens culinaris agglutinin, has been associated with HCC diagnosis[102,103]. In the LT context, an AFP-L3% level >50 ng/ml combined with Milan criteria improved HCC recurrence prediction, when compared with Milan criteria alone[71]. Interestingly, AFP-L3% has been suggested as a highly specific marker of HCC in patients with low AFP level[103], which could overcome the limitation of AFP usefulness as a biomarker of HCC recurrence in patients with AFP-negative HCC. However, more studies are needed for this promising biomarker.

Liquid biopsy has attracted much attention as a feasible and not noninvasive tool to identify tumoral markers in peripheral blood for diagnosis, monitoring and prognosis of cancer overcoming tissue biopsy limitations. Circulating tumoral cells and tumoral cell free nucleic acids in peripheral blood could be advisory of micrometastasis, and their utility has been explored in HCC diagnosis and prognosis[104]. Very few data is available about the potential role of these circulating tumoral components as preoperative predictors of HCC recurrence after LT, and it is still a controversial issue. Although circulating HCC cells have been detected before LT, they have not been associated with HCC recurrence after LT[105]. Regarding circulating nucleic acids, AFP mRNA

expression in peripheral blood has been suggested as a surrogate of circulating tumoral cells and has been associated with an increased risk of HCC recurrence after LT[106]. However, their utility is controversial and some authors consider AFP mRNA to be nonspecific for HCC micrometastases.

Some other circulating RNA have been explored, but none of them has been widely recognized as valuable marker of HCC recurrence, probably because none of them are specific for HCC[104]. Circulating tumor DNA has been isolated in patients with HCC, and has been associated with microvascular invasion[107]. However, much effort is still needed in order to consider these circulating tumor components as valuable markers in clinical practice since some limitations still need to be overcome. Although the complex methodology to isolate these tumoral components has improved dramatically, their extremely low frequencies in peripheral blood require more sensitive and cost effective techniques. Also, HCC-specific biomarkers should be validated and evidence of their association with HCC recurrence after LT should be proven.

Finally, different micro (mi)RNA signatures in liver tissue have been associated with HCC recurrence after LT[108,109]. However, the necessity of liver tissue samples limits their application preoperatively, and circulating miRNAs are at present being explored. Several circulating miRNA have been suggested as potential biomarkers for HCC diagnosis[110], vascular invasion and prognosis[111-112]. To date, to the best of our knowledge, there is no data about the association of miRNAs with HCC recurrence after LT, and future studies are warranted in order to explore the utility of these promising biomarkers in preoperative prediction of HCC recurrence after LT".

And we have included these new references:

102 Taketa K, Endo Y, Sekiya C, Tanikawa K, Koji T, Taga H, Satomura S, Matsuura S, Kawai T, Hirai H. A collaborative study for the evaluation of lectin-reactive alpha-fetoproteins in early detection of hepatocellular carcinoma. *Cancer Res* 1993; **53:** 5419-5423 [PMID: 7693340]

103 Kuromatsu R, Tanaka M, Tanikawa K. Serum alpha-fetoprotein and lens culinaris agglutinin-reactive fraction of alpha-fetoprotein in patients with hepatocellular carcinoma. *Liver* 1993; **13**: 177-182 [PMID: 7690873]

104 Li J, Han X, Yu X, Xu Z, Yang G, Liu B, Xiu P. Clinical applications of liquid biopsy as prognostic and predictive biomarkers in hepatocellular carcinoma:circulating tumor cells and circulating tumor DNA. *J Exp Clin Cancer Res* 2018; **37**: 213 [PMID: 30176913 DOI: 10.1186/s13046-018-0893-1]

105 Wang S, Zheng Y, Liu J, Huo F, Zhou J. Analysis of circulating tumor cells in patients with hepatocellular carcinoma recurrence following liver transplantation. *J Investig Med* 2018; **66**:1-6 [PMID: 29632031 DOI:10.1136/jim-2017-000655]

106 Marubashi S, Dono K, Nagano H, Sugita Y, Asaoka T, Hama N, Miyamoto A, Takeda Y, Umeshita K, Monden M. Detection of AFP mRNA-expressing cells in the peripheral blood for prediction of HCC recurrence after living donor liver transplantation. *Transpl Int* 2007; **20:** 576-582 [PMID: 7425725]

107 Ono A, Fujimoto A, Yamamoto Y, Akamatsu S, Hiraga N, Imamura M, Kawaoka T, Tsuge M, Abe H, Hayes CN, Miki D, Furuta M, Tsunoda T, Miyano S, Kubo M, Aikata H, Ochi H, Kawakami YI, Arihiro K, Ohdan H, Nakagawa H, Chayama K. Circulating Tumor DNA Analysis for Liver Cancers and Its Usefulness as a Liquid Biopsy. *Cell Mol Gastroenterol Hepatol* 2015; **1**:516-534 [PMID: 28210698 DOI: 10.1016/j.jcmgh.2015.06.009]

108 Liese J, Peveling-Oberhag J, Doering C, Schnitzbauer AA, Herrmann E, Zangos S, Hansmann ML, Moench C, Welker MW, Zeuzem S, Bechstein WO, Ulrich F. A possible role of microRNAs as predictive markers for the recurrence of hepatocellular carcinoma after liver transplantation. *Transpl Int* 2016; **29**:369-380 [PMID: 26697811 DOI: 10.1111/tri.12733]

109 Han ZB, Zhong L, Teng MJ, Fan JW, Tang HM, Wu JY, Chen HY, Wang ZW, Qiu GQ, Peng ZH. Identification of recurrence-related microRNAs in

hepatocellular carcinoma following liver transplantation. *Mol Oncol* 2012; **6:** 445-457 [PMID: 22552153 DOI: 10.1016/j.molonc.2012.04.001]

110 Bharali D, Jebur HB, Baishya D, Kumar S, Sarma MP, Masroor M, Akhter J, Husain SA, Kar P. Expression Analysis of Serum microRNA-34a and microRNA-183 in Hepatocellular Carcinoma. *Asian Pac J Cancer Prev* 2018; **19**:2561-2568 [PMID: 30256056]

111 Cho HJ, Kim SS, Nam JS, Kim JK, Lee JH, Kim B, Wang HJ, Kim BW, Lee JD, Kang DY, Kim JH, Jae YM, Hwang JC, Shin SJ, Lee KM, Cho SW, Cheong JY. Low levels of circulating microRNA-26a/29a as poor prognostic markers in patients with hepatocellular carcinoma who underwent curative treatment. *Clin Res Hepatol Gastroenterol* 2017; **41**:181-189 [PMID: 27839726 DOI: 10.1016/j.clinre.2016.09.011]

112 Zhang J, Lin H, Wang XY, Zhang DQ, Chen JX, Zhuang Y, Zheng XL. Predictive value of microRNA-143 in evaluating the prognosis of patients with hepatocellular carcinoma. *Cancer Biomark* 2017; **19**:257-262 [PMID: 28436387 DOI: 10.3233/CBM-160357]

- Another limitation of pre-transplant serum biomarkers to be considered by the authors is that the vast majority of studies published in the field did not implement a methodology to control for competing risks. When considering HCC recurrence as a time-dependent outcome, a patient who experience early death after LT, not related to HCC, may never have a chance to recur. Please comment.

This limitation has been included in the section "limitations of pretransplant serum biomarkers", page 16, as follows: "Another limitation of the different studies reviewed here, relies on the analyses of HCC recurrence as a time-dependent variable, such as recurrence or disease-free survival, without accounting for competing risk, such as death. So, patients who died early after LT, whose dead was not related to HCC may never have had the chance to experience HCC recurrence"

- A paragraph delineating further directions may be welcomed. In opinion of the authors, What would be the role of cutting edge biomarkers such as cell free DNA, miRNAs… in the near future?

As commented above, we have included a brief comentary about these cutting edge biomarkers in the new section "Other potential serum biomarkers"

- I would recommend the authors to conclude the manuscript by claiming for an international consensus in this setting, which may provide with practical recommendations to implement serum biomarkers in local practice algorithms.

We have included a final paragraph claiming for multicenter prospective studies and international consensus as follows:

"For those reasons and taking into account the limitations highlighted here, multicenter prospective studies are demanded and an international consensus is mandatory in order to provide practical recommendations to guide the implementation of serum biomarkers combined with morphological criteria to better stratify patients at high or low risk of HCC recurrence after LT".

- Minor English polishing is required.

We have sent the manuscript for language editing

Reviewer code: 00054465

This is a nice review of the current state and outcomes of liver resection and transplantation for hepatocellular cancer. Most of the data was retrospective review of biomarkers and the published outcomes of using the Milan criteria for transplantation. The authors conclude that using multiple biomarkers may be stronger data to predict the probability of recurrence of cancer after transplantation. A well analyzed conclusion that NOW REQUIRES A PROSPECTIVE STUDY.

Thank you very much for your interest and positive comments in this manuscript. We agree with the necessity of multicenter prospective studies and we have added a final paragraph claming for these studies and for international consensus about combination of serum biomarkers and morphological criteria as follows:

"For those reasons and taking into account the limitations highlighted here, multicenter prospective studies are demanded and an international consensus is mandatory in order to provide practical recommendations to guide the implementation of serum biomarkers combined with morphological criteria to better stratify patients at high or low risk of HCC recurrence after LT".