Submission of a revised manuscript entitled: “Collagen proportionate area correlates to hepatic venous pressure gradient in non-abstinent cirrhotic patients with alcoholic liver disease” by Restellini et al.

Dear Editor,

Please find attached a revised version of our manuscript with corrections highlighted.

You will find below a point-by- point reply to all the reviewer’s comments:

We would like to thank the reviewers for helping us improving the quality of our manuscript, which we hope is now suitable for publication in World Journal of Hepatology.

Yours sincerely,

Pr Laurent Spahr

REVIEWERS' COMMENTS TO THE AUTHOR:

Reviewer 1 (03646639)

1. The average child pugh score of the patients in this study is more than 9. It would be of more interest if authors should focus more on non-invasive evaluation of hepatic fibrosis and portal pressure.

As reported in the limitation section, only a minority of patients had liver stiffness measurement precluding any comparisons with CPA and HVPG. However, the usefulness of liver stiffness is limited in this population as ascites, a frequent complication of cirrhosis, limits the performance of liver stiffness measurement.

2) The authors should show what composite clinical outcome is.

As reported page 7, our composite clinical outcome included: "liver related death or liver transplantation, as well as clinically relevant episodes including ascitic decompensation, overt episodes of hepatic encephalopathy and PHT-related bleeding"

3) I would encourage the authors to exclude the healthy candidates who underwent transjugular liver biopsy in this study. There may be some ethical concerns raised from liver biopsy on healthy cases.

It is our policy to perform a liver biopsy early in patients eligible for living donation in agreement with our institutional ethical committee. The transjugular route has been

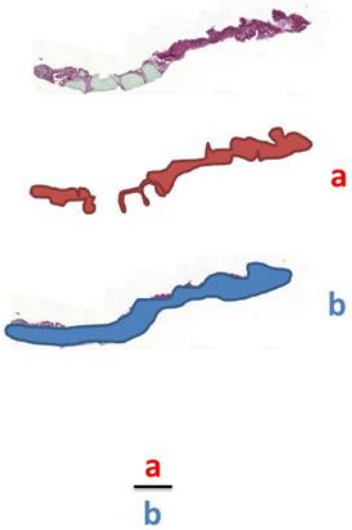
preferred with regards to patient comfort and reduced risk of serious complications according to our local experience. A sentence has been added page 6.

"It is our policy to perform a liver biopsy early in patients eligible for living donation in agreement with our institutional ethical committee"

Reviewer: 2 (02945170)

1. Why were the sections stained with picrosirius red? The CPA were seemed too low in cirrhosis patients, what were the reasons?

Computer-assisted digital image analysis of a liver tissue specimen is named collagen proportionate area (CPA). This technique must use picrosirius red in order to perform quantitative measurement of liver fibrosis. The collagen surface stained with PicroSirius is referred to the tissue area, producing a "fibrosis ratio" (= a / b) or a collagen proportional area (CPA).



$$\frac{a}{b}$$

We agree CPA were seemed low in cirrhotic patients but biopsies were fragmented reflecting the material we can get in clinical practice in cirrhosis.

2) The information about the septal width, number of nodules and nodules size in biopsy slides and the relationship with HVGP should be discussion.

Histological features are reported on Table 2. We do not have information on septal width, number of nodules and nodule size.

Reviewer: 3 (03647931)

1. I have one question to the authors, what is the medical indication of transjugular liver biopsy in healthy donors who normally undergo a percutaneous liver biopsy?

It is our policy to perform a liver biopsy early in patients eligible for living donation in agreement with our institutional ethical committee. The transjugular route has been preferred with regards to patient comfort and reduced risk of serious complications according to our local experience. A sentence has been added page 6.

"It is our policy to perform a liver biopsy early in patients eligible for living donation in agreement with our institutional ethical committee"

Reviewer: 4 (03665102)

1. The results are intriguing: CPA positively correlated with HVPG only in active drinkers, although the two groups were homogeneous. Only HVPG, as extensively demonstrated, was a predictor of further liver complications.

However, in my modest opinion, the physiopathological significance and the clinical impact of this study are not very clear: the discussion should be improved in order to clarify these aspects.

We agree with the reviewer 4 our results are intriguing and require additional confirmation. However as discussed in page 13 and 14, our results support that active alcohol consumption may influence portal hemodynamic in addition to existing architectural changes due to cirrhosis. Accordingly, an oral administration of 0.5 g/kg of ethanol increases both HVPG and azygos blood flow in patients with alcoholic cirrhosis and thus may precipitate variceal bleeding [ref 13]. This observation is consistent with the higher value of HVPG in active alcohol drinkers who developed a clinical complication of PHT during follow-up as compared to those without clinical decompensation [25]. In addition, our findings suggest that active alcohol drinking may negatively impact on hepatic microcirculation and intrahepatic resistance. We were not able to identify an influence of histological lesions on parameters such as CPA and HVPG. We speculate that major architectural changes of cirrhosis present in all patients may have blunted the possible role of lesions such as marked steatosis or inflammation on the HVPG.

Additional sentences have been added in the discussion

"CPA positively correlated with HVPG only in active drinkers. Our results support that active alcohol consumption may influence portal hemodynamic in addition to existing architectural changes due to cirrhosis"

2. Furthermore, there are several methodological flaws in this study: the percentage of patients with decompensated liver cirrhosis and especially with

clinical manifestations of portal hypertension was very high; a control group with compensated cirrhosis, without complications of portal hypertension, or with chronic hepatitis with moderate to severe liver fibrosis, but without cirrhosis, was not included;

We agree with the reviewer comment. A comparison with patients having a compensated cirrhosis or with chronic hepatitis with moderate to severe liver fibrosis, but without cirrhosis could have added some value to our results. However, the composition of our study population is strongly influenced by recruitment in a tertiary care hospital with predominance of decompensated patients with advanced chronic liver disease. Nevertheless, we believe that our results are valid, as we also provide data in healthy subjects submitted to the same CPA and HVPG measures demonstrating striking differences.

3. Determination of liver stiffness was available only in few patients and, for this reason, it was useless

We have addressed this point as follows on p. 13-14.

"only a minority of patients had liver stiffness measurement precluding any comparisons with CPA and HVPG. However, the usefulness of liver stiffness is limited in this population as ascites, a frequent complication of cirrhosis, limits the performance of liver stiffness measurement"

4. The history and the amount of alcohol consumption, the length of the withdrawal period were not indicated.

This point has been corrected in the methods section:

"Both active alcoholic patients and abstinent patients were eligible for inclusion. Abstinent patients were defined as patients who did no drink any glass of alcohol for the last 6 months before inclusion. Abstinence or relapse status was self-reported"

5. Minor points: page 10: 4/41 in active drinkers and 3/20 in abstinent patients died as expected, the prevalence of clinical manifestations of portal hypertension was higher in patients with higher fibrosis density, although the difference was not statistically significant (Table 3). The authors could subcategorize their patients not only according to fibrosis density, but also according to their drinking habits.

The main purpose of our study was to compare low versus high fibrosis density. Due to the limited number of patients, we are worried that subgroups analysis may not be appropriate, especially since patients characteristics were not different between the group of alcohol active and non-active users.

Reviewer: 5 (03563089)

Lack of some histological images to go hand-in-hand with the rest of the outstanding results. If added, would enhance the quality of the paper even better.

We fully agree with this comment and provide in the new version an illustration of typical liver biopsy samples stained with Sirius red and measurement of CPA (see new Figure 1)

Reviewer: 6 (00053433)

1. For the sake of clarity, authors should provide details about the duration of abstinence in abstinent patients.

Abstinence or relapse status was self-reported. Abstinent patients were defined as patients, who were strictly abstinent from alcohol for the last 6 months before the inclusion. Definition has been added in the text Page 6:

"Both active alcoholic patients and abstinent patients were eligible for inclusion. Abstinent patients were defined as patients who did no drink any glass of alcohol for the last 6 months before the inclusion. Abstinence or relapse status was self-reported."

2. In a multivariate model including active and inactive alcohol users, only HVPG was independently associated with fibrosis density. This finding is relevant and should be stressed in the discussion section.

We thank the reviewer 2 for this comment. Indeed, within subjects with active alcohol use or abstinence, in multivariate analysis including HVPG, drinking status and sex, only HVPG was independently associated with fibrosis density (OR 1.2 per unit increase in HVPG, 95% CI [1.1-1.4], p=0.01). This result emphasizes the strong correlation between HVPG and fibrosis density using a 5% cut-off value as depicted in Figure 1A. An additional sentence has been added in the discussion section:

"In multivariate analysis, only HVPG was independently associated with fibrosis density (OR 1.2 per unit increase in HVPG, 95% CI [1.1-1.4], p=0.01) "

However, in a subgroup analysis, this correlation was conserved only in active drinkers (see new figure 2 B). Discussion has been focused on this interesting difference, emphasizing the key role of active alcohol consumption.

3. It should be clarified how many abstinent subjects resumed alcohol consumption during follow-up. If all of those patients resumed alcohol abuse, this could severely compromise associations with clinical outcomes.

As reported on page 11, a return to regular/moderate alcohol consumption (≤ 20 gr/day) was reported in only 2 patients on the 20 patients, who were qualified as abstinent at baseline. It was a mild alcohol consumption and none of the 2 patients had composite outcome. Considering this small number of cases, it shouldn't compromise association with our clinical outcomes.

4. In the group of patients abstinent from alcohol, neither HVPG nor CPA was associated with the development of a composite clinical outcome. However, the exact number of cases with hepatic decompensation within this group has not been mentioned, and one could speculate that it would be just a few.

In addition, if all abstinent subjects resumed alcohol consumption during follow-up, both facts could have precluded the identification of association between HVPG/CPA and clinical outcomes. This possibility should be briefly discussed.

As mentioned above, only 2 patients who were qualified as abstinent at baseline returned to regular/moderate alcohol consumption (≤ 20 gr/day) during follow-up.

However, we agree there may still be information bias as the information on abstinence was self-reported. Additional sentence has been added in the limitation section.

"Third, information on abstinence was self-reported leading to a risk of information bias that could possibly preclude association between HVPG/CPA and clinical outcomes in abstinent patients"

Our composite outcome including ascitic decompensation, portal hypertensive bleeding, or episode of overt hepatic encephalopathy occurred in 32 patients encompassing 20 abstinent patients. Only 2 out of those 20 patients resumed alcohol consumption during follow-up. It was a mild alcohol consumption and none had the criteria of composite outcome. Considering this small number of cases, it shouldn't bias the association with clinical outcomes

5. The number of patients with clinical complications due to PHT should be indicated in each subgroup of Figure 2.

We agree with the reviewer and we created a supplementary Table to address this issue (supplementary Table 1.)

Supplementary Table 1**: **clinical complications due to portal hypertension in alcohol abstinents and alcohol drinkers subgroups

Variable	Alcohol abstinents (n=21)	Active alcohol drinkers (n=31)
Ascites	17/21 (80%)	15/31 (48%)

GI bleeding	0/ 20	0/31
HE	6/19 (32%)	5/31 (16%)
Death	3/20 (15%)	4/33 (12%)
Liver transplantation	1/20 (5%)	2/31 (6.4%)
TIPS	3/20 (15%)	3/31 (9.7%)

Footnote: number representing patients with available data. For definitions, see text.

Reviewer: 7 (00006459)

1. This small-medium size study is adequate to support the conclusions made. Please add clarity to the description of the liver biopsy method on page 8, regarding how the liver tissue was obtained. There is detail on how blood pressure was measured.

Our biopsy method has been previously reported in ref 19. In the method section (page 7-8), we briefly reported that transjugular biopsies were performed using both a TJL-101-ET needle set 8 Cook Europe, Bjaeverskov, Denmark) and a 8F curved catheter (Cordis Europa, Amsterdam, The Netherlands). The liver biopsy specimen was placed into formalin 10%, then fixed and embedded in a paraffin wax block to be processed for light microscopy. The histopathological specimens were thoroughly examined by an expert in liver pathology (LRB) using standard high-power field views.

2. In the abstract, why is there no mention of the fibroscan data?

As reported in results section, due to technical limitations (morphotype, ascites) only few patients benefited from a transient elastography (n=12, 12/41 patients in the group of active alcohol drinkers, and 0/20 in the abstinent group) precluding any comparison and limiting pertinence to include these data in the abstract.