Author's response to reviews

Title: Lymphatic marker podoplanin/D2-40 in human advanced cirrhotic liver-Reevaluations of microlymphatic abnormalities

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Author's response to reviews: see over
Dear Prof. Norton,

We wish to submit our revised manuscript entitled “Lymphatic marker podoplanin/D2-40 in human advanced cirrhotic liver – Reevaluation of microlymphatic abnormalities” for consideration to be published in *BMC Gastroenterology*

Thank you very much for giving us the opportunity to respond to the referees and to incorporate their suggestions into the manuscript. I would also like to thank the referees for their review of our manuscript and the very insightful comments.

In this cover letter, we attach a point-by-point response to the concerns of the reviewers, in which we also specified the changes made in the manuscript. To facilitate the reviewers and editors in checking the revisions, we have highlighted all the changes by underlines. We have also rechecked our manuscript to ensure that it conforms to the journal style.

On behalf of all of the authors, I do hope that these amendments and clarifications are satisfactory.

Yours sincerely,

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Reviewer: Armin Thelen

Comments
1. The most criticism of the study is the low number of analyzed tissue specimens (only 5 in each group). That is not important for the analysis of the specificity of the antibody but it is a disadvantage for the analysis of the comparison of the vessel density between the different study groups. Therefore, it may be helpful to address this shortcoming of the study in the discussion section. Discussions have been streamlined focusing on the main findings and their implications.

Response: We thank the reviewer for the useful comments. Indeed we should include the small number of samples as a shortcoming of our study. We have added the following sentence in Discussion:

“A limitation of the present study is that we investigated a small number of samples (five in each group), which may by a disadvantage in the analysis and comparison of vessel density between study groups. A larger number of samples should be examined to confirm the present finding.” (Page 20 lines 6-9)

2. First paragraph of the section “Materials and methods”: It is not clear to me which groups of tissue specimens come from patients who underwent resection and who underwent liver transplantation as both are described as tissues from cirrhotic livers. Authors may specify this.

Response: We thank the reviewer for pointing out this important omission. We have added the details of the third group in Material and Methods as follows:

“Cirrhotic liver specimens were obtained from another group of five patients (five males; aged 58–65 years, mean 61.2 years) with HCC and cirrhosis of Child–Pugh grade C, who underwent liver transplantation (Child C-LC group).” (Page 7 lines 7-10)
Reviewer: sinan akay

Comments
Although there was no proliferation marker investigated in the article, the word “proliferation” is used for lymphatic endothelial cells.

Response: The reviewer is correct to point out that use of the word “proliferation” is inappropriate to describe the present result. We have changed the wording as follows: “The data obtained in the present study suggest that the factors stimulating the proliferation of lymphatic and blood vessel endothelial cells associated with increased density of lymphatic and blood vessels might be different.” (page 18 lines 12)

Advice on publication: Although the article includes patients with cirrhosis, results of the study are descriptive and may be it will be more convenient to publish this article in a preclinical journal. The discussion of the article does not contain any discussion about the formation or reabsorption of ascites.

Response: We agree with the reviewer that our manuscript provides insight on basic scientific evidence based on findings obtained from patients with different stages of cirrhosis. However, the findings explained some of the clinical characteristics observed in different stages of cirrhosis, and have clinical implications. Since the wide scope of BMC Gastroenterology includes all aspects of prevention, diagnosis and management of gastrointestinal and hepatobiliary disorders, as well as related molecular genetics, pathophysiology, and epidemiology, the broad readership includes both basic scientists and clinicians. We feel that our article will be of interests to readers of BMC Gastroenterology. We agree that the issue of ascites should be addressed. We have included the following discussion in the revised manuscript:

“The most common cause of ascites in cirrhosis is elevated pressure in the hepatic circulation (portal hypertension). The elevated pressure causes leakage of fluid from the lymph vessels in and around the liver into the abdominal cavity. The role of lymphatic abnormalities on the formation or reabsorption of ascites should be examined in further studies.” (page 16 lines 18 to page 17 line 4)
Reviewer: Patricia Lalor

Major Compulsory Revisions
i) The manuscript describes an interesting study to determine the validity of using an antibody raised against podoplanin to identify lymphatic vessels in human liver samples, and goes on to generate data describing how the density of lymphatics changes with disease. The authors assert that this is the first study to quantify the density of lymphatic vessels in cirrhosis and demonstrate expression of podoplanin in cirrhosis. Staining with Cd34 is used throughout to differentiate ‘vascular’ from ‘lymphatic’ endothelium.

I would suggest that given the broad readership of this particular journal the authors should expand the introduction to discuss the availability and specificity of different markers for the different components of the vascular tree in the normal and diseased liver. This is particularly important in the context of CD34 expression for example which is ‘mostly’ present on ‘vascular’ endothelium in the liver but has been reported on lymphatics in some studies (some of which include expression on tumors which arise on a cirrhotic background), does stain sinusoidal endothelium when it becomes ‘capillarised’ in disease (in support of some of the data shown in periportal images in this manuscript), and has also been reported on various precursor cell populations in the liver.

Response: We thank the reviewer for the useful comment. Following the advice, we have added the expression patterns of different markers in Introduction as follows:
“LYVE-1 expression is attenuated in cirrhotic nodules and absent in HCC liver compared to the normal sinusoidal counterpart [13]. …. CD34 is a type 1 transmembrane sialomucin mostly present on vascular endothelium in the liver but has been reported on lymphatics in some studies. CD34 is absent from most sinusoidal endothelial cells in normal liver but expression increases during capillarization in chronic inflammatory disease and in the sinusoidal-type vasculature within hepatocellular carcinomas [15]. Sinusoidal capillarization indicated by CD34 expression correlated with dedifferentiation of the liver tissue during the course of cirrhosis [16].”

The authors observations on the extent of CD34 expression are therefore not novel and they should temper their discussion relating to links between CD34 and podoplanin expression. For example in Fig 1c the CD34 staining at low power nicely highlights positivity on central vessels and portal vessels in normal tissue, but there is also a suggestion of some weak stain on a structure that may be sinusoidal at the centre of the lower half of the panel. Thus clearer discussion of the true identity of cells bearing increased CD34 expression in cirrhotic samples
Response: We appreciate the reviewer for the insightful comment. This report focuses on podoplanin/ D2-40 as a lymphatic marker in human normal and cirrhotic liver. CD34 has been researched extensively, and its expression in normal and diseased tissues has been well studied and reported. We used CD34 in this study as a “vascular” marker (including specialized vasculatures such as capillarization) for the purpose of comparison only. While we clearly described the results of both CD34 and D2-40 in Results, we did not discuss CD34 expression but concentrate on lymphatic vessels in Discussion.

In Fig. 1c, we think that “Positive immunoreactivity of CD34 was observed in central vessels and portal vessels, as well as terminal portal venules.” (page 12, lines 7-8; Legend to Fig. 1a).

**ii) The authors should check their statement that they are the first to determine lymphatic vessel density in cirrhosis. This may indeed be the case using this particular marker but I believe there may be other studies characterising density using other means including vascular casts. This should be clarified.**

Response: We agree with this comment. We have modified the sentences as follows: “The present report describes, for the first time, study demonstrated that D2-40 mAb is a selective marker of lymphatic endothelium in normal and cirrhotic liver tissues,...” “This is the first report to describe the analyses of the density of lymphatic vessels in liver cirrhosis.”

**iii) Similarly it would be useful to have a little more clinical data for the ‘normal’ samples used. It is suggested these are non-involved samples from tumour patients I believe, and thus there may be potential for modulation of vascular phenotype in fibrotic samples (however I acknowledge that the morphology on the histological staining images shown looks good).**

Response: Yes, the “normal” samples were uninvolved portions of resected metastatic liver carcinoma (primary colonic carcinoma in all cases). Since these were archived samples, we were not able to retrieve detailed clinical data including liver function tests. However, all specimens had normal macromorphology and appeared normal on histological studies, as also agreed by the reviewer.

- **Minor Essential Revisions**
  * i) Given potential for sections used during image analysis to contain various*
vascular compartments across the different zones of the lobule, and variations in size of portal areas with advanced cirrhosis it would be helpful to add detail as to how the scoring was performed (ie how were areas chosen for scoring, are the same amount of vessels included in each ‘representative’ field, were only portal fields considered ??)

Response: For control samples, the portal area was chosen at random. For cirrhotic samples, three different portal regions and regenerative fibrotic areas were selected at random.

ii) Western blot figure suggests GAPDH blotting used for densitometric comparison of samples but methods section suggests β-actin was used. Should be clarified

Response: We apologize for the error. We have corrected the text as follows: “The relative D2-40 signal intensity was obtained by dividing the intensity of D2-40 signals by that of β-actin-GAPDH signals.” (page 11 line 16)

iii) Electron micrographs are beautiful but quite difficult for a non-expert to interpret purely on morphological appearance, a few additional labels might be helpful.

Response: We thank the reviewer for the comment. We have added additional labels in the electron micrographs as follows: “PV: portal venule; a: capillary artery; Ly: lymphatic vessel ;P: portal vein” I would like to add ”PV(e): portal vein, c(e): capillary arterial endothelial cell, and Ly(e): lymphatic endothelial cell”.

iv) Labelling on figure panels is a bit confusing as each page bears a figure number (1-9) whilst the correct figure numbers are also stated in black text on the panels. Should be corrected for final version.

Response: In fact, there are only four figures. Figure 1 has 3 parts; A, B and C, and Fig. 4 has 2 parts; A and B, each shown on a separate file to avoid confusion. We have clearly labeled each figure as follows: Fig. 1A (a to d), Fig. 1B (e to h), Fig. 1C (i to l).

Quality of written English: Needs some language corrections before being
published

Response: We have checked the whole manuscript carefully for spelling, grammatical and syntactical errors.