Author's response to reviews

Title: Microvessel density by automated analysis from regenerative nodule to small hepatocellular carcinoma - approach with CD105 and CD34 immunoexpression.

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Author's response to reviews: see over
Dear Editors,

Thank you for your contribution on our manuscript MS 1279903796107264, entitled “Microvessel density by automated analysis from regenerative nodule to small hepatocellular carcinoma - approach with CD105 and CD34 immunoexpression.” Your expertise has undoubtedly helped to raise the level of our work.

Since most of the Referees suggested that an extensive review of the English language was performed, our group decided to send the manuscript to Edanz Editing and the version included in this email is the product of their revision. A modification in the title was also suggested, so, if still possible, we would like to replace the previous title for: “Microvascular density of regenerative nodule to small hepatocellular carcinoma by automated analysis using CD105 and CD34 immunoexpression”.

This way, we hope that many of the understanding issues have been solved. Please find below the answers to your questions and comments, divided by referee for easier understanding and analysis:

**Referee 1 (GeorgeTsirakis):**

**Reviewer’s report:**

In the present manuscript, the authors investigated the immunohistochemical expression of CD105 and CD34 in various pathological liver tissues, as markers of neoangiogenesis. They found that CD34-MVD was increasing from RN to HCC, whereas CD105-MVD was higher in RN. Moreover, in tissues of RN and DN, CD105 expression was higher than CD34. These observations suggest that the expression of CD105 is closely related to cirrhosis, whereas CD34 is related to carcinogenesis. This is a quite well written manuscript.

Minor Essential Revisions
1. MVD should be steadily abbreviated as microvascular or microvessel density, not with both, suggesting that in title, key words and discussion “microvessel” should be replaced by “microvascular”.

Suggestion accepted. In title, key words and discussion “microvessel” was replaced by “microvascular”.

2. In introduction, it is written that angiogenesis is increased in some pre-malignant lesions in cervical, lung and in adenoma-carcinoma colon sequence. I would also added that this is also happens in the evolution of MGUS to non-active and consequently to active multiple myeloma [Vacca A, et al. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol. 1994Jul;87(3):503-8].

Suggestion accepted (please see “Background” section, page 5 of the manuscript, second paragraph).

3. I think that in methods section, the materials used are not completely clear. I suggested that all patients had cirrhosis (and if this is the case then it should be mentioned). Moreover, I did not understand if the biopsies were obtained from the initial or the transplanted liver.

Substantial rewriting was performed and English language was revised for a better understanding. The sentence in manuscript now appears in “Methods” (Page 6) section as:

“The samples were obtained from 28 patient liver cirrhotic explants who underwent surgery between 2000 and 2007. The explant specimens were buffered-formalin fixed and paraffin-embedded using standard histology methodology to ensure the viability of tissues for further immunohistochemical studies.”

4. In results, the values of CD105 and CD34 could be removed since they are shown in the tables. I would keep only the p values.

Change made as indicated by the reviewer.
5. In tables or in their legends, I would like to see the p values. The p values were included in the legends of the tables (page 18 of the manuscript).

6. In discussion, it is written that soluble CD105 was higher in the serum of cirrhotic patients compared to healthy population, suggesting that cirrhosis is a state of increased angiogenesis [30]. Nevertheless, the same authors showed that serum levels of soluble CD105 was even higher when cirrhosis co-existed with HCC, compared to cirrhosis and cancer alone. If the patients of the study had cirrhosis, I would expect increased histological expression of CD105 in HCC biopsies. This has to do with the fact that serum levels of soluble CD105 have already been directly correlated to bone marrow MVD in Multiple Myeloma and with other markers of angiogenesis in various myeloid malignancies [Pappa C, et al. Emerging roles of endoglin/CD105 and angiogenic cytokines for disease development and progression in multiple myeloma patients. Hematol Oncol.2013 Apr 11.,Tsirakis G, et al. Clinical significance of CD105 in angiogenesis and disease activity in multiple myeloma. Eur J Intern Med. 2012 Jun;23(4):368-73., Calabrò L, et al. Differential levels of soluble endoglin (CD105) in myeloid malignancies. J Cell Physiol. 2003 Feb;194(2):171-5].

Please comment.

The results displayed in the referred liver paper are probably due to the fact that endoglin was measured in the serum, and so, other factors may have contributed to the elevation in seric endoglin in patients with hepatocellular carcinoma and cirrhosis. We would like to emphasize that in such paper, the diagnosis of cirrhosis and hepatocellular carcinoma was based on clinical aspects and regular laboratory and ultrasound analysis, so biopsy was not always performed. Our study, on the other hand, was carried on samples with representation of the whole nodule diagnosed by histopathological analysis. Therefore, in our opinion, our methodology was more adequate to the analysis of tissue endoglin, which was the focus of our study. Finally, our study demonstrated that DMV-CD105 is also elevated in HCC as well as in other solid tumors and hematopoietic neoplasms.
Referee 2 (Freda Passam):

Minor essential revisions
In the current study Paschoal and colleagues have measured the microvessel density in regenerative nodules, dysplastic nodules and hepatocellular carcinoma samples and described that CD105 MVD is more expressed in regenerative nodules whereas CD34 MVD higher in hepatocellular carcinoma. Overall the study is limited in sample numbers as far as the hepatocellular carcinoma samples are concerned. It has been described before by other groups in larger sample sizes that CD105 is useful in assessing MVD of hepatocellular carcinoma, which carries predictive value (Ho, World J Gastroenterol 2005, Yao, Ann Clin Lab Sci, 2007).

The measurement of endoglin in the regenerative and dysplastic hepatic nodules is new. What is unclear is:
1. How could the knowledge of increased endoglin vs CD34 MVD guide the selection of antiangiogenic treatment?

Further studies are still required for such definition. The main pitfall for CD105 blockage is the fact that this molecule is linked to many other cellular processes including regeneration of damaged tissue, and so manipulation of its levels should be carefully evaluated.

2. Do the authors have information on the outcomes of these patients?

The outcome of the patients was not under the scope of this paper.

3. Reduced endoglin in the tumour vasculature has been associated with increased metastatic potential (anderberg, J Exp Med 2013). Were the cases of cirrhosis with reduced endoglin MVD related to higher hepatocellular carcinoma transition?

This parameter was not under the scope of this paper. However, independently of the presence or absence of carcinoma in cirrhotic livers, the average MVD-CD105 was the same.
Referee 3: Eleni Mayson

MAJOR COMPULSORY REVISIONS

1) MVD measurement:
   o I think it would be helpful to explain in detail how the MVD is calculated by the software, given that this is the major endpoint of measurement. I have a haematological background, and so I am unfamiliar with what method this software uses to calculate the MVD.

   In this study we used a pre-established and largely discussed and validated method, and so extensive discussion and detailing of the image method in our paper would deviate the scope of our analysis. The detailed method can be found in the referred paper by Vagner Bernardo et al: Reproducibility of immunostaining quantification and a description of a new digital image processing procedure for quantitative evaluation of immunohistochemistry in pathology, Microsc. Microanal. 15: 353-365, 2009. The author of the paper referred was the responsible for our analysis as well. Nevertheless, a brief description of the methodology was added to the “Microvascular density” section, in “methods”, page 7 of the manuscript.

   o What are some of the limitations of using this software? How sensitive is it? Is it expensive? What QC (quality control) exists and how readily available is this software? Is it standardized across institutions?

   The Image Pro Plus is a largely used software for image analysis, extensively validated and standardized worldwide. It is commercially available and informations on quality parameters are available at http://image-pro-plus.software.informer.com/.

2) Tissue used
   o What measures were taken to ensure viability of tissue prior to processing?

   The following information was added in to the “Methods” section of the manuscript, page 6:
“The explants specimens were 10% buffered-formalin fixed and paraffin-embedded using standard histology methodology to ensure the viability of tissues for further immunohistochemical studies.”

3) Antibodies used
o What internal and external QC is there with the author’s methods?
o What is the sensitivity and specificity of each antibody?
o What do the positive and negative controls consist of?
o What are some of the limitations of the method used?

The positive and negative controls were added to the “Immunohistochemistry” section, in “Methods”, page 7, as follows:
“Negative controls consisted of the reaction performed without primary antibodies and positive controls consisted of placenta and granulation tissue for CD34 and CD105, respectively”.

Since the antibodies used are commercially available, information on the topics above regarding sensitivity, specificity, quality controls and limitations of its use are available on the respective data sheets at:
CD34:  http://www.dako.com/br/ar49/p235316/prod_products.htm and

4) Discussion
o The authors state that CD105 is more specific than CD34 because it reflects neovascularisation associated with hypoxic tissue. Does the viability of explanted tissue (and the possibility of hypoxic damage) affect these results?
The very short period of time between the organ removal and its fixation has not been referred in the literature as sufficient for interfering with CD105 expression.

o CD34 and CD105 has demonstrated prognostic significance in non-hepatic tumours, but what firm evidence is there that it reflects prognosis in hepatic tumours?
Our study was not prognosis-based and we did not analyze this parameter.
o What is the evidence that MVD quantification confers prognostic significance in hepatic tumours, given that they are highly vascular tumours in vivo?
Our study was not prognosis-based and we did not analyze this parameter.

o Do in vitro results of these markers, correlate with in vivo prognosis? If so, what aspect of prognosis? I think the authors need to reword the conclusion and discussion to clarify their findings based on the data presented in their study. They can then substantiate their findings with evidence from other studies.
Extensive revision of the English language was performed in order to correct and possibly improve understanding of our analysis. As prognosis was not assessed in our study, discussion on this parameter was kept at minimal level not to shift the scope of the paper.

Referee 4 (Marie-Christine Kyrtonis):

Major Compulsory Revisions
The message of the study should be clearer, even if it is only a negative one. The reader understands, as is presented the study that CD-105 is not useful for differential diagnostic purposes and not safe for therapeutic applications. What about prognostic purposes?
The prognosis analysis was not the scope of our study. We hope that after the extensive language revision and rewriting, the message of the study has become clearer.

Minor Essential Revisions
In the abstract, the results are not understandable. The “Results” of the Abstract have been rewritten, as the reviewer indicated (page 3):
“The median MVD for CD34 was higher in HCC than in DN and RN (p<0.01), and was higher in DN compared with RN (p=0.033). In contrast, MVD with CD105 was higher in RN, and the difference was significant in RN and DN compared with HCC (p=0.019 and p=0.012, respectively). When MVD with
CD34 and CD105 were compared within a single group, there was a significant predominance of CD105 in RN and DN (p<0.01). In addition, MVD-C34 in HCC predominated compared with MVD-CD105, but the difference was not statistically significant (p=0.128).

In the “Methods”, the final MVD calculation should be better explained as well as the assessment of normality. Extensive rewriting and insertion of details were performed in the “Microvascular Density” section, in “Methods”, page 7.

The presentation of results should be improved. Extensive rewriting of this section and insertion of details were performed, page 8.

Are representative pictures of CD105 and CD34 staining in HCC, RN and DN available? Suggestion accepted. Please check the figures 3 to 6 uploaded in this new version.

There are some language mistakes especially in the “Methods” part. Extensive rewriting of this section was performed and the authors sent the manuscript to Edanz Editing to improve the English language.

Should any further questions or suggestions be replied, please do not hesitate in contacting us.

Yours faithfully,

Juliana Passos Paschoal