Author's response to reviews

Title: Lower serum prohepcidin levels associated with lower iron and erythropoietin requirements in hemodialysis patients with chronic hepatitis C

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Author's response to reviews: see over
Dear Christna Chap,

We are pleased to receive your letter mentioning you might consider accepting our manuscript for publication provided with appropriate changes requested by the reviewer accordingly. We made changes in our manuscript in the light of your and the reviewer’s suggestions. We hope you will find the changes satisfactory. We apologise for delaying for submission of revised version of manuscript.

“As attached, please find 2 revised versions of the manuscript.

Version-1 includes revisions according to the suggestions of the reviewers in red color, for your convenience.

Version-2 is the finalized format of the work”.

We are looking forward to hearing from you soon,

Sincerely yours,
Response to Reviewer 1 (Elisio Costa):

We are pleased to learn that the reviewer found our manuscript interesting. We made following changes in our manuscript in the light of the reviewer’s suggestions.

1. **Reviewer addressed that the pathophysiological changes in iron cycle and in inflammation, associated with HD and with hepatit C virus infection, need to be improved in the introduction section.**

   **Reply.** As requested by the reviewer, we improved the introduction section of the manuscript. The following sentences and references were added to the background section;

   “In HD patients, compared to healthy controls higher levels of prohepcidin and hepcidin associated with chronic inflammation were reported. (Costa E et al. *Acta Haematol* 2009, Costa E et al. *Am J Nephrol.* 2008). High levels of hepcidin in these patients was found to be related to an increased inflammation and resistance to rhEPO therapy (Costa E et al. *Acta Haematol* 2009, Costa E et al. *Am J Nephrol.* 2008). Serum levels of prohepcidin, the precursor molecule of hepcidin, were found lower in patients with chronic HCV infection and these concentrations of this molecule were found negatively associated with total iron stores in non-uremic patients with chronic HCV infection (Miura K et al. *Hepatology* 2008, Fujita N et al. *Mol Med* 2007, Nishina S et al. *Gastroenterology* 2008). However, changes in hepcidin regulation have not been examined previously in HD patients with chronic HCV.”

   (Background section, page 3, lines 9 to 16).

2. **A revision of other works that studied the iron cycle, the inflammation and the levels of hepcidin (prohepcidin) in HD patients, with and without hepatitis C, must be included in this section.**

   **Reply.** We included the works that studied the role of inflammation and the levels of hepcidin (prohepcidin) in HD patients. (Background section, page 3, lines 9 to 16).
3. The induction of hepcidin expression by inflammation is an effect believed to be dependent on cytokine p2. Aims: the authors intend to evaluate the association between hepcidin, inflammation and iron parameters. However, the authors evaluated the serum levels of prohepcidin, and some author’s don’t found correlation between serum levels of hepcidin and of prohepcidin. This must be discussing in the paper.

Reply. As the reviewer requested, we discussed the association between prohepcidin and hepcidin in the discussion section as follows: "In the present study, we measured a serum prohepcidin level, which is one of the limitations. The detection and quantification of hepcidin in serum have been hampered by several technical difficulties. However, Costa et al reported significant positive correlation between prohepcidin and hepcidin serum levels ($r=0.624$, $p<0.0001$) (Costa E et al. Acta Haematol. 2009; 122(4):226-9). There is also evidence that prohepcidin levels are reliable indicator of hepcidin levels and activity (Dallalio G et al. Br J Haematol 2003; 122: 996–1000). Due to the lack of available laboratory methods for quantifying circulating hepcidin in clinical samples due to the small peptide size, serum prohepcidin levels are used as an indicator of hepcidin levels, which are easily measured with the widely available ELISA kits.” (Discussion section, page 10, lines 14 to 20).

4. Patients: It is not clear how the authors selected patients. In fact, the % of diabetic patients is very low in this sample. The HD patients with hepatitis C virus infection were selected firstly, and secondly HD patients without hepatitis C? The two groups of HD patients were matched by age, sex, and time under HD?

Reply. In our clinic, among 165 HD patients, 30 HD patients with chronic hepatitis C were selected according to inclusion criteria written in the manuscript. After selection of the HCV positive patient group, the controls for each case were chosen from HCV negative patients, who were matched for age, sex and time under HD, in the same center. We added this information into the methods section. (Methods section, page 3, lines 23 to 26).

5. Statistical analysis: the author’s don’t referred the statistical tests used in order to evaluate the variables that presented a non-normal distribution. Moreover, on the
tables, the authors must use median values and percentile 25 and percentile 75 to present the results of variables with a non-normal distribution. The authors also say that correlations have been performed using Pearson´s correlation. However, most of the variables have a non-normal distribution... the authors have transformed the non-normal variables in normal variables?

Reply. According to reviewer’s suggestion we used median values and percentile 25 and percentile 75 to present the results of variables (hs-CRP, iron and total iron need) with a non-normal distribution. In correlation analysis between numerical parameters for non-normal distributed variables, Pearson’s correlation test was used with transformed data. Multiple linear regression model was used to identify the independent determinants of outcome variable after adjustment for potential confounding factors for whole HD, HCV positive and negative groups. We thank to reviewer for this reminding. This was also added to the statistical analysis section of the manuscript. (Statistical analysis section, page 5, lines 24 and 25; page 6, lines 2 to 5).

6. Results: in tables, SD is greater than median value in some parameters, i.e. hs-CRP, and total iron need. These results must be presented as median (P25-P75).

Reply. As the reviewer requested, the mentioned results were presented as median (P25-P75). (Tables 1 and 2).

7. The ratio neutrophil/lymphocyte must be included in the text.

Reply. As requested, neutrophil/lymphocyte ratio was included in the text. (Results section, page 6, lines 20 and 21)

8. Discussion: some points must be included in discussion section, namely, the possible reasons why HD patients show an enhanced inflammatory process;

Reply. In HD patients, inflammation is also a well known feature and actually in our study all the inflammation markers including hs-CRP, IL-6 and TNF-α were found higher compared to healthy controls. The causes of highly prevalent state of inflammation in HD patients are multiple, including decreased renal function, volume overload, comorbidity and intercurrent
clinical events (Stenvinkel P et al. Kidney Int. 2005;67(4):1216-33). We added these points into the manuscript. (Discussion section, page 9, lines 25 to 27).

9. The differences in rhEPO doses required to achieve the hemoglobin values;
   
   **Reply.** We added the difference in rhuEPO doses between study groups into the manuscript. (Discussion section, page 9, lines 7 to 9, page 10, lines 23 and 24).

10. why the authors measured the serum levels of prohepcidin, and not directly the levels of hepcidin
   
   **Reply.** As we mentioned above, in the present study, we measured a serum prohepcidin level, which is one of the limitations. The detection and quantification of hepcidin in serum have been hampered by several technical difficulties (small size of hepcidin, limited availability of the antigen, isolation of hepcidin from urine involves complex, time-consuming procedures). However, Costa et al reported significant positive correlation between prohepcidin and hepcidin serum levels \((r = 0.624, p <0.0001)\) (Costa E et al. Acta Haematol. 2009;122(4):226-9). There is also evidence that prohepcidin levels are reliable indicator of hepcidin levels and activity (Dallalio G et al. Br J Haematol 2003; 122: 996–1000). Due to the lack of available laboratory methods for quantifying circulating hepcidin in clinical samples due to the small peptide size, serum prohepcidin levels are used as an indicator of hepcidin levels, which are easily measured with the widely available ELISA kits.” We added this comment into the discussion section of the manuscript (Discussion section, page 10, lines 15-23).

11. the levels of prohepcidin in all HD patients is very similar to that found in controls. This result must be discussed because some authors described that prohepcidin are higher in HD patients (Am J Nephrol 2008;28:677-683);
   
   **Reply.** “In recent studies, serum levels of prohepcidin and hepcidin were found to be significantly higher in HD patients compared to healthy controls (Costa E et al Acta Haematol 2009, Tessitore N et al. Nephrol Dial Transplant. 2010, Costa E et al. Am J Nephrol. 2008). In the present study, we found no difference in serum prohepcidin levels between HD patients and controls. Due to the case control nature of the present study, half of
the patients were HCV positive who had lower serum prohepcidin levels than the HCV negative patients. For that reason the mean serum prohepcidin levels of the HD patients was similar to healthy controls (142±23 ng/mL vs 137±20 ng/mL) (p=0.417). However, serum prohepcidin levels of HCV negative HD patients were significantly higher than the healthy controls [137±20 ng/mL (p=0.020)] which is consistent with previous reports” (Discussion section, page 9, lines 12 to 19).

12. serum levels of EPO seems to induce a decrease in hepcidin gene expression and inflammation an increase in hepcidin gene expression. This must also been discussed.

Reply.
We previously have discussed the role of inflammation (IL-6, hs-CRP and TNF) on prohepcidin in HD patients according to our results (Discussion section, page 9, lines 20 and 21, page 10, lines 1 to 8). However, we did not measure serum EPO and look at hepcidin expression. For that reason, we could not add any extra information into the text (Discussion section, page 9, lines 21 and 22).

Response to Reviewer 2 (Vivekanand Jha):
We made following changes in our manuscript in the light of the reviewer’s suggestions.

1. In the introduction, they say they wanted to investigate whether "iron plays a role in alterations of iron metabolism in HD patients with HCV infection", how the measurement of prohepcidin will help that is not clarified.
   Reply. We revised the introduction section to make more clear for comprehension (Background section, page 3, lines 9 to 16).

2. HCV infection has been diagnosed only by antibody screening. Did they confirm this using NAT both in antibody positive and negative cases? If not, why? What is the prevalence of this infection in their unit?
Reply. As we mentioned in the methods section, the HCV antibody status was examined using the third generation of HCV enzyme immunoassay. The sensitivity and specificity of 3rd generation of ELISA test is quite high and was reported as 98.9% and 100%, respectively (Colin C et al, J Virol Hepat, 2001). For that reason, we did not used NAT to confirm the test. In our clinic, we have been following-up 165 HD patients and of these patients 43 (26%) had chronic hepatitis C. We excluded 5 patients due to positive hepatitis B virus surface antigen (HBsAg), 2 patients due to end-stage liver disease and 6 patients due to infection or recent hospitalization within the past 4 weeks.

3. A number of statistical tests have been applied, but it is unclear what do they conver. For example, the association between prohepcidin levels and different parameters in hep c +ve and -ve patients are totally different. What is the significance of these variations? Do these have any clinical significance? All these have not been discussed.

Reply. We tried to discuss the difference in associations of prohepcidin with iron parameters, IL-6 between HCV positive and HCV negative HD patients (Discussion section, page 8, lines 22 to 25, page 9, lines 7 to 9, page 9, lines 12 to 22, ). Main clinical significance of these differences is to explain the lower requirement of rhEPO and iron in HCV positive patients, which is emphasized in the manuscript (Discussion section, page lines 24 and 25).

4. Page 7 says "multiple regression analysis was performed to predict prohepcidin...". Multiple regression can only show association and not predict anything. What were the parameters entered in this model? What type of modeling was done?

Transplant 2010;25:2685). We rewritten the regression analysis section more detailed as requested. (Results section, page 7, lines 15 to 18, lines 26 to 28; page 8, line 1 and 8 to 11).

5. One striking finding in the study is the lack of any difference between the prohepcidin levels in HD patients and healthy controls. What is the explanation of this finding, since almost all astudies show elevated leveld in HD population.

Reply. As response to concerns of the reviewer 1, we added following paragraph to the manuscript.

“Due to the case control nature of the present study, half of the patients were HCV positive who had lower serum prohepcidin levels than the HCV negative patients. For that reason the mean serum prohepcidin levels of the HD patients was similar to healthy controls (142±23 ng/mL vs 137±20 ng/mL) (p=0.417). However, serum prohepcidin levels of HCV negative HD patients were significantly higher than the healthy controls [137±20 ng/mL (p=0.020)] which is consistent with previous reports”. (Discussion section, page 9, lines 12 to 18)

6. Table 4 shows discordance between the levels of inflammatory markers. For example, IL-6 is higher in HCV-ve patients but hsCRP and TNF-a are in the opposite direction. The difference would be significant if the number of patients was more.

Reply. In the present study, only significant difference was found in serum IL-6 levels between HCV positive and HCV negative which is consistent with the previous reports (Mendoza EC et al. J Infect Dis 1996;174:842-844, Woitas RP, et al. J Immunol 1997;159:1012-1018). However, the other inflammatory markers were found similar. It’s not possible to mention the causes and clinical meaning of this finding.

7. Table 1,2 and 3,4 could be easily combined.

Reply. As the reviewer requested Table 1,2 and 3,4 were combined.

8. The correlation between prohepcidin and ferritin is not a novel finding, and the scatterplots can be done away with.
Reply. We removed the scatterplots.

9. The discussion does not try to dissect out the significance of the finding. They repeatedly mention hepatic iron stores which is not a subject of this study at all. This is a mere associative study and even that is weak. Hence making any kind of assumptions is premature. This has not been discussed.

Reply. Because of the case control design of the study, the results cannot infer a causal relationship. This is the second limitation of the study. We also mentioned this issue in the “limitation of the study section” (Discussion section, page 10, lines 20 to 22).

10. There is more literature on hepcidin levels in Hep C infected patients that the authors have not discussed. In fact some studies have shown elevated prohepcidin and IL-6 levels in these subjects. The authors have not discussed the possible mechanism of their finding of lower levels, especially in dialysis patients, as even decline in GFR leads to accumulation of hepcidin.

Reply. There is convincing evidence reporting that serum hepcidin levels were repeatedly demonstrated to be significantly lower in patients with hepatitis C (Girelli D et al. J Hepatol. 2009;51(5):845-52, Miura K et al. Hepatology. 2008;48(5):1420-9). However, Lee SH et al showed increased serum IL-6 and prohepcidin levels in patients with hepatitis C (Lee SH et al. The Korean Journal of Hepatology 2010;16:288-294). We discussed this issue and added this reference as the reviewer requested. (Discussion section, page 8, lines 22 to 25). We also discussed the possible mechanisms of lower prohepcidin levels in dialysis patients with hepatitis C. (Discussion section, page 9, lines 12 to 22)

Concerns to Reviewer 3 (Antonio Lupo):

Major Compulsory Revisions
1. I think that authors should include clinical data regarding HCV, such as: liver biopsy histological characteristics, HCV patients’ genotype, previous interferon therapies… and they should exclude important clinical HCV related biases.

Reply. As we mentioned previously, none of the HCV positive patients had signs of hepatic cirrhosis, namely clinical manifestations (ascites, jaundice, collaterals and splenomegaly) and ultrasonographical examination (Methods section, page 4, lines 9 to 12). However, we had not performed liver biopsy, which is a very invasive method. In our country, genotypes other than genotype 1 are quite rare in patients with chronic HCV infection. In a previous study, genotype 1 was observed in 97.1% of Turkish patients (Altuglu I, et al. Int J Infect Dis. 2007). Due to the very high incidence of genotype 1 in Turkish population with chronic HCV infection, HCV genotyping test was not performed to patients in this study population.

2. In order to better understand the pro-hepcidin biological activity in these patients could be interesting to take a look, in a subset of patients, to macrophage or liver ferroportin levels and to measure the degree of correlation between pro-hepcidin and ferroportin.

Reply. We thank the reviewer for this valuable contribution however we are now unable to take into consideration such hypothesis because of the limitations of the study budget.

3. The described close relationship between inflammation level and lower iron requirement in HCV positive HD patients is not really clear (See conclusion “HCV positive HD patients have low levels….. inflammation which might account for iron accumulation/or lower iron and ESA requirement in these patients”). Please explain better. Is it just a matter of IL-6? As shown in Table 4, there is no difference in hs-CRP and TNF-alpha levels between HCV pos and HCV neg patients.

Reply. We have previously reported that anti-HCV positive HD patients required less EPO and iron supplementation in order to reach target hemoglobin levels (Altintepe L et al Clin Nephrol 2004). However, lower serum prohepcidin levels were also reported in patients with chronic HCV infection and those concentrations of this molecule were found negatively associated with total iron scores and contribute to the low rhuEPO resistance and the need for
low doses in these patients (Miura K et al *Hepatology* 2008, Fujita N et al *Mol Med.* 2007, Nishina S et al *Gastroenterology* 2008). (Discussion section, page 9, lines 7 to 9, line 20 and 21). As we already addressed in the discussion section, lower serum IL-6 levels may be responsible for the decreased prohepcidin levels in HCV positive HD patients (Discussion section, page 10, lines 5 to 8). We revised the sentence “HCV positive HD patients have low levels of serum prohepcidin which might account for iron accumulation/or lower iron and rhuEPO requirement in these patients” (Discussion section, page 10, lines 24 and 25).

**Minor Essential Revisions**

1. **Discussion need to be more detailed.** Perhaps some considerations about the study could be included in it (e.g., the weaknesses and potential biases of the study, the selection of included patients etc).

As requested by the reviewer, discussion was improved and the limitations and potential biases of the study, the selection of included patients were added into this section (Discussion section, page 10, lines 15-23).

2. **The title seems not really focused.**

The title is revised as “LOWER SERUM PROHEPCIDIN LEVELS ASSOCIATED WITH LOWER IRON AND ERYTHROPOIETIN REQUIREMENTS IN HEMODIALYSIS PATIENTS WITH CHRONIC HEPATITIS C”.

Once more, we thank to contributions of reviewers on our paper.