ANSWERING REVIEWERS

August 25, 2016

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 28276-revised.doc).

Title: Presepsin teardown – pitfalls of biomarkers in the diagnosis and prognosis of bacterial infection in cirrhosis

Author: Maria Papp, Tamas Tornai, Zsuzsanna Vitalis, Istvan Tornai, David Tornai, Tamas Dinya, Andrea Sumegi and Peter Antal-Szalmas

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 28276

We are grateful to the reviewers for their positive opinion, useful recommendations and for suggesting our paper for publication. The manuscript has been improved according to the suggestions of reviewers and editor.

Answers to editor's comments:

1. We replaced the already existed author contributions section to the required place
2. We provided the required documents in PDF format regarding the approved grant application forms, ethics approval, informed consent statement, conflict-of-interest statement, and data sharing statement.
3. "Biostatistics statement" information is included now in the "Materials and Methods" section affirming that a biomedical statistician performed statistical review of the study. Certificate of statistical review signed by a biostatistician is provided in PDF format as well.
4. We replaced the already existed core tip section to the required place
5. We added audio core tip in the appropriate format.
6. We reformatted all the reference numbers according to the requested form.
7. We wrote the "Comments" section according to the editor's guidelines and also added the peer-review.
8. We provided decomposable forms of the Figures. Additionally, number of patient subgroup was also indicated in Figures that were missing in the original version of the manuscript.
9. We add PubMed citation numbers and DOI citation to the reference list and list all authors.
10. We revised the manuscript according to the reviewers' requests including.

Answers to reviewer comments:

Reviewed by 03355965

"Papp et al in this article aimed to assess the performance of presepsin in the diagnosis and prognosis of cirrhosis associated bacterial infections in comparison to CRP and PCT. Have Authors considered if there is any differences in presepsin values between gram-positive and gram-negative infections? Have Authors analyzed any case of fungal infections? Other Authors used PCT and MR-proADM as markers of bacterial infections and reported interestingly differences depending on the pathogens causing infections: gram-positive vs. Gram-negative or yeast infections. Please compare your results with these other studies by Angeletti S et al. citing these authors. Authors

should provide a Table with the median value of PCT, CRP and presepsin found in patients with infections, patients with infections and organ failure and patients without infections to evidence any differences existing and give the reader an immediate look about it. Authors should clarify which control population used for ROC curve analysis and should provide a ROC curve comparison between PCT, CRP and presepsin. Makin ROC curve comparison is possible to obtain a graphical representation that is the best way to indicate the ROC curve goodness of one marker over the others. Please follow the example of the article by Angeletti S et al."

Thank you for comments. We answer them point-by-point.

*"**Have authors considered if there is any difference in presepsin values between gram-positive, gram-negative infections? Other Authors used PCT and MR-proADM as markers of bacterial infections and reported interestingly differences depending on the pathogens causing infections: gram-positive vs. gram-negative or yeast infections. Please compare your results with these other studies by Angeletti S et al. citing these authors."***

We really agree with the remark of the ***Reviewer*** that it is important to investigate whether there are any differences in the presepsin levels depending on the pathogens causing infections: Gram-positive vs. Gram-negative or yeast infections since some of the acute phase proteins (APP). For example, procalcitonin (PCT) or mid-regional pro-adrenomedullin (MR-proADM) are interestingly different ***(Angeletti et al. APMIS 2015)***. Level of PCT is known to be higher in patients with sepsis caused by Gram-negative than Gram-positive strains ***(Kocazeybek B et al. Chemotherapy 2003, Charles PE et al. BMC Infect Dis 2008, Brodská H et al. Clin Exp Med 2013, Leli C et al. Dis Markers 2015)***. Some reports also highlighted differences in circulating cytokine levels in bloodstream infections according to Gram specificity, i.e. Gram-negative infections leaded to higher increase in the level of interleukin (IL)-6, TNF-alpha or IL-10 ***(Xu XJ et al. Intensive Care Med 2013)***. On the contrary, levels of other APPs, such as C-reactive protein (CRP), soluble (s)CD14, sCD163 or soluble urokinase plasminogen activator receptor (SuPAR) are not in relation to the Gram specificity of the infection ***(Burgmann H et al. Clin Immunol Immunpathol 1996, Huttunen R et al. J Intern Med 2011, Tornai T el al. Liver Int 2016).***

Regarding association of presepsin level to the Gram specificity of the infection, no association was reported in previous studies ***(Endo S et al. J Infect Chemother 2012, Enguix-Armada A et al. Clin Chem Lab Med 2015, Plesko M et al. Neoplasma 2016).*** In agreement with these data, presepsin levels did not differ between infections caused by Gram-negative or Gram-positive strains in our study. This was already stated in the original version of the manuscript: "Bacteria were Gram-negative in 52.6% and Gram-positive in 47.4% of culture-positive cases. Considering the type of infectious episodes, presepsin level was not different according to the location or Gram specificity of the infection (data not shown)". (*Result Section – Study Population*, Page 12 and *Association between presepsin levels and bacterial infections*, Page 13).

Here we present the median (and the corresponding 25-75 percentile) presepsin levels according to the Gram specificity of the infection for the *Reviewer*. Presepsin levels did not differ significantly in Gram negative and Gram positive infections (median, IQR: 1241 (871- 1884) vs. 852 (661-2467) pg/mL, p=0.780).

We did not discuss this finding, however, in view of pertinent data available in the literature. In the revised version of the manuscript, now we did it (*Discussion Section*, Page 17): **"It is acknowledged, that level of certain APPs are different according to the pathogens causing infections, while others are not. In a landmark study of *Angeletti S et al.* level of PCT and mid-regional pro-adrenomedullin MR-proADM were found to be significantly higher in patients with sepsis caused by Gram-negative than Gram-positive strains[42]. These data are also confirmed by other studies[43–45]. Some reports also highlighted differences in circulating cytokine levels in bloodstream infections according to Gram specificity, i.e. Gram-negative infections leaded to higher increase in the level of interleukin (IL)-6, TNF-alpha or IL-10[46]. On the contrary, levels of other APPs, such as C-reactive protein, soluble**

(s)CD14, sCD163 or soluble urokinase plasminogen activator receptor (SuPAR) are not in relation with the Gram specificity of the infection[47–50]. In the present study, presepsin level was not different according to Gram specificity of the infection, which is in agreement with previous literature findings[51–53].” We also added this information to conclusions (*Discussion Section*, Page 20): **“Level of presepsin is not associated with the pathogens causing infections.”**

“Have authors analyzed any fungal infections?”

In our cohort of patients with cirrhosis we did not detect any cases of invasive fungal infections. According to the request of the ***Reviewer*** we added this information to the *Result Section* (*Study Population*, Page 13). **“No cases of invasive fungal infections were detected.”** Inclusion of our patient population with infection and as well as sampling procedure were performed at time point of hospital admission as we stated it in *Patients and Methods* Section (Page 9). Therefore, it is not surprising that invasive fungal infections were not represented in this cohort of 75 patients with infection and liver cirrhosis. Early invasive fungal infections are infrequent in patients with liver disease and do not exceed 1% even in the intensive care setting ***(Theocharidou E et al. Clin Microbiol Infect 2015)***. Fungal infections are associated with >6-day hospital admission, >5-day inappropriate antibiotic use, central venous catheter, prior surgery ***(Bartoletti M et al. J Hepatol 2014)***.

“Authors should provide a Table with the median value of PCT, CRP and presepsin found in patients with infections, patients with infections and organ failure and patients without infections to evidence any differences existing and give the reader an immediate look about it.”

We fully agree with the **Reviewer** that it is important to provide PCT, CRP and presepsin levels in different subgroups of patients altogether making it possible to give the reader an immediate look about them. To fulfill this request we tailored the pertinent part of the *Table 1* (Page 24) instead providing these data in a separate table. Our reasons were the followings: PCT, CRP and presepsin levels in patients with and without infections have already been existed in the original version of the *Table 1*. Thus we had to add only APPs data of patients with infections according to presence or absence of organ failure. With this approach we were able to avoid redundancy in Tables. We have already six Tables.

Acute phase proteins, median [IQR]			Non-Infected	Infected		P-value
Presepsin (pg/mL)	overall		477 [332-680]	1002 [575-2149]		< 0.001
	OF absent	present		710 [533-1277]	2357 [1398-3666]	< 0.001
CRP (mg/L)	overall		4.6 [1.8-8.8]	30.1 [11.3-57.4]		< 0.001
	OF absent	present		25 [9.6-40.5]	52.2 [23.4-84]	0.027
PCT (μmol/L)	overall		0.1 [0.1-0.2]	0.4 [0.1-1.2]		< 0.001
	OF absent	present		0.2 [0.1-0.5]	1.7 [0.6-5.3]	< 0.001

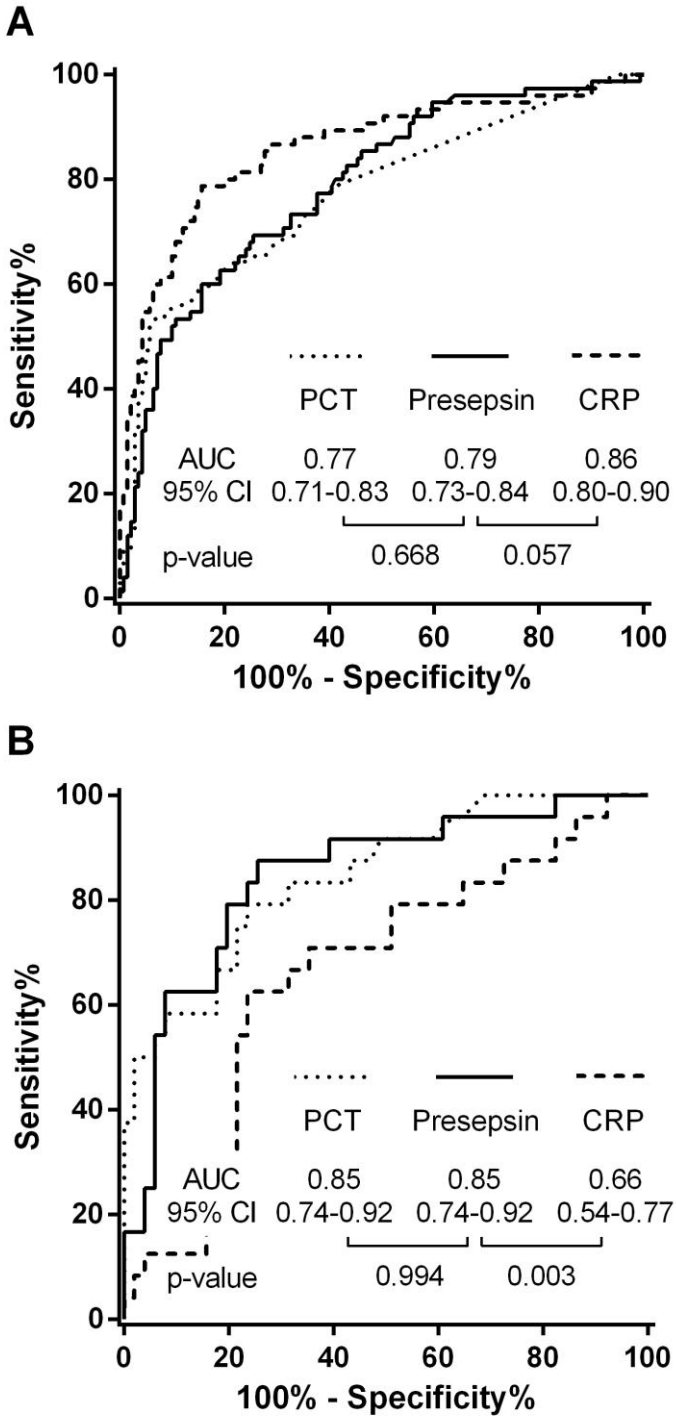
“Authors should clarify which control population used for ROC curve analysis and should provide a ROC curve comparison between PCT, CRP and presepsin. Makin ROC curve comparison is possible to obtain a graphical representation that is the best way to indicate the ROC curve goodness of one marker over the others. Please follow the example of the article by Angeletti S et al.”

We performed two ROC analyses in our study. First analysis was performed in the whole group of patients (n=216). And we assessed the diagnostic accuracy of these three APPs in the identification of bacterial infections. The control group comprised patients without bacterial infection (n=141). Second analysis was performed among patients with ongoing bacterial infection (n=75). And we assessed the

ability of these biomarkers to identify patients with organ failure during infectious episode. The control group comprised patients without organ failure during infectious episode (n=51).

Results of ROC analyses are presented in the original version of the manuscript (*Result* Section – *Accuracy of presepsin level in the diagnosis of bacterial infections compared to classic acute phase proteins*). In the pre-final version, graphical presentations of the ROC curves were included. However, we wanted to avoid presenting redundant data as text and as graph simultaneously, and therefore the graphs were deleted. We fully agree with ***Reviewer***, however, that it is more representative to have a graphical presentation at hands about ROC curves. Accordingly, we included the ROC curves (**Figure 3**) (Page 34) in the revised version of the manuscript. In the figure legend we clearly indicated the groups where the ROC analysis were performed as we explained above.

Figure 3. Receiver-operating characteristic curves of presepsin, procalcitonin and C-reactive protein for the identification of bacterial infection overall (A) or bacterial infection complicated by organ failure (B). ROC analysis were performed (A) in the whole cohort (n=217) or (B) in patients with bacterial infection (n=75). The control group comprised (A) patients without bacterial infection (n=141) or (B) patients with bacterial infection without organ failure (n=51).
OF: organ failure, AUC: area under curve, CI: confidence interval, CRP: C-reactive protein, PCT: procalcitonin


A
Sensitivity%
100% - Specificity%
PCT
Presepsin
CRP
AUC
95% CI
0.77
0.71-0.83
0.79
0.73-0.84
0.86
0.80-0.90
p-value
0.668
0.057
B
Sensitivity%
100% - Specificity%
PCT
Presepsin
CRP
AUC
95% CI
0.85
0.74-0.92
0.85
0.74-0.92
0.66
0.54-0.77
p-value
0.994
0.003

Answers to reviewer comments:
Reviewed by 02943023

"This study suggests that presepsin is a promising biomarker during diagnostic procedure of bacterial infections in cirrhosis for enhancing diagnostic capacity of CRP and reflecting more accurately the severity of infections. Performance of presepsin is equal to PCT in these clinical settings. However, procalcitonin but not presepsin is a biomarker for predicting infection-related short-term mortality in patients with cirrhosis. I think this study is very well written with valuable results to publish. I just would like to ask couple of things. 1. Did you analyze the presepsin level according to the presence of 'acute-on- chronic liver failure'? 2. What do you think the reason for only procalcitonin but not presepsin is a biomarker for predicting infection-related short-term mortality in patients with cirrhosis? 3. Do you have any suggestion for more aggressive or preemptive antibiotic therapy according to presepsin level in cirrhotic patients? It will be great if you answer about questions in the 'Discussion' section of the manuscript. Thank you so much."

Thank you for comments. We answer them point-by-point.

Question 1: ***"Did you analyze the presepsin level according to the presence of 'acute-on-chronic liver failure'?"***

For this study we included patients between May 2010 and April 2011. European definition of acute-on chronic liver failure (ACLF) was not established that time. Presence and grade of organ system failure(s) [OF] were determined retrospectively based on the available clinical and laboratory data after accessibility of CLIF-C Organ Failure Score (***Arroyo V. et al. J Hepatol 2015***) (*Material and Methods* Section – Data Collection, Page 9). In *Figure 1*, we presented presepsin levels according to this classification (Page 29). And also in the *Results* Section (Association between presepsin levels and bacterial infections, Page 13-14). "Presepsin level was associated with the severity of the infection. Twenty-four infections (32%) were complicated with at least one OF. Presepsin level was significantly higher in patients with OF as compared to those without (2358 pg/mL [1398-3666] vs. 710 pg/mL [533-1277], $p<0.001$) (Figure 1)."

Question 2: ***"What do you think the reason for only PCT and not Presepsin is a biomarker for predicting infection-related short-term mortality in patients with cirrhosis?"***

From a *biological point of view* PCT has a different profile than presepsin or other acute phase proteins (APPs) and pro-inflammatory cytokines. (1) Procalcitonin belongs to a different class of molecules, which may be called "hormokines" indicating cytokine-like behavior of the molecule during inflammation and infection ***[Müller et al. J Clin Endocrinol Metab. 2001]***. (2) It is produced by parafollicular cells (C cells) of the thyroid and by the neuroendocrine cells of the lung and the intestine and not primarily in the liver. Instead of one, it represents several organ involved into the pro-inflammatory response ***[Matwiyoff GN et al. Inflamm Res, 2012]*** (3). Lastly, it has been demonstrated that PCT poses harm to the host. Administration of PCT to septic animals greatly increases mortality, and several toxic effects of PCT have been elucidated by *in vitro* experimental studies as well. Antibodies have been developed that neutralize the harmful effects of PCT, and their use markedly decreases the symptomatology and mortality of animals that harbour a highly virulent sepsis analogous to that occurring in humans ***[Nylen ES et al. Crit Care Med 1998]***. Presepsin represents activation of the monocyte-macrophage system of which more than 80% is located in the liver. Activated monocytes and macrophages can pose harm to the host with the production of excessive amount of inflammatory cytokines. They are involved, however, in the resolution of the inflammatory process, and promote clearance of tissue debris during inflammation in the presence of

local microenvironmental anti-inflammatory signals such as interleukin-10 (M2-type, pro-resolution, anti-inflammatory liver macrophages) ***[Sica A et al. J Clin Invest. 2012]***. In this regard, activation of macrophages and monocytes is not only a representative of immune cell activation that may contribute to tissue damage, but also activation of processes involved in tissue repair.

From a *statistical point of view* however, we emphasize that presepsin was a predictor of poor outcome/short-term mortality in the univariate analysis. However, in the multivariate analysis presepsin did not retain independent prognostic capacity. In multivariate analysis/modeling a sample size of n=100 is considered sufficient to avoid type 2 error. In our study we had 75 patients with bacterial infection, that could have resulted in a type 2 error. It is also important to consider the effect size. The smaller the effect size of a biomarker the higher is the number of patients that should be included in a study to retain independence after adjusting for different clinical factors. It is likely that Presepsin has smaller effect size than PCT.

According to the suggestion of the ***Reviewer***, we extended the previous succinct explanation about the revealed differences in the impact of APPs on short-term mortality. We deleted the related sentence from the *Discussion* (Page 20). "This finding suggests that the increase in PCT particularly represents the exaggerated inflammatory processes in cirrhosis-associated infections") and added the followings: **"From biological point of view this finding might be explained by the fact that PCT has a different profile. It belongs to a distinctive class of molecules, so-called "hormonkines" [81]. Procalcitonin has a cytokine-like behavior during inflammation and infection. It is produced primarily in neuroendocrine cells of various organs and represents involvement of several instead of one organ into the pro-inflammatory response [82]. Lastly, it has been demonstrated that PCT has various toxic effects and pose harm to the host. Administration of PCT to septic animals greatly increases mortality. Antibodies directed against PCT are able to ameliorate harmful effects of PCT with a marked decrease symptomatology and mortality of sepsis [83]. Presepsin represents activation of the monocyte-macrophage system during inflammatory process. Macrophages have a dual effect: production of excessive amount of inflammatory cytokines can cause tissue damage but involvement in the resolution of the inflammation promote tissue repair. This latter process is driven by M2-type macrophages in the presence of local microenvironmental anti-inflammatory signals such as IL-10 [84].**

Question 3: ***"Do you have any suggestion for more aggressive or preemptive antibiotic therapy according to presepsin level in cirrhotic patients?"***

Biomarker tailored antibiotic therapy during bacterial infection is still an unmet need in clinical practice. We really agree with the *Reviewer* thus this is a remarkable issue and should be evaluated in case of emerging new biomarkers by all means. The point is that several concerns are raised regarding antibiotic stewardship using biomarkers in cirrhosis, but only some of them can be answered based on findings of present study.

(1) Antibiotic regime is different based on Gram specificity of the infection. Thus association of the biomarker level to the pathogens causing infections is of importance in this regard. At present, there are only a few biomarkers that are able to distinguish between Gram-negative and -positive infections, and based on previous and our data presepsin seems no to be one of them. Please see the answer of *Reviewer 03355965*.

(2) The choice of initial empirical antibiotics is based on the type, severity and origin of infection, recent antibiotic use and on the local epidemiological data about antibiotic resistance. Out of these considerations, in our patient cohort presepsin was associated with the severity of infection. In ROC analysis the best cut-off value of presepsin for identifying severe infection was 1206 pg/mL. Since bacterial infection complicated by OF is associated with significant mortality in cirrhosis, it is rational that empiric antibiotic regimen should have a broad spectrum in this group of patients. And thereafter,

if the causative organism is identified (about 50% of cases), antibiotic regimen should be narrowed to decrease the likelihood of emergence of antibiotic resistance. Based on our data, however, it is early to suggest this approach. On the one hand, in our study diagnostic accuracy of presepsin decreased in advanced disease stage or in the presence of acute complications (e.g. renal failure) supporting that a single diagnostic cut-off value of a biomarker has certain caveat in cirrhosis. On the other hand, negative (NPV) and not the positive predictive value (PPV) of this presepsin level was high (92.7% vs. 61.8%). The rate of severe infection in patients with presepsin level <1206 pg/mL was rare. This cut-off for presepsin provided a level of security to exclude and not certainty of presence of severe infection.

(3) It is an intriguing issue whether in cirrhotic patients with severe infection success of therapy and duration of antibiotic treatment can be evaluated and individually adapted by presepsin measurement. Unfortunately, our study design (single-point measurement, retrospective) is inappropriate to address this important issue.

English language editing was performed by a native English speaker. Certificate is attached as well.

Linguistic edits and other changes in the content of the text were highlighted **in red**.

Please find attached the "highlighted" version of the manuscript with the detailed changes that were made according to the reviewers' comments.

The following required accompanying documents (in a pdf format) are resubmitted along with our revised paper (28276-revised_Highlighted_FINAL, 28276-revised_CleanCopy):

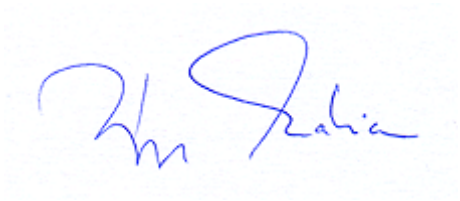
28276_1_Institutional_Review_Board_statement_WJG2016
28276_2_Informed_consent_statement_WJG2016
28276_3_Certificate_of_statistical_review_WJG2016
28276_4_Conflict_of_interest_statement_WJG2016
28276_5_Data_sharing_statement_WJG2016
28276_6_Language_edit_letter_WJG2016
28276_7_Approved_Grant_Application_Format_WJG2016
BPG_Copyright_Assignment_28276

We would like to thank you again for the helpful comments and for considering our paper. We do hope that the changes that have been made, have improved the quality of the manuscript also with regards to the presentation of the data.

All authors have fulfilled the criteria of authorship and seen and approved the final version of the revised manuscript and they have authorized the first author to grant on behalf of all authors to transfer exclusive copyright to World Journal of Gastroenterology in case of acceptance.

We do hope that the new data presented could be of interest to the readers of the World Journal of Gastroenterology.

Sincerely yours,



Maria Papp, MD, PhD
Institute of Medicine
Department of Gastroenterology
University of Debrecen
Nagyerdei krt. 98, Debrecen
H-4032 Hungary
Phone/ Fax: +36 52 255 152

e-mail: papp.maria@med.unideb.hu
drpappm@yahoo.com