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Editor-in-Chief

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Dear Editor-in-Chief

Please find enclosed the edited manuscript in Word format (file name: WJG 204-Revision 2.doc).

Title: Measurement of calprotectin in ascitic fluid to identify elevated polymorphonuclear cell count.

Author: Emanuel Burri, Felix Schulte, Jürgen Muser, Rémy Meier, Christoph Beglinger

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 204

The manuscript has been improved according to the excellent and helpful suggestions of reviewers. All changes within the text have been highlighted in yellow. Please find a detailed point-by-point list of all changes made to the manuscript keyed to the reviewer comments below.

Comments by the Editorial Office

Format has been updated.
A Comments section has been added.
References have been added.

Comments by Reviewer 1

A power calculation does not appear to have been done so why did the authors stop after 130 samples?

This was an exploratory study to demonstrate the ability of calprotectin measurement in ascitic fluid to detect elevated polymorphonuclear cell count. Our goal was to prove, that the concept of measuring a polymorphonuclear inflammation protein rather than polymorphonuclear cells to detect elevated cell count in ascites would be feasible. We intended to lay the groundwork for future studies to further investigate the use of ascitic calprotectin. We are aware that in order to fully validate this test in different clinical settings, a larger number of patients would be required.

In the Methods section under Statistical Analysis, we have introduced a sentence explaining that no formal sample size calculation was done because of the exploratory nature of the study (page 9, lines 1f). This is now also specifically mentioned in the limitations of the study under the section Discussion (page 15, lines 24f).

Comments by Reviewer 2

The authors need to discuss the role of reagent method in the diagnosis of SBP and add the relevant articles (Riggio O, Angeloni S. World J Gastroenterol. 2009 Aug 21;15(31):3845-50.)

As suggested by the reviewer, we have expanded the discussion on reagent methods and have included the relevant references mentioned in the article by Riggio et al (page 14, lines 23f).

The authors have used point of care testing of ascitic fluid calprotectin. Will it be possible to apply in other set up? They need to discuss the cost efficacy as well?

The point of care testing device we used in our study (Quantum Blue) is commercially available by Bühlmann Laboratories in Switzerland. It is being widely used for the rapid measurement of fecal calprotectin. For the measurement of ascites, the dilution series and preparation of the biological samples is slightly different. The basic technique of quantitative measurement of calprotectin is identical. In theory, measurement of calprotectin using this point of care device would also be possible in other settings, e.g. to analyze synovial fluid in order to distinguish between reactive and infectious arthritis. The measuring device itself costs 2500 US dollar, while the cartridges cost 20 US dollars per analysis (page 8, lines 6f). Although we have not carried out a formal cost efficacy analysis, the use of this point of care device is likely to be less expensive than traditional methods, especially if salary costs for laboratory personnel are taken into account. We have added a section on cost effectiveness in the Discussion (page 15, lines 23f).

Comments by Reviewer 3

Among the contributing authors there is the abbreviation LR, who is said to have participated in the acquisition of the data, but who I am unable to identify among the authors listed.

We would like to thank the reviewer for that remark. This is a mistake on our side. LR did not take part in this study and thus is not mentioned in the authors list.

Page 5 lines 9-10: “.. patients with refractory ascites were ...” Why did the authors use “refractory ascites”, which has a precise meaning, while above they said “All patients with ascites were eligible for the study? In the Results section it is also said: “Most pts were referred for diagnostic paracentesis”. Did the patients have true refractory ascites? Please comment

We prospectively included all patients referred for paracentesis. In 24 patients, the procedure was performed more than once, either because of true refractory ascites (N=6) or in order to check for PMN cell count in patients with SBP under treatment, to drain temporary ascites during hepatic decompensation, or in malignant disease.

We have eliminated the sentence “Samples from patients with refractory ascites were collected at every paracentesis” to avoid misunderstandings in terms of patient inclusion (page 6, line 9).

Page 6 lines 9-10: “blood samples were also obtained at the same time” Why? There are no blood results mentioned in the text.

Blood samples were drawn to calculate the serum-ascites albumin gradient (SAAG), to classify patients according to the Child-Pugh Turcotte Class, and to calculate the The Model for End-Stage Liver Disease (MELD) score.

Page 9 lines 2-3: 71 patients were included in the final analysis. The sum of patients: 54 with liver cirrhosis, 11 with malignant ascites, etc. etc. is more than 71. Please verify.

As suggested by the reviewer, we have clarified the wording of this part of the manuscript (page 10, lines 13f). A total of 71 patients were included in the final analysis: 54 patients with liver cirrhosis (2 of them also had malignant ascites), 11 with malignant ascites (including 2 patients with liver cirrhosis), 3 patients with heart failure and 5 patients with portal hypertension from metastatic liver disease (no malignant cells in ascites). $54 + 11 + 3 + 5 = 73$, but since 2 patients have liver cirrhosis and malignant ascites, the final patients count is 71.

Page 9, last two lines: “ ... POC tests (median 0.38 µg/mL (IQR is lacking) 0.38 – 5.62 (range 0.38 -13.31).” Are these numbers correct? I’m wondering, if the median is 0.38, how it is possible for the range to be 0.38-13.31 Is it perhaps because more than half of these patients have calprotectin values of 0.38, which is the lowest value? Please comment.

The point of care device is using lateral flow techniques to measure ascitic calprotectin. The limits of measurement for this device are 0.375 µg/mL to 3.8 µg/mL. Below and above those cut-offs, calprotectin concentrations can no longer be exactly measured and results become unreliable. In a majority of the samples measured, the PMN count was low. Therefore, calprotectin concentrations in ascites were also low, often below the lower cut-off point. This is why the distribution of calprotectin concentrations, and hence the median value, are shifted towards the lower cut-off point. The lowest measurable value is 0.375 µg/mL.

There has been a mistake with the upper value of the interquartile range. We have changed it from 5.62 to 0.56 (exact value 0.562) (page 9, line 25). Additionally we have added “IQR” before 0.38-0.56 (page 10, line 33)

Page 10: lines 8-11: “Immediately following paracentesis samples (N=17),.... “ In these 17 patients there is a real gain in time to make the diagnosis, but the number is too small. Therefore, how many of these patients have PMN >250/ μ L of ascites? How many of these patients are cirrhotic?

We fully agree with the reviewer, that the full advantage of a point of care test would be to use it as a real bedside test, as we did in the patients mentioned above. However, in order to maintain a high quality from a methodological point of view and to standardize point of care measurement as much as possible, we decided to primarily measure all samples in a central laboratory by experienced technicians, who were blinded to any information regarding the patients history or laboratory values.

The ancillary study including 17 samples was done merely to demonstrate the feasibility of real-life bedside measurement of ascitic calprotectin with the point of care test and to assess the comparability with measurements done in a laboratory. It was not the goal to validate the test itself and we agree, that the small number of patients does not allow such conclusion. Of the 17 samples, 11 were from cirrhotic patients and 2 had PMN >250/ μ L.

Page 12, lines 7-11: “It is clinically significant that calprotectin a readily-available bedside test.” The number of the bedside POC testing patients is too small (17 cases) to draw firm conclusions on its utility; “...and encourage its use as a diagnostic test for SBP screening” (only 4 patients were found positive for SBP (still too few); why for screening?

Of the study population all 130 samples were not only measured with the established ELISA test, but also with the point of care (POC) test. As mentioned above, the reason why measurements were performed in a central laboratory by experienced technicians otherwise not involved in patient care, was to ascertain a high methodological quality of the study. However, the methods used were identical to the ones performed by EB at the study site.

Current guidelines from the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Disease (AASLD) recommend paracentesis be performed in all patients with ascites to rule out abdominal infection, both in patients being hospitalized but also in outpatient settings. Accordingly, PMN cell count is routinely performed in those patients to screen for SBP. The test characteristics of ascitic calprotectin provide high sensitivity, both with the ELISA and the POC (both measured in 130 samples) and therefore encourage its use as a screening test for patients with ascites. Further studies will show, if measurement of calprotectin in ascites can fully substitute PMN counting.

We agree that the number of patients with SBP is too small to fully validate this test. This is acknowledged under the limitation in the Discussion section. However, we showed that ascitic measurement reliably identifies PMN count >250/ μ L.

Page 12, lines 7-11: “.... especially in conjunction with the use ...” Why in conjunction? Are two calprotectin tests proposed? Please clarify.

As suggested by the reviewer, we rephrased the sentence to clarify its meaning (page 10, lines 10f).

This section is sometimes repetitive (repetition of the Introduction section) and should be shortened.

As suggested by the reviewer, we have substantially changed the Discussion to avoid repetition and shortened the text.

Figure 2: At the end of this legend, 3A and 3B have to be changed to 2A and 2B (is that correct?)

We thank the reviewer for that remark. As suggested, we changed the labeling (page 19, line 12)

Table 2: It is not clear to me why the ranges of the Total cell count and of the PMN count do not coincide with those given in the Results section, page 9, Ascitic fluid cell count subsection.

We thank the reviewer for pointing that out. Total cell count and PMN count given on page 9, line 10 describe the range (minimum and maximum) of all values measured for the whole study population. It illustrates the widespread distribution of cell counts. Table 2 on page 26 gives median total cell count and median PMN count in patients with and without elevated PMN cell count. We have changed Table 2 to give maximal and minimal values instead of percentiles for those two parameters (page 26).

As the Authors said in the last two lines of the manuscript, the main aim of this work is to diagnose SBP in cirrhotic pts with ascites. Attempts to make a diagnosis of SBP at bedside are welcomed, but in this study there were few patients with liver cirrhosis, those found with PMN count >250 very few and those with SBP rare (only 4 cases), therefore the AA should be more cautious in suggesting the calprotectin test in all pts with ascites. It would be more prudent to suggest this assay, above all the POC bedside test, in those hospitals where appropriate instrumental equipment or qualified personnel for the PMN count is not available.

As mentioned above, we acknowledge the fact that the number of SBP patients is too small to fully validate measurement of ascites as a diagnostic test for SBP. However, our study shows, that ascitic calprotectin reliably identified PMN >250/uL, both with the ELISA and the point of care test. As suggested by the reviewer, we have changed the wording of the Conclusion accordingly (page 16, lines 5f).

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,
Emanuel Burri