Format for ANSWERING REVIEWERS

November 20, 2013

Dear Editor,

The Authors would like to thank the Editor and the Reviewers for their valuable comments and suggestions on how to improve the manuscript.
Please find enclosed the edited manuscript in Word format (file name: 6595-edited.doc).

Primary title: Serum and urine metabolomic fingerprinting in inflammatory bowel diseases - ^{1}H NMR-based study
Title after revision: Serum and urine metabolomic fingerprinting in diagnostics of inflammatory bowel diseases

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Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 6595

1 The format of the manuscript has been improved according to the reviewers' suggestions:

a) The format of all quotations in the text has been corrected.
b) The format of all references has been corrected.
c) All abbreviations appearing in the tables or figures have been listed and defined.
d) The titles of the figures have been modified.
e) The current title is shortened (it has less than 12 words) and does not contain any abbreviations – "Serum and urine metabolomic fingerprinting in diagnostics of inflammatory bowel diseases".
f) Some minor corrections in the text have been made, e.g. the abbreviation ^{1}H NMR was defined in the abstract and in the main text, etc.
g) The format of figures has been changed so now the fonts and lines can be edited or moved.
h) The COMMENTS section is included.

2 The revision has been made according to the suggestions of the reviewers:

I. Reviewer No. 00044334:

(1) "I wonder if the metabolite markers elevated in IBD samples are truly specific for IBD but not in nonspecific intestinal inflammation such as diverticulitis or infections colitis. To this, they should compare these data to those from nonspecific intestinal inflammatory diseases."

Indeed, it is hard to say if the metabolite markers elevated in IBD samples were truly specific for IBD. Unfortunately, we did not perform our study in patients with other type of colitis, additionally, to the best of our knowledge no such a study has ever been done. Taking into account these fair objections as well as following the suggestion of another reviewer we decided to focus our findings on a comparison between the group of patients with active IBD and the group with IBD in remission.

(2) "The changes in metabolite markers between IBD and controls need to be reasonably explained based on their potential pathogenetic mechanisms."

An attempt at a biological interpretation of metabolomic results was given in the Discussion section. Consequently, 10 papers were added in references (items 43-60).

(3) "Did the authors perform a validation study using independent another cohort of IBD samples?"

The validation study using independent another cohort of IBD samples was not performed.

(4) "How did the authors eliminate medication signals? What about immunomodulators or biologics?"

Firstly, spectra with signals generated by medicaments were recorded. As the spectra were analysed the signals originating from acetaminophen, 5-aminosialicylate and azathioprine (the information about the last one was added in the text) were eliminated from analysis by excluding particular ppm regions. Among the patients included in the study, no one received mercaptopurine, methotrexate or biologics.

(5) "Their current findings do not support the conclusion that 1H NMR-based metabolic fingerprinting of human serum and urine combined with ... could be a very useful tool in IBD diagnostics."

We have modified the conclusions in the abstract ("NMR-based metabolomic fingerprinting of serum and urine has the potential to be a useful tool in distinguishing patients with active IBD from those in remission") as well as the final conclusions of the manuscript ("The results of our study demonstrate that ^{1}H NMR-based metabolic fingerprinting of human serum and urine combined with multivariate data analysis could be a useful tool in distinguishing patients with active IBD from those in remission that is of great importance in IBD monitoring. At the same time, the results indicate that this diagnostic method has rather a weak potential in the differential diagnosis of IBD.").

II. Reviewer No. 02571956: No comments.

III. Reviewer No. 02685459:

(1) "While the study is correctly performed from the technical point of view, I rather disagree with the main claims of this manuscript. Actually, most findings were already demonstrated in previous studies. For example, Schicho et al (ref 24) already showed the interest of 1H NMR metabolomics to characterize IBD, and they already tried to find biomarkers characteristic of such disease. In fact, the only new finding of the present manuscript is the differentiation between IBD patients in active phase or in remission. This is interesting and relevant for publication, but this main message is hidden a number of claims which are not new or erroneous. For example: -the main claim of the manuscript ("core tip", p.4, and see also p. 17) is that this study demonstrates the usefulness of 1H NMR metabolomics as a diagnostic tool for IBD. This is not true, especially because the manuscript shows that no difference is observed between CD and UC patients...which adds nothing to previous studies. Moreover, the potential usefulness of the method was already highlighted by Schicho et al, so it should not form the main claim of the present manuscript -the main conclusion (p.4 and in the conclusion) should therefore be more modest, and centered on the ability of 1H NMR to differentiate between patients in active or remission phase. Moreover, this is only possible with urine (not with serum) -the results clearly claim (p. 3) that "the most significant differences in metabolomic profiles were found between the group of patients with active IBD and healthy control subjects"....OK, but this was already demonstrated by Shicho et al. [...] In particular, the authors should center the manuscript on the main message (ability to distinguish between active/remission phases) and be careful to give full credit to previous authors when their main claims correspond to results that were previously published."

Following these suggestions we have focused our manuscript on the comparison between the group of patients with active IBD and the group with IBD in remission. The structure of the abstract, the results and discussion sections has been revised, so these findings are specified as the most important. We have also modified the conclusions contained in the abstract ("NMR-based metabolomic fingerprinting of serum and urine has the potential to be a useful tool in distinguishing patients with active IBD from those in remission") as well as the core tip and the final conclusions of the manuscript ("The results of our study demonstrate that ^{1}H NMR-based metabolic fingerprinting of human serum and urine combined with multivariate data analysis could be a useful tool in distinguishing patients with active IBD from those in remission that is of great importance in IBD monitoring. At the same time, the results indicate that this diagnostic method has rather a weak potential in the differential diagnosis of IBD."). At the same time, we would like to preserve the elements of the manuscript referred to the CD and UC comparison as well as IBD vs. healthy control subjects. To date there are just a few papers that have evaluated the usefulness of metabolomic based diagnostic tests of serum (the first one of 2012) and urine in patients with IBD, moreover, only part of them refers to the differential diagnosis between CD and UC, at the same time, the conclusions of these studies are at least in part contradictory.

(2) "No attempt was made to interpret (from the biological point of view) the results observed between sample groups. I know this is sometimes difficult, but the authors should try to give some biological interpretation to their metabolomic results."

An attempt at a biological interpretation of metabolomic results was given in the Discussion section. Consequently, 10 papers were added in references (items 43-60).

(3) "Finally, a number of minor point should also be corrected":

- "The NMR parameters are given in a paragraph called "serum and urine sample preparation"...the title does not seem appropriate"

  The title was changed to "Sample handling and NMR spectroscopy"

- "NMR parameters: why using a 20 ppm spectral width, as the peaks are spread over 9 ppm only?"

  The wider spectral weight SW was used for all the spectra to enable a better phase and baseline correction. It has no influence on the obtained results.

- "When the acquisition time is given, the unit should be added (seconds)"

  The appropriate units were added.

- "How was the choice of the pulse sequence made? (cpmgpr1d vs noesypr1d or zgpr?)"

  Only cpmg pulse sequence among those mentioned (cpmgpr1d, noesypr1d, zgpr) provides effective filtering of signals originating from macromolecular compounds. In terms of urine samples the choice of noesypr1d sequence is due to better water signal suppression than standard zgpr. The pulse sequences were selected according to the well-established protocol of Beckonert et al. [Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat Protoc* 2007; 2: 2692-2703]

- "What were the parameters of the cpmg pulse sequence? (number of echoes, duration of the

cpmg train)"

The one-dimensional (1D) Carr-Purcell-Meiboom-Gill (CPMG) spectrum with water presaturation was acquired to filter out any broad resonances arising from the presence of proteins and to enable observation of low molecular weight compounds (number of loops = 80, spin echo delay = 400 □s). Each spectrum consisted of 128 scans and was stored in 64k data points. After Fourier transformation of spectra, the baseline and phase were manually corrected using Topspin 1.3 software (Bruker, GmBH, Germany).

- "Page 9: "Serum samples are much more stable" instead of "stabile"

The phrase was corrected.

- "No information is given about the bucketing procedures: how was it performed? with identical or variable bucket size?"

No special bucketing procedure was used. The whole study is based on the concept of fingerprint analysis, therefore after careful alignment of NMR resonances all spectrum data points were utilized for multivariate modeling.

- "In Figure 1, it seems that different vertical scales were employed for different parts of the spectra (noise level different from right to left). This should be indicated in the legend."

The information about the zoom level was added in Figure 1 (aromatic region was zoomed 16x).

(4) "The table and figure legends should be more complete, so that the reader can understand what the figure is about without referring to the text."

All abbreviations appearing in the tables or figures were listed and defined. The titles of the figures were slightly changed according to the reviewers' suggestions.

3 References and typesetting were corrected

We believe that the quality of the revised version of the manuscript has been improved making the paper suitable for publication in the *World Journal of Gastroenterology*.

Sincerely yours,

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