

Dear Editors:

Manuscript NO: 75509

Title: Intestinal mucosal barrier in functional constipation: Dose it change?

Thank you very much for giving us the precious opportunity to revise our manuscript. We appreciate the comments and suggestions from the reviewers. These comments are all highly insightful and enable us to greatly improve the quality of our manuscript. We fully agree, and have studied the reviewers' comments very carefully and have tried our best to improve the manuscript. We also revised the manuscript according to the science editor's comments and suggestions. The followings are our point-by-point responses to the original reviewer's remarks underneath each comment. Revised portions are marked in yellow in the revised version of the manuscript.

All the best.

Yours Sincerely,

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Dear reviewer 1 (02440884):

Thanks a lot for giving us the valuable opportunity to revise our manuscript. We sincerely appreciate the valuable comments and suggestions, which are very helpful for revising and improving our paper. We have studied your comments very carefully and tried our best to improve the manuscript. Our point-by-point responses to the original remarks are provided underneath each comment.

Comments to the Author:

1. Is there any change in ultrastructure, in particular is there an increase in the length of the zonula occludens in functional constipation?

Response:

This is indeed an intriguing and worthwhile question. According to the comments, we consider that “zonula occludens” refers to “tight junctions”. In this study, we observed that tight junctions (*i.e.*, zonula occludens) in FC patients presented an intact and continuous ultrastructural appearance without obvious changes. However, due to the existing experimental conditions and possible differences from section making, we did not measure the length of the zonula occludens. Tight junctions locate at the most apical region of intercellular junctions and are responsible for intercellular sealing. Therefore, we consider that paracellular permeability may primarily depends on the size of intercellular space rather than the length of tight junctions. Moreover, recent observations suggest that tight junctions can be very dynamic rather than unchanged [1-3], which might bring uncertainty to the length measurement. On the other hand, we carefully reviewed the literature on ultrastructure of tight junctions, but there is no information available about the length. From the perspective of ultrastructure, current studies still focus on the continuity of tight junctions and width of intercellular space [4-6]. In summary, we used an observational approach consistent with the existing studies and the results should be convincing. We hope that future studies could explore an optimal

method to evaluate the length of tight junctions, which will lead to a better understanding of their functions, especially in pathological conditions.

References

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- [2] Matter K, Balda MS. Signalling to and from tight junctions. *Nat Rev Mol Cell Biol.* 2003, 4(3):225-236.
- [3] Sasaki H, Matsui C, Furuse K, et al. Dynamic behavior of paired claudin strands within apposing plasma membranes. *Proc Natl Acad Sci U S A.* 2003, 100(7):3971-3976.
- [4] Martinez C, Lobo B, Pigrau M, et al. Diarrhoea-predominant irritable bowel syndrome: an organic disorder with structural abnormalities in the jejunal epithelial barrier. *Gut.* 2013, 62(8):1160-1168.
- [5] Zhu H, Xiao X, Shi Y, et al. Inhibition of miRNA-29a regulates intestinal barrier function in diarrhea-predominant irritable bowel syndrome by upregulating ZO-1 and CLDN1. *Exp Ther Med.* 2020, 20(6):155.
- [6] de Oliveira SS, de Oliveira IM, De Souza W, et al. Claudins upregulation in human colorectal cancer. *FEBS Lett.* 2005, 579(27):6179-6185.

2. In addition to occludin, the claudin proteins are important transmembrane proteins of the zonula occludens. The molecules are mentioned in the Introduction of the manuscript. It comes not clear to the reader that the investigations are focused on occludin and claudins are not further studied. This point should be addressed by additional experiments or some explanations in the body of the Introduction and/or Discussion.

Response:

We sincerely thank you for this kind suggestion. We are sorry that we did not evaluate claudins in the present study. The claudin family contains multiple members in humans with different functional properties and intricate interplay^[1-3], which makes it difficult to analyze the effect of a single claudin. To counterbalance this shortcoming to a certain extent, we used multiple

methods to evaluate the intestinal mucosal barrier in FC patients. And we will focus on intestinal claudins in future studies. In accordance with the kind suggestion, corresponding explanations have been added in the 2nd paragraph on P 5 (Introduction part), 2nd paragraph on P 14 and 3rd paragraph on P 16 (Discussion part), highlighted in yellow.

References

- [1] Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology*. 2001; 120: 411-422.
- [2] Markov AG, Aschenbach JR, Amasheh S. Claudin clusters as determinants of epithelial barrier function. *IUBMB Life*. 2015; 67: 29-35.
- [3] Furuse M, Sasaki H, Tsukita S. Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol*. 1999; 147: 891-903.

Dear reviewer 2 (02567669):

Thank you very much for giving us the precious opportunity to revise our manuscript. We sincerely appreciate the valuable comments and suggestions, which are very helpful for revising and improving our paper. We have studied your comments very carefully and tried our best to improve the manuscript. Our point-by-point responses to the original remarks are provided underneath each comment.

Comments to the Author:

My only concern relates to the functional tests of gut permeability: Zonulin family proteins and intestinal fatty acid binding protein in the serum (and potentially in the stool). Have the authors done these tests, too? If yes, I suggest to include these data into the present manuscript.

Response:

We sincerely appreciate your kind comment and good suggestion. In fact, at the beginning of the study, we selected two commonly used serological

markers based on previous studies ^[1-4], *i.e.*, D-lactic acid and zonulin. However, considering that zonulin is closely related to small intestinal permeability ^[5], but the remaining tissue samples collected in the present study were derived from the colonic mucosa. Therefore, we did not include this part of the experimental data in the manuscript. To make our article more complete, we fully agree with your good suggestion, and add the data related to serum zonulin to the present manuscript, please see the 5th paragraph on P 12 (Results part), 3rd paragraph on P 15 and 1st paragraph on P 16 (Discussion part), highlighted in yellow. There were no significant differences in serum D-lactic acid or zonulin levels between FC patients and healthy controls, which were consistent with other results of the study. Thus, our data are convincing. We are sorry that we did not detect intestinal fatty acid binding protein (I-FABP) and acknowledge that this is a shortcoming of the article. Nonetheless, this does not seem to influence the conclusion of the paper, because a number of previous studies also used one or two serum indicators to assess intestinal mucosal barrier function and obtained reliable results ^[6-9]. We will focus on this indicator in future research. It has been interpreted in the 3rd paragraph on P 16 (Discussion part), highlighted in yellow.

References

- [1] Demircan M, Cetin S, Uguralp S, et al. Plasma D-lactic acid level: a useful marker to distinguish perforated from acute simple appendicitis. Asian journal of surgery. 2004, 27(4):303-305.
- [2] Murray MJ, Gonze MD, Nowak LR, et al. Serum D(-)-lactate levels as an aid to diagnosing acute intestinal ischemia. Am J Surg. 1994, 167(6):575-578.
- [3] Wood Heckman LK, DeBoer MD, Fasano A. Zonulin as a potential putative biomarker of risk for shared type 1 diabetes and celiac disease autoimmunity. Diabetes Metab Res Rev. 2020, 36(5):e3309.
- [4] Barbaro MR, Cremon C, Caio G, et al. 247 Zonulin Serum Levels Are Increased in Non-Celiac Gluten Sensitivity and Irritable Bowel Syndrome With Diarrhea. Gastroenterology. 2015, 148(4).

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[6] Zhu H, Xiao X, Shi Y, et al. Inhibition of miRNA-29a regulates intestinal barrier function in diarrhea-predominant irritable bowel syndrome by upregulating ZO-1 and CLDN1. *Exp Ther Med.* 2020, 20(6):155.

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[8] Singh P, Silvester J, Chen X, et al. Serum zonulin is elevated in IBS and correlates with stool frequency in IBS-D. *United European Gastroenterology Journal.* 2019, 7(5):709-715.

[9] Yang C, Zhang X, Wang S, et al. Small intestinal bacterial overgrowth and evaluation of intestinal barrier function in patients with ulcerative colitis. *Am J Transl Res.* 2021, 13(6):6605-6610.

Dear science editor:

Thank you very much for giving us the precious opportunity to revise our manuscript. The comments and suggestions are very valuable and helpful for revising and improving our paper. We have made careful revisions or explanations point by point.

Comments to the Author:

1. Whether the authors detected Zonulin family proteins and intestinal fatty acid binding protein in the serum. If so, please provide relevant data, which will make your article more complete.

Response:

We sincerely appreciate your kind comments. According to your and the reviewer's suggestions, we have provided data relevant to serum zonulin levels in the manuscript and made explanations in detail. Please see our response to the reviewer 2.

2. In figure 3, ZO-1 expression in healthy controls is slightly lower than that in patients with functional constipation. The lack of statistical difference in the author's results may be caused by the small sample size.

Response:

We sincerely thank you for your careful review and kind comment. We agree with you, and to counterbalance the small sample size due to the limited tissue availability, we performed a comprehensive evaluation of the intestinal mucosal barrier in FC patients. Future studies in a large sample should be conducted to validate our results. Relevant explanations have been added in the limitations of the study, please see the 3rd paragraph on P 16, highlighted in yellow.