Dear Dr. Ya-Juan Ma，

Thank you very much for your evaluation about our manuscript. We thank the reviewers for the time and effort that they have put into reviewing the previous version of the manuscript. Their suggestions have enabled us to improve our work. Based on the instructions provided in your letter, we uploaded the file of the revised manuscript.

Appended to this letter is our point-by-point response to the comments raised by the reviewers. All of the revisions that we make to the revised manuscript have been cited in the response letter and highlighted in yellow.

We would like also to thank you for allowing us to resubmit a revised copy of the manuscript.

We hope that the revised manuscript is accepted for publication in World Journal of Gastroenterology.

Best regards,
Shukun Yao

Reviewer 1：

COMMENTS TO AUTHORS
This study proposed to measure leptin levels and analyse the relationship of leptin with clinical features, visceral sensitivity, mast cells, and nerve fibres. My specific queries and comments are below: 1. Abstract: The characteristics of the patients and controls are not well described such as age, sex, characteristics (type/severity) of baseline disease and presence of other comorbidities. Were the controls age- and sex matched with the patients? What were the criteria adopted to stablish the diagnosis of IBD-D?

Response:

Thank you for your careful thinking and suggestion. We have modified the abstract according to your comments.

METHODS: Forty-two patients with diarrhea-predominant irritable bowel syndrome (IBS-D) fulfilling the Rome Ⅲ criteria and 20 age- and sex-matched healthy controls underwent a clinical and psychological evaluation using validated questionnaires (including IBS Symptom Severity Scale, IBS-specific Quality of Life, Hamilton Anxiety Scale and Hamilton

Depression Scale), along with colonoscopy, colonic mucosal biopsy, and visceral sensitivity testing. Serum leptin levels were assayed using enzyme-linked immunosorbent assay. Mucosal leptin expression and localization were evaluated using immunohistochemistry and immunofluorescence. Mucosal leptin mRNA levels were quantified using quantitative real-time reverse transcription polymerase chain reaction. Mast cell counts and activation rates were investigated with toluidine blue staining. Correlation analyses between these parameters were performed.

RESULTS: There were no statistically significant differences in age, gender, or body mass index between the IBS-D group and the control group. The median IBS Symptom Severity Scale score in the IBS-D group was 225.0 (range 100-475). IBS-D patients had significantly increased anxiety (IBS-D: median, 6.5, interquartile range (IQR), 3.3; control: median, 2.0; IQR, 2.0; $P<0.001$) and depression (IBS-D: median,7.0; IQR, 3.0; control: median, 3.0; IQR, 2.0; $P<0.001$) scores. IBS-D patients had significantly lower first sensation threshold (IBS-D: median, 50.6; IQR, 25.9; control: median, 80.5; IQR, 18.6; $P<0.001$), defecation sensation threshold (IBS-D: median, 91.5; IQR, 29.3; control: median, 155.0; IQR, 21.1; $P<0.001$) and maximum tolerable threshold (IBS-D: median, 163.2; IQR, 71.2; control: median, 226.2; IQR 39.3; $P<0.001$), and their mucosal leptin expression (IOD IBS-D: median, 4424.71; IQR, 4533.63; control: median, 933.65; IQR, 888.10; $P<0.001$), leptin mRNA levels (IBS-D: median, 1.1226; IQR, 1.6351; control: median, 0.8947; IQR, 0.4595; $P = 0.009$), and mast cell activation rates (IBS-D: median, 71.2%; IQR, 12.9% vs. control group: median, 59.4%; IQR, 18.88%; $P < 0.001$) were significantly increased. The colocalization of leptin and leptin receptors was observed on mast cells and PGP9.5-positive nerve fibers in the intestinal mucosa. Also, leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, but negatively correlated with the defecation sensation threshold and the maximum tolerance threshold during visceral sensitivity testing (adjusted $P<0.0038$).

2. Page 6, Measures (line 11). Could the authors add the reference related to the validated questionnaire?

Response:

Thank you very much for your suggestion. The "validated references" include IBS Symptom Severity Scale (IBS-SSS), IBS-specific Quality of Life questionnaire (IBS-QOL), Hamilton Anxiety Scale (HAMA) and Hamilton Depression Scale (HAMD). These questionnaires have their own references in the manuscript.

3. Were the specific questionnaires validated in the Chinese population? Could the authors add the reference related to the specific questionnaires?

Response:

Thank you very much for your suggestion. These questionnaires are widely used in China. According to your suggestion, we have added the references that the questionnaires used in the Chinese population.

Measures

Questionnaires

Clinical status was evaluated with the use of validated questionnaires. The IBS Symptom Severity Scale,[22, 23] a 5-item self-reporting questionnaire designed to measure disease severity, was given to each participant to complete. Questionnaire items included information regarding abdominal pain, bloating, satisfaction with bowel habits, and overall interference with quality of life. The total score of the questionnaire ranges from 0 to 500.

The IBS-specific Quality of Life questionaire[24, 25] was used to evaluate changes in patient quality of life. This scale evaluates 34 broad well-being factors based on variables including feelings of dysphoria, social interactions, body image, and health worries. The Hamilton Anxiety Scale[26, 27] and Hamilton Depression Scale[27, 28] were used to measure anxiety and depression, respectively.

23 Bao C, Zhang J, Liu J, Liu H, Wu L, Shi Y, Li J, Hu Z, Dong Y, Wang S, Zeng X, Wu H. Moxibustion treatment for diarrhea-predominant irritable bowel syndrome: study protocol for a randomized controlled trial. BMC

Complement Altern Med 2016 2016-10-24; 16(1): 408 [PMID:27776494 DOI: 10.1186/s12906-016-1386-4]

25 Tang YR, Yang WW, Liang ML, Xu XY, Wang MF, Lin L. Age-related symptom and life quality changes in women with irritable bowel syndrome. World J Gastroenterol 2012 2012-12-28; 18(48): 7175-7183 [PMID:23326122 DOI: 10.3748/wjg.v18.i48.7175]

27 Zhao JM, Wu LY, Liu HR, Hu HY, Wang JY, Huang RJ, Shi Y, Tao SP, Gao Q, Zhou CL, Qi L, Ma XP, Wu HG. Factorial study of moxibustion in treatment of diarrhea-predominant irritable bowel syndrome. World J Gastroenterol 2014 2014-10-07; 20(37): 13563-13572 [PMID:25309087 DOI: 10.3748/wjg.v20.i37.13563]

4. Please, Could the authors revise the tables' titles?

Response:

Thank you very much for your suggestion. We have revised the tables' titles according to your comments.

5. In the tables, legends should contain sufficient information to provide an adequate understanding of the table by the reader without reference to the text.

Response:

Thank you very much for your suggestion. We have revised the table legends according to your suggestion.

Table 1 Demographics, clinical characteristics and visceral sensitivity of IBS-D patients and healthy controls.

Table 2 Correlation between leptin level and clinical and experimental parameters in IBS-D patients.

6. Authors should be more careful with the details of the figures. They should be improved as well as the legends.

Response:

Thank you very much for your suggestion. We have revised the figures according to your suggestion. We will provide high resolution pictures for review.

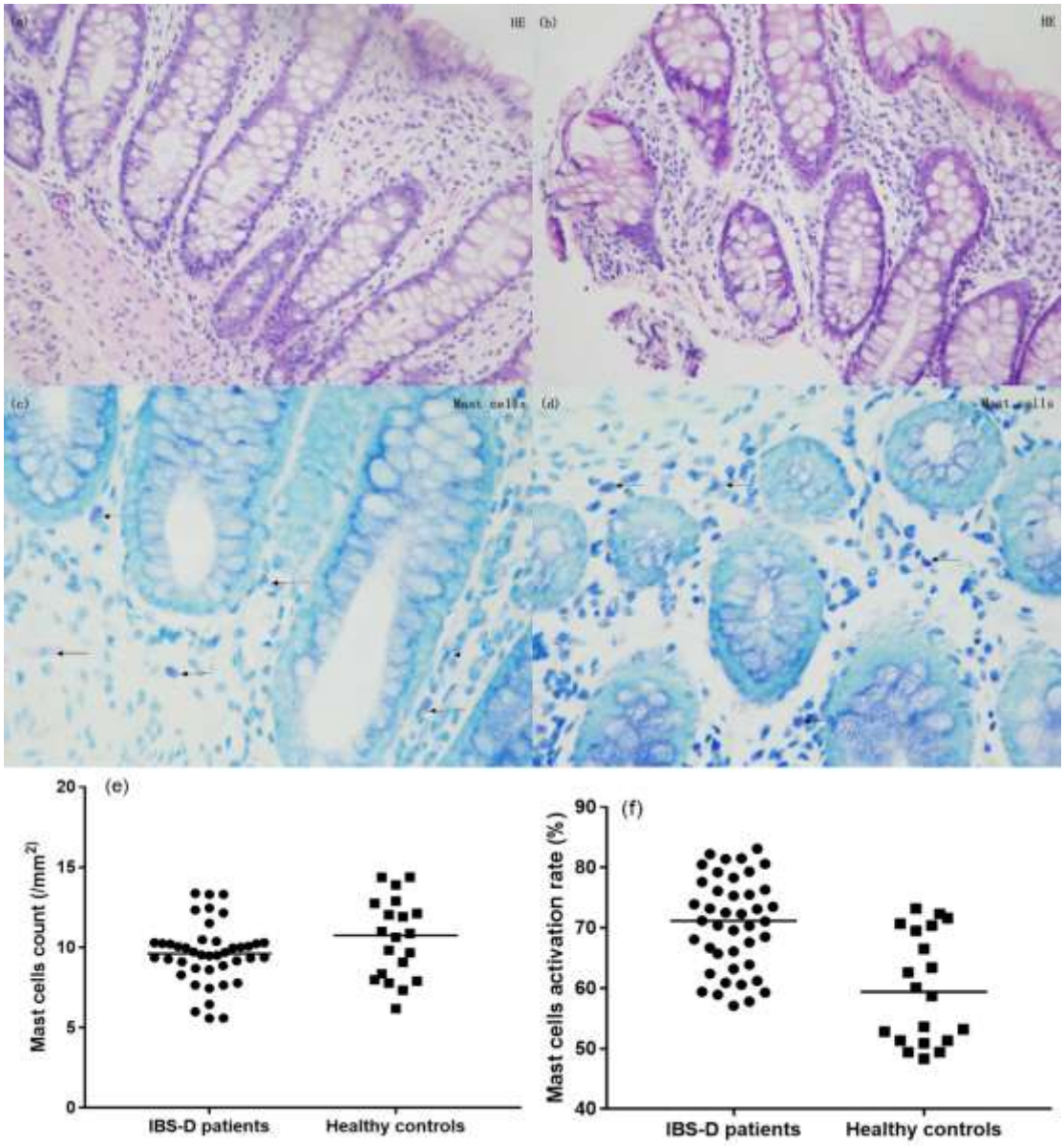

Figure 1 Intestinal mucosal histology by haematoxylin and eosin staining, mast cells toluidine blue staining, mast cells count (/mm²) and mast cells activation rate (%) in IBS-D patients and controls. (a): Normal colonic mucosal H&E histology of IBS-D patients (×200); (b): Normal colonic mucosal H&E histology of healthy controls (×200); (c): Mast cells toluidine blue staining of IBS-D patients (× 400, arrows represent mast cells); (d): Mast cells toluidine blue staining of healthy controls (× 400, arrows represent mast cells);

(e): Mast cells count not significantly different between the two groups (P = 0.164). (f): Mast cells activation rate significantly increased in IBS-D patients (P < 0.001). Line in the scatter plots means the median.

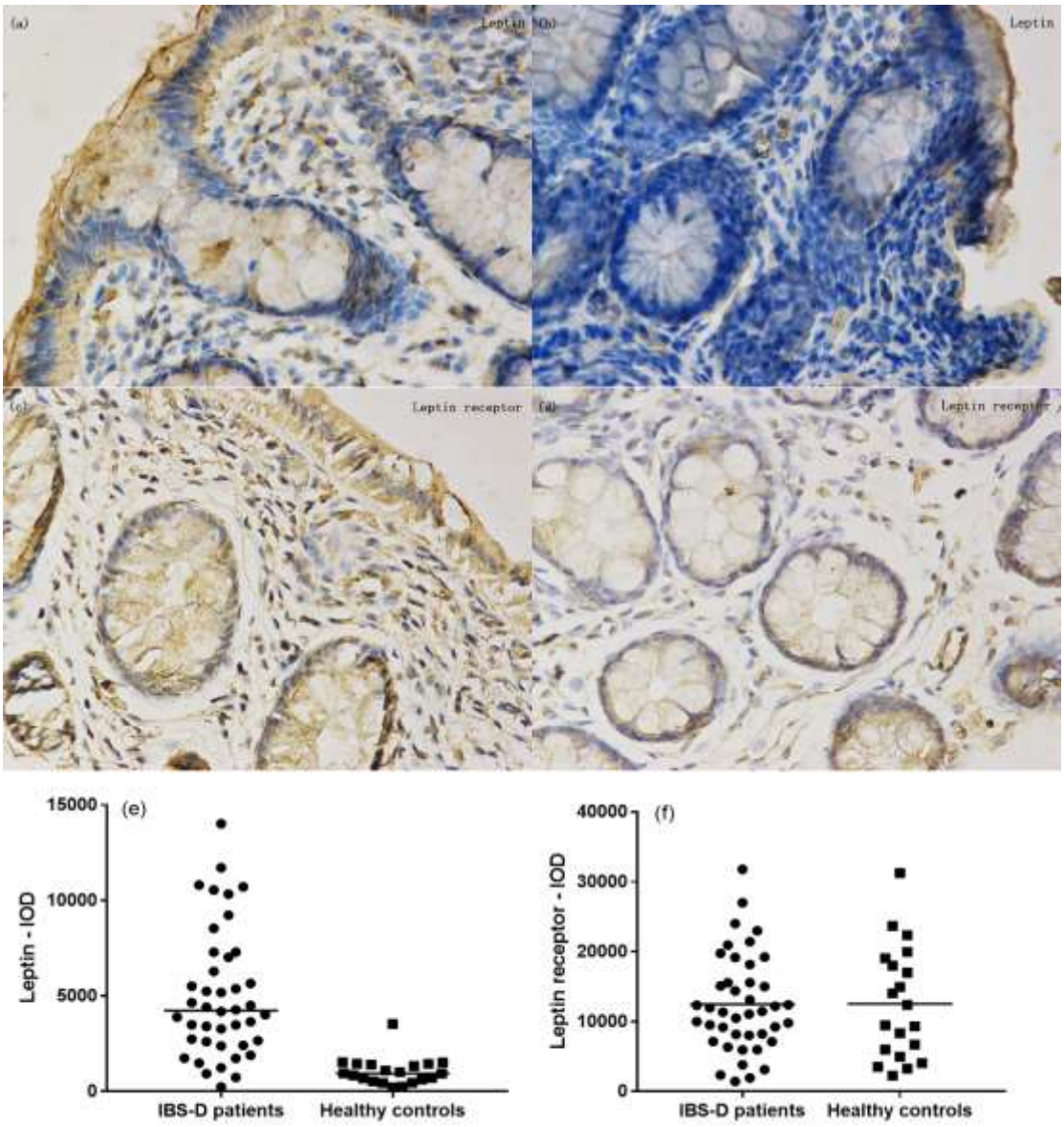

Figure 2 Immunohistochemical staining of leptin and leptin receptor in IBS-D patients and healthy controls. (a): Colonic mucosal leptin expression in IBS-D patients (×400); (b): Colonic mucosal leptin expression in healthy controls (×400); (c): Colonic mucosal leptin receptor expression in IBS-D patients (×400); (d): Colonic mucosal leptin receptor expression in healthy controls (×400); (e): Colonic mucosal leptin immunoreactivity significantly increased in IBS-D patients than the healthy controls; (f): Colonic mucosal

leptin receptor immunoreactivity not increased significantly in IBS-D patients than the healthy controls. Line in the scatter plots means the median. IOD, integral optical density.

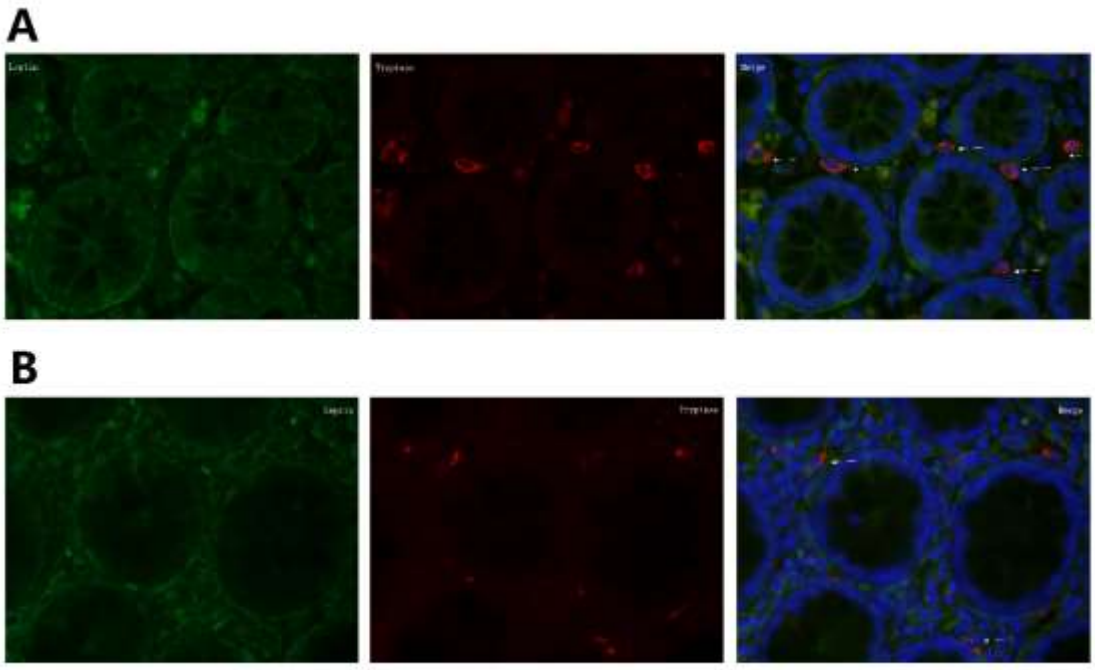

Figure 3 Double-labeling immunofluorescence of leptin and tryptase in IBS-D patients and healthy controls. A: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in IBS-D patients (×400, arrows represent colocalization of leptin and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in healthy controls (×400, arrows represent colocalization of leptin and tryptase).

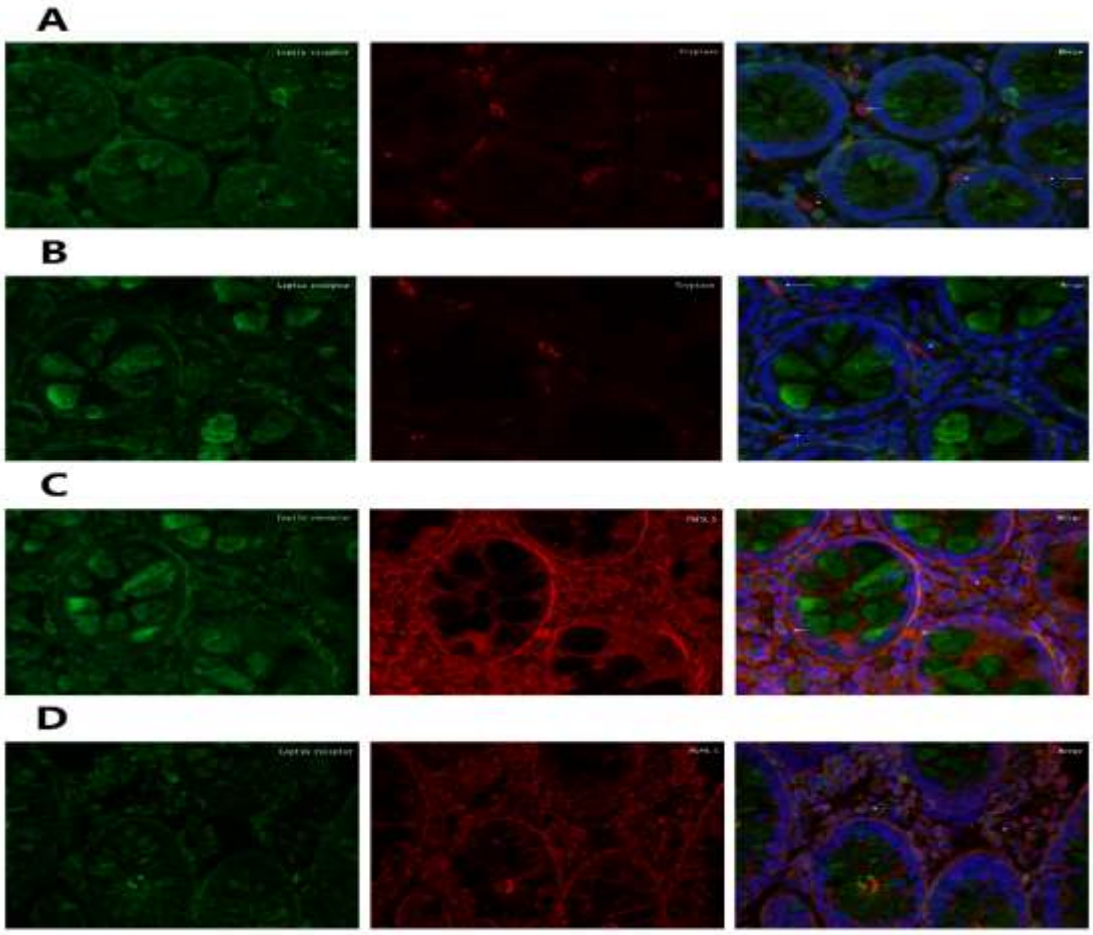

Figure 4 Double-labeling immunofluorescence of leptin receptor with tryptase and PGP9.5 in IBS-D patients and healthy controls (× 400 magnification). A: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in IBS-D patients (×400, arrows represent colocalization of leptin receptor and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in healthy controls (×400, arrows represent colocalization of leptin receptor and tryptase); C: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP 9.5 in IBS-D patients (×400, arrows represent colocalization of leptin receptor and PGP 9.5); D: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP 9.5 in healthy controls (×400, arrows represent colocalization of leptin receptor and PGP 9.5). PGP9.5, protein gene product 9.5

7. There is a limited discussion regarding the strengths, weakness and limitations of the study.

Response:

Thank you very much for your suggestion. We have revised the discussion according to your suggestion.

The present study to investigate the possible role of leptin in the pathogenesis of IBS-D. Biopsies from IBS-D patients revealed a significant upregulation of both leptin protein and mRNA levels. The increased leptin protein or mRNA expression closely correlated with IBS symptom severity, anxiety, depression, and visceral sensation thresholds, which may provide a putative basis for the development of IBS symptoms. To our knowledge, this was the first study to preliminarily confirm that leptin may play a certain role in the pathophysiology of IBS-D.

There were several limitations in this study. First, because of the nature of our research foundation,[32, 54] the present study focused on patients with the IBS-D subtype. Therefore, the findings may not be generalizable to constipation-predominant IBS (IBS-C), mixed-type IBS (IBS-M), and unsubtyped IBS (IBS-U) patients. Second, leptin has been shown to increase during the process of inflammation.[55] However, we cannot be sure that all postinfective IBS patients were excluded from the study. Because these two subgroups may be different in pathogenesis, future experiments should study these two types separately. Third, due to lack of previous data related to our study, we did not estimate sample size and power, which may reduce the reliability of our conclusion. Fourth, in qRT-PCR, we used only one reference gene (GAPDH), which may influence the accuracy of the results. Finally, we cannot make a cause and effect inference on the basis of our findings; additional studies of the effects of leptin on mast cells and nervous system as well as larger clinical studies are needed.

8. The manuscript should be described in a more concise and substantial manner.

Response:

Thank you for your suggestion, which is very important to me. Because of your suggestion, I found the deficiency in our work. We will improve our writing skills in our future work.

9. Reviewer conclusion: Accept but needs revision (both major and minor).

Response:

Thank you for your construction comments for improving our manuscript.

Reviewer 2：

COMMENTS TO AUTHORS
In their study, the authors investigate the expression of leptin and the leptin receptor in serum and mucosal biopsies of IBS-D patients and healthy controls. Based on mainly immunohistochemical stainings the authors relate the leptin profiles to mast cells and neurons and correlate the findings to visceral hypersensitivity measurements, symptom scores and QoL. Major remarks 1. The authors should provide much more details on the immunohistochemical results. In my opinion the stainings are not clear at all or mistakes are present in the legends. The stainings presented not always support the statement/conclusions of the authors in my opinion. For instance in figure 2 (and all other figures) the authors should mention which samples are from IBS-D patients and which from controls. If I understood it correctly the leptin immunoreactivity shown in part a is from an IBS-D patient showing less intense staining than part b which is a control according to the legend?

Response:

Thank you very much for your careful thinking and suggestion. We have revised the manuscript according to your suggestion. Besides, We will provide high resolution pictures for review.

Figure 2 Immunohistochemical staining of leptin and leptin receptor in IBS-D patients and healthy controls. (a): Colonic mucosal leptin expression in IBS-D patients (×400); (b): Colonic mucosal leptin expression in healthy controls (×400); (c): Colonic mucosal leptin receptor expression in IBS-D patients (×400); (d): Colonic mucosal leptin receptor expression in healthy controls (×400); (e): Colonic mucosal leptin immunoreactivity significantly

increased in IBS-D patients than the healthy controls; (f): Colonic mucosal leptin receptor immunoreactivity not increased significantly in IBS-D patients than the healthy controls. Line in the scatter plots means the median. IOD, integral optical density.

2. Also the mast cell activation rate should be demonstrated in much more detail. How did the authors score this parameter: using a present/absent or a more continuous scale, or using a scoring system, … It is not clear from figure 1 what exactly in the stainings accounts for the difference in activation rate.

Response:

Thank you very much for your suggestion. According to your comments, we added details about mast cell activation rate. Besides, we will provide high resolution pictures.

Mast cells were stained with toluidine blue. The slides were first soaked in 0.5% toluidine blue，and then differentiated with acetone. Afterwards, five 400x magnification fields (field area 0.237 mm^2) were chosen at random and scanned. Mast cells were identified with light microscopy by their metachromatic cytoplasmic granules. Finally, mast cell degranulation was assessed based on unclear or irregular cell membranes and the presence of extruded secretory granules. The mean mast cell numbers were expressed per millimetre square of the mucosal area (/mm^2). The percentage of degranulated mast cells (mast cell activation rate), was calculated in each section (degranulated mast cells/the total number of mast cells ×100%).

3. Please also clarify the co-localisations in figures 3-4. Indicate with arrows and/or asterisks which mast cells show co-staining with leptin and which mast cells don't. If the mast cell activation rates are different I wonder whether tryptase is a reliable marker to study this research question. One could wonder whether the tryptase stainings per se were not different between controls and IBD patients.

Response:

Thank you very much for your careful thinking and suggestion. We have revised the manuscript according to your suggestion. Besides, we will provide high resolution pictures.

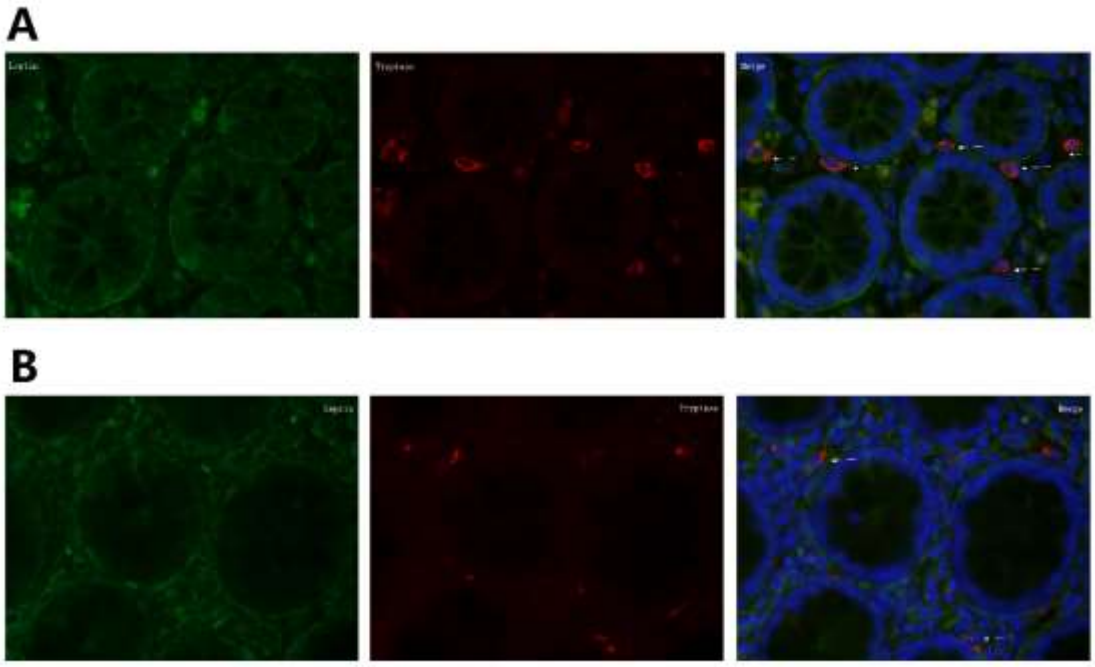

Figure 3 Double-labeling immunofluorescence of leptin and tryptase in IBS-D patients and healthy controls. A: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in IBS-D patients (×400, arrows represent colocalization of leptin and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in healthy controls (×400, arrows represent colocalization of leptin and tryptase).

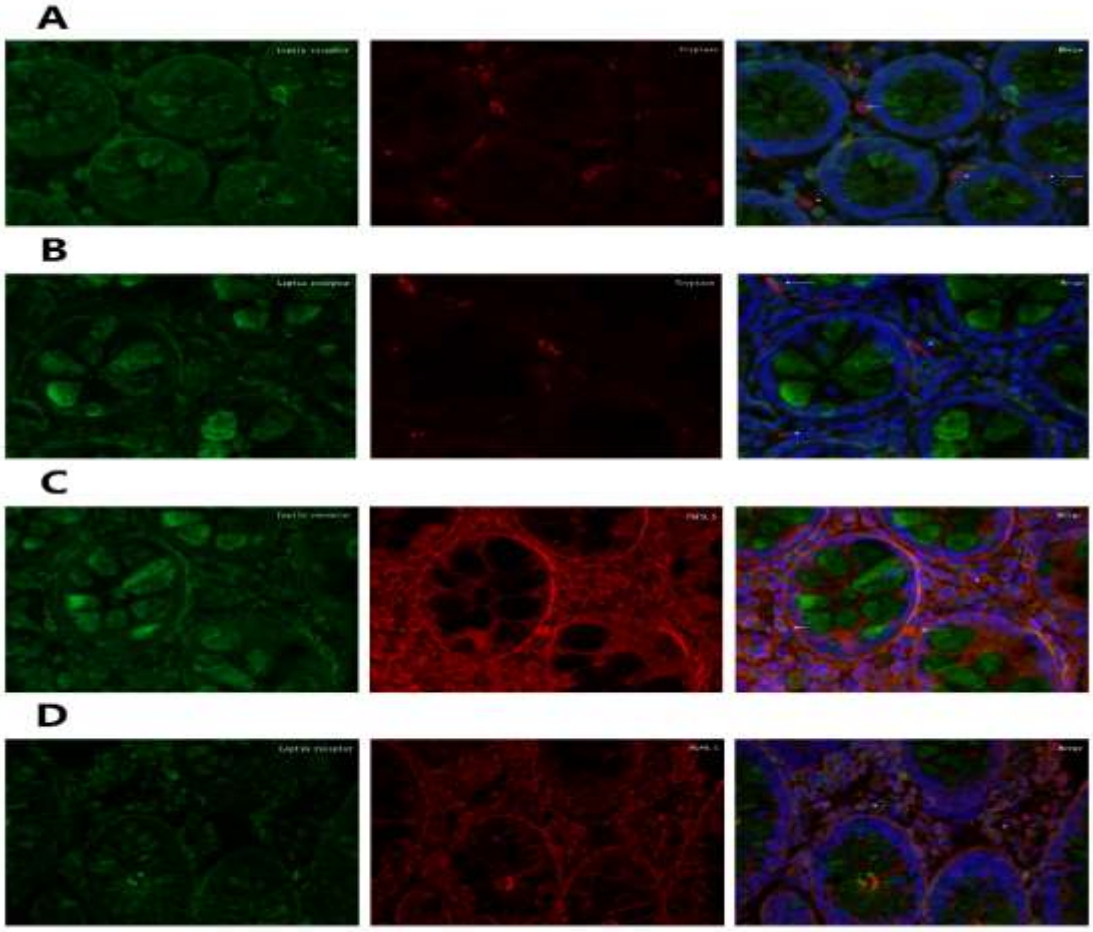

Figure 4 Double-labeling immunofluorescence of leptin receptor with tryptase and PGP9.5 in IBS-D patients and healthy controls (× 400 magnification). A: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in IBS-D patients (×400, arrows represent colocalization of leptin receptor and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in healthy controls (×400, arrows represent colocalization of leptin receptor and tryptase); C: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP 9.5 in IBS-D patients (×400, arrows represent colocalization of leptin receptor and PGP 9.5); D: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP 9.5 in healthy controls (×400, arrows represent colocalization of leptin receptor and PGP 9.5). PGP9.5, protein gene product 9.5

4. Looking at table 1 the authors indicate that the IBS scales were not determined in the controls, why not ?

Response:

Thank you very much for your careful thinking. IBS-SSS applies only to IBS patients rather than healthy controls.

5. The authors conclude that the increased levels of mucosal leptin interact with mast cells and the nervous system. This seems an overstatement to me as the immunohistochemical stainings are the only basis for this statement and they are not that clear to me. Besides the authors only used PGP 9.5 as a neuronal marker, why didn't they study more specific neuronal markers investigating for instance the colocalisation with afferent nerve fibers? Please change the word ''interacting' in your final conclusion in abstract and discussion.

Response:

Thank you very much for your careful thinking and suggestion, which is very important to me. Because of your suggestion, I found the deficiency in our work. The conclusion that the increased levels of mucosal leptin interact with mast cells and the nervous system was speculated based on references and our preliminary results. We will validate the conclusion using basic experiments in subsequent studies. The enteric nervous system (ENS) includes both afferent and efferent nerve fibers, so we used PGP 9.5 instead of neuronal markers specific for afferent nerve fibers. Our future work will use more specific neuronal markers to distinguish afferent nerve fibers from efferent nerve fibers. We have revised the text according to your comments.

CONCLUSION: Increased levels of mucosal leptin, may interact with mast cells and nervous system contributing to the pathogenesis of IBS-D

There were several limitations in this study. First, because of the nature of our research foundation,[32, 54] the present study focused on patients with the IBS-D subtype. Therefore, the findings may not be generalizable to constipation-predominant IBS (IBS-C), mixed-type IBS (IBS-M), and unsubtyped IBS (IBS-U) patients. Second, leptin has been shown to increase during the process of inflammation.[55] However, we cannot be sure that all postinfective IBS patients were excluded from the study. Because these two

subgroups may be different in pathogenesis, future experiments should study these two types separately. Third, due to lack of previous data related to our study, we did not estimate sample size and power, which may reduce the reliability of our conclusion. Fourth, in qRT-PCR, we used only one reference gene (GAPDH), which may influence the accuracy of the results. Finally, we cannot make a cause and effect inference on the basis of our findings; additional studies of the effects of leptin on mast cells and nervous system as well as larger clinical studies are needed.

6. Were the observers for the histological data analysis blinded for the two groups as well as the investigator investigating the visceral hypersensitivity ?

Response:

Thank you very much for your question. The observers for the histological data analysis were blinded for the two groups as well as the investigator investigating the visceral hypersensitivity. We changed our manuscript according to your suggestion.

All the tests were performed by the same investigator between 1 and 3 PM in a blind fashion.
The quantification of immunoreactivity was performed by two operators in a blind fashion.
Specimens were examined by two operators in a blind fashion using a Nikon Eclipse 90i microscope (Nikon Instruments Co., Ltd., Tokyo, Japan).

7. Do the authors have any data on the power of their study ?

Response:

Thank you very much for your careful thinking and suggestion. Due to the lack of previous data related to our study, we did not estimate sample size and power. We have added it as one of our limitations in the discussion.

Minor remarks:
1. The authors used only one reference gene (GAPDH) where normally three reference genes are used according to the guidelines. Could the authors at least show that the reference gene remained stable in their analyses.

Response:

Thank you very much for your careful thinking and suggestion, which is very important to me. Because of your suggestion, I found the deficiency in our work. Referring to our previous study[1], we used only one reference gene rather than three. We will avoid this problem in our future study. Besides, we have added it as one of our limitations in the discussion.

References:
1 Xu XJ, Zhang YL, Liu L, Pan L, Yao SK. Increased expression of nerve growth factor correlates with visceral hypersensitivity and impaired gut barrier function in diarrhoea-predominant irritable bowel syndrome: a preliminary explorative study. Aliment Pharmacol Ther 2017 2017-01-01; 45(1): 100-114 [PMID:27862119 DOI: 10.1111/apt.13848]

2. In the discussion the authors link the leptin expression to CRH and cortisol while this is not studied at all in their paper.

Response:

Thank you very much for your careful thinking and suggestion. We speculated the linkage of leptin expression to CRH and cortisol based on references listed in our manuscript. This was a speculation we raised to try to explain why leptin levels were changed in IBS-D patients, and we will validate it in our further studies.

3. Page 15 comments – background line 5 'Leptin instead of leptin'

Response:

Thank you very much for your suggestion. We have revised the comments – background according to your suggestion.

Leptin not only exerts significant biological effects, such as appetite control, by signaling satiety and increasing energy expenditure, but also through modulation of the immune system and gastrointestinal function.

4. Page 23 legend figure 1. 'Metachromatically instead of metachromatically'

Response:

Thank you very much for your suggestion. We have revised the legend figure 1.

Figure 1 Intestinal mucosal histology by haematoxylin and eosin staining,

mast cells toluidine blue staining, mast cells count (/mm²) and mast cells activation rate (%) in IBS-D patients and controls. (a): Normal colonic mucosal H&E histology of IBS-D patients (×200); (b): Normal colonic mucosal H&E histology of healthy controls (×200); (c): Mast cells toluidine blue staining of IBS-D patients (× 400, arrows represent mast cells); (d): Mast cells toluidine blue staining of healthy controls (× 400, arrows represent mast cells); (e): Mast cells count not significantly different between the two groups ($P = 0.164$). (f): Mast cells activation rate significantly increased in IBS-D patients ($P < 0.001$). Line in the scatter plots means the median.