RESEARCH ARTICLE

REVISED Study of Antibodies to Cytolethal Distending Toxin B (CdtB) and Antibodies to Vinculin in Patients with Irritable Bowel Syndrome [version 4; peer review: 2 approved]

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Abstract

Background: Irritable bowel syndrome (IBS) is a common gastrointestinal disorder, categorized into various subtypes. Post-infection IBS may be attributed to the release of cytolethal distending toxin B (CdtB), which cross-reacts with the adhesion protein vinculin responsible for normal intestinal contractility.
Objective: This study aims to identify anti-CdtB and anti-vinculin levels in IBS patients compared to healthy control.
Subjects and methods: This retrospective case-control study was conducted on 100 subjects with IBS, as determined by a questionnaire based on Rome III criteria, recruited from the outpatient clinics of the Tropical Medicine at Mansoura University Hospital from January 2019 to January 2020.
Results: The optical density (OD) results of the anti-vinculin and anti-CdtB levels were significantly elevated in patients with IBS (1.58±0.496 OD, 2.47±0.60 OD) when compared to control subjects (1.13±0.249 OD, 2.1±0.24 OD), respectively with P=0.001 for both. Anti-vinculin level was significantly higher in the IBS-D subtype than the other subtypes (P=0.001) while, Anti-CdtB was significantly elevated in IBS-C, IBS-D subgroups compared to control subjects (P=0.001).
Conclusion: Findings of the present study support the hypothesis that IBS results from post-infectious disorders initiated by bacterial enteritis. A hypothesis could be applied to all IBS subgroups. On the other hand. These biomarkers might reflect the post-infectious state's

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Any reports and responses or comments on the

severity.

Keywords
irritable bowel syndrome, anti-vinculin, anti-CdtB, Rome IV

article can be found at the end of the article.

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REVISED **Amendments from Version 3**

The OD was added to all sections of the study as it was the correct used unit.

Any further responses from the reviewers can be found at the end of the article

Introduction

Irritable bowel syndrome (IBS) is a common gut disorder that affects approximately 11% of the global population.[1,2] IBS mainly manifests in subjects with abdominal pain with bowel habit changes in the absence of either radiological evidence of associated pathological conditions or detectable chemical and physiological abnormalities. The diagnosis of this clinical condition relies upon Rome criteria.[3–7]

The Rome working team recommended classifying subjects with IBS into different sub-groups depending on their bowel habits changes predominance. The IBS sub-groups included IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), Mixed IBS (IBS-M), and Un-subtyped IBS.[3]

To understand the pathogenesis of irritable bowel syndrome (IBS), previous studies have developed a rat model utilizing infection with *Campylobacter jejuni* in order to elicit a post-infection phenotype resembling human post-infection IBS (PI-IBS) characterized by apparent changes in the composition of small intestinal microbiota.[8,9] In these studies, progression to IBS was accompanied by the detection of a specific bacterial toxin named cytolethal distending toxin B (CdtB), a potential factor attributing to the pathogenesis of PI-IBS. Experimental studies suggested a low incidence of IBS when infected with a mutant strain of *C. jejuni* that lacks CdtB.[8,10]

Furthermore, the development of antibodies to CdtB was associated with altering gut microbiota associated with reducing specific interstitial cells of Cajal (ICC).[11,12] These findings were linked to the ability of anti-CdtB to cross-react with vinculin, a host cell adhesion protein present in interstitial cells of Cajal and the myenteric ganglia that control the normal activity of the intestinal tract, including phase III of inter-digestive motor activity.[13] Absence or decrease in phase III contractions results in small intestinal bacterial overgrowth in animal models and human patients with IBS.[14,15] In this sense, autoimmunity may profoundly affect the host immune response to infections with *C. jejuni*, subsequently leading to IBS.[16,17] Based on these data, it has been suggested that loss of vinculin in the neuromuscular system of the gastrointestinal tract (GIT) may be associated with the affection of the gut in animal models of post-infection *C. jejuni.* Detection of circulating levels of anti-CdtB and anti-vinculin by enzyme-linked immunosorbent assay (ELISA) has been used to identify patients with IBS-D,[18] and to differentiate it from other IBS subtypes.[19] However, it should be noted that the idea of a specific IBS microbiome is someone controversial with larger studies analysing mucosal microbiomes showing now distinct signature.[20]

The present study aims to detect and quantify anti-CdtB and anti-vinculin levels in subjects with IBS and their possible role in diagnosing different IBS subtypes.

Methods

This was a retrospective case-control study comprising 100 adult patients aged >18 years with IBS, recruited from the Tropical Medicine Department's outpatient clinics at Mansoura University Hospital from January 2019 to January 2020, and 100 healthy subjects with matched gender and age as a control group.

Selection and exclusion criteria

Patients were recruited and IBS determined by a questionnaire-based upon the Rome III criteria, then classified according to their predominant stool composition over 25% of the time: into IBS-C (hard or lumpy stools), IBS-D (loose and watery stools), or IBS-M (a mix of both types).[19] Exclusion criteria included patients with hepatic, renal, or autoimmune diseases, those with history of inflammatory bowel disease, gastrointestinal surgeries, thyroid disorders, diabetes mellitus, and patients with a history of taking antibiotics in the last 30 days.

Laboratory methods

A 10 ml blood sample was obtained from each subject, which was then divided into three aliquots. Two aliquots were used to determine complete blood counts, and one aliquot was utilized for serum separation to assess complete liver function tests, including alanine transaminase, aspartate transaminase, total bilirubin, total albumin, and the kidney

function test creatinine. The third aliquot was overlaid on heparin for plasma separation, and the remaining sera were stored at -20°C to be used for evaluation of anti-vinculin antibodies by laboratory prepared ELISA and anti-CdtB antibodies by commercial ELISA (Creative Diagnostics. 45-16 Ramsey Road Shirley, NY 11967, USA).

ELISA for anti-vinculin

Anti-vinculin levels were measured in separated plasma using human vinculin protein in a concentration of 1.2 µg/ml (Novoprotein Scientific, Summit, New Jersey, USA) as an antigen. The vinculin was used to coat wells of the plate following overnight incubation in the wells at 4°C with 100 mmol/l borate buffered saline AQ4 at a pH of 8.2 (Sigma-Aldrich). The reaction was blocked by using BSA 3% and incubating for one hour at room temperature, then washing three times with 0.05% PBS and Tween 20 (pH 7.4). Plasma was added after a 1:32 dilution in saline, then antibodies for vinculin (R and D Systems Cat# MAB6896, RRID:AB_10992930), were added as positive control and incubated for one hour at room temperature followed by washing three times with 0.05% PBS and Tween 20 (pH 7.4). Horseradish peroxidase-conjugated secondary antibodies (Millipore–Merck) were added and incubated for one hour at room temperature. After washing, a tetramethylbenzidine substrate solution (BioRad) was used for detection using a micro-plate reader (stat Fax-1200; Awareness Technology, Florida, USA). Optical densities (ODs) were read at 370nm, and the results were interpreted as OD.[12]

ELISA for anti-CdtB (creative diagnostics)

The ELISA was used to determine the anti-CdTB of *C. jejuni* using the recombinant C*ampylobacter* CdtB protein (https://www.creativebiomart.net/description_436265_12.htm). The protein was used as antigens immobilized at the wells of the 96 microplates overnight at 4 C with a concentration of 1.2 µg/mL prepared in borate buffer saline to obtain PH 8.2. Negative wells were prepared by adding only borate buffer saline. After overnight incubation, the reaction was blocked by adding bovine albumin with a concentration of 3% prepared in phosphate buffer and incubated at room temperature for one hour. Then the plate was used to determine anti-CdtB in the serum samples with dilution 1:512, and anti-CdtB antibodies) were used as positive controls (https://www.creative-diagnostics.com/search.aspx?pageid=1&keys=CdtB&status=0&fl=ELISA%257e&flt=2,&cid=4). The plate was incubated for one hour at room temperature. The wells were then washed three times with phosphate buffer, and then horseradish peroxidase-conjugated secondary antibodies were added to the wells and incubated for one hour at room temperature. TMB turns blue in peroxidase reaction and finally turns yellow under the action of acid. Optical densities (ODs) were read at 450. The OD values were used for the data analysis.

Statistical analysis

Data are reported as means and standard deviation (SD) or counts and percentages when appropriate. Comparisons between groups were made using t-tests, Mann-Whitney tests, Chi-square, or Fisher exact tests dictated by data type and distribution.

One-way analysis of variance (ANOVA) was used to test differences between more than two groups. P-value < 0.05 was considered significant for all statistical analyses in this study. All analyses were performed using the Statistical Package of Social Sciences (SPSS) version 22 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

This study included 100 patients with IBS (49 males and 51 females) aged 46.6 ± 6.8 years and 100 healthy controls with a statistically insignificant difference between patients and control regarding age and sex ($P = 0.8$ and $P = 0.6$, respectively). Patients were classified according to Rome III criteria into 40 patients with IBS-C, 26 patients with IBS-D, and 34 patients with IBS-M (Figure 1). Laboratory investigations, including ALT, AST, albumin, total bilirubin, hemoglobin, total leucocytes count, platelets, and creatinine, showed non-significant differences between patients and control subjects ($P = 0.6$, $P = 0.5$, $P = 0.7$, $P = 0.6$, $P = 0.99$, $P = 0.99$, and $P = 0.58$) respectively (Table 1).

The OD of the anti-vinculin and anti-CdtB levels were significantly elevated in IBS patients (1.58 ± 0.496ng/ml and 2.47 ± 0.60 ng/ml) respectively compared to the control subjects (1.13 ± 0.249 ng/ml and 2.1 ± 0.24 ng/ml) respectively with $P = 0.001$ for both (Table 2).

Anti-vinculin levels were also significantly higher in different IBS subgroups compared to control subjects, with the anti-vinculin level being significantly elevated in the IBS-D subtype when compared to the other subtypes with $P = 0.001$. Similarly, anti-CdtB showed significant elevation in IBS-C and IBS-D compared to control subjects ($P = 0.001$), with a significantly higher level detected in IBS-D than IBS-C ($P = 0.001$). However, the level of anti-CdtB in IBS-M was detected at a non-significant lower level compared to control subjects ($P = 0.2$), but at a significantly lower level when compared to IBS-C and IBS-D ($P = 0.001$) (Table 3).

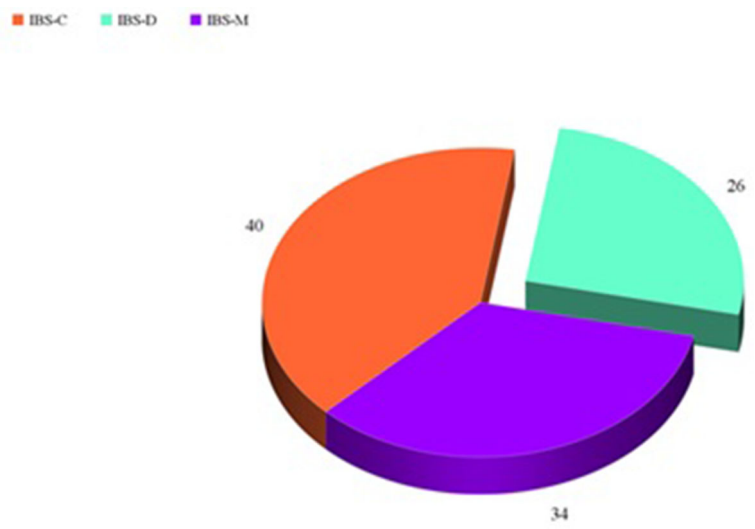

Figure 1. Distribution of patients according to Rome III criteria.

Table 1. Comparison of demographic and laboratory findings between patients and control subjects.

Parameter	Patients with IBS (n = 100)	Healthy Control (n = 100)	*P*
Age *(Mean ± SD)*	50.1 ± 6.6	46.6 ± 6.8	0.8
Sex • **Male** *(No/%)* • **Female** *(No/%)*	49 (49%) 51 (51%)	49 (49%) 51 (51%)	0.6
Hemoglobin *(Mean ± SD) gm/dl*	13.19 ± 1.7	13.16 ± 1.7	0.99
Total leucocytes count *(Mean ± SD) × 10^3/mm^3*	13.2 ± 1.7	13.1 ± 1.8	0.99
Platelets *(Mean ± SD) × 10^3/mm^3*	134.15 ± 56.32	141.35 ± 34.04	0.003
Creatinine *(Mean ± SD) mg/dl*	0.98 ± 0.25	0.96 ± 0.28	0.58
ALT *(Mean ± SD) IU/l*	28.85 ± 4.7	29.2 ± 4.4	0.6
AST *(Mean ± SD)*	27.26 ± 4.32	27.7 ± 4.05	0.5
Albumin *(Mean ± SD) gm/dl*	4.00 ± 0.51	4.1 ± 0.52	0.7
Total bilirubin *(Mean ± SD) mg/dl*	0.82 ± 0.10	0.9 ± 0.11	0.6

Table 2. Comparison of anti-vinculin and anti-CdtB in patients with IBS versus control subjects.

Parameter	Patients with IBS *(Mean ± SD)*	Control Subjects *(Mean ± SD)*	*P*
Anti-vinculin	1.58 ± 0.496	1.13 ± 0.249	0.001
Anti-CdtB	2.47 ± 0.60	2.1 ± 0.24	0.001

Discussion

There is an extreme necessity for the utilization of accessible and reliable, low-cost biomarkers to avoid unnecessary routine use of colonoscopy in diagnosing IBS in low-risk population with age <50 years, no history of GIT bleeding, nocturnal passage of stool, weight loss, familial history of inflammatory bowel diseases or colorectal cancer, recent bowel

Table 3. Comparison of anti-vinculin and anti-CdtB between different subgroups of IBS and control subjects.

Parameter	IBS-C (N = 40) *(OD mean ± SD)*	IBS-D (N = 26) *(OD mean ± SD)*	IBS-M (N = 34) *(OD mean ± SD)*	Control (N = 100) *(OD mean ± SD)*	*P*
Anti-vinculin	1.33 ± 0.49	1.84 ± 0.42	1.68 ± 0.42	1.13 ± 0.25	P1 = 0.001 P2 = 0.001 P3 = 0.001 P4 = 0.01 P5 = 0.001 P6 = 0.001
Anti-CdtB	2.52 ± 0.46	2.98 ± 0.6	2.03 ± 0.67	2.1 ± 0.24	P = 0.001 P1 = 0.001 P2 = 0.001 P3 = 0.001 P4 = 0.2 P5 = 0.001 P6 = 0.001

. P1 comparing IBS-C and IBS-D.
. P2 comparing IBS-C and IBS-M.
. P3 comparing IBS-D and IBS-M.
. P4 comparing IBS-M and control subjects.
. P5 comparing IBS-IBS-C and control subjects.
. P6 comparing IBS-D and control subjects.

habits changes, and/or the presence of abdominal masses or lymaphadenopathy.[1,20] Previous studies reported that anti-CdtB and anti-vinculin might be valuable noninvasive biomarkers to identify IBS patients[21,22] in different populations. However, these biomarkers have not been sufficiently evaluated in Egyptian patients.

In the current study, both anti-vinculin and anti-CdtB demonstrated significantly elevated levels in IBS patients when compared to the control subjects, a finding that mirrors those from a previous study by Talley et al.[23] However, data reported by Rezaie et al.[16] depicts significant elevation in levels of both biomarkers only in IBS-M and IBS-D, but not IBS-C. This discrepancy in findings may be attributed to the difference in etiology of different IBS subtypes,[4] as it is hypothesized that most cases of post-infectious IBS manifest as IBS-D or IBS-M, with a minority of patients manifesting as IBS-C.[9] Another factor may make the microbiome profile difference between IBS patient subgroups; bacterial species producing methane are decreased in IBS-D and IBS-M[23] and increased in IBS-C.[24] Patients included in the present study, particularly those in the IBS-C subgroup, may represent patients who develop IBS following infections associated with their microbiota profile changes. These findings need extensive longitudinal studies to be confirmed.

Anti-vinculin and anti-CdtB levels in this study were significantly elevated in patients with IBS-D, a concordance finding with Pimentel et al., who reported that anti-CdtB and anti-vinculin distinguished IBS-D from IBD, other organic GI diseases and healthy control. In addition, Bayoumy et al.[24] reported that anti-vinculin could be an important biomarker for IBS-D diagnosis among Egyptian patients. Cytolethal distending toxin represents a virulence factor for bacterial pathogens such as *Escherichia coli, Salmonella, Shigella*, and *Campylobacter jejuni*, by causing epithelial barrier breakdown and suppression of the acquired immune response to invading pathogens, resulting in an amplified pro-inflammatory response with consequent persistence of bacterial infection.[16] Development of anti-CdtB antibodies occurs in response to secretion of cytolethal distending toxin following infection with bacterial pathogens. Molecular mimicry accounts for the potential cross-reaction between anti-CdtB and vinculin with resultant anti-vinculin autoantibody production leading to injury to interstitial cells of Cajal (ICC) with the development of IBS.[12] Based on the suggestion of an association between the metabolic syndrome and liver affection and IBS, this study group performed liver function tests as a simple evaluation of liver affection. However, liver enzymes were normal in IBS patients' studied group, in contrast to reports by Lee et al.[26]

In the present study there was no history of previous infection with *C. jejuni*. However, the elevated levels of antiCdtB and antivinculin can be used as biomarkers for diagnosis of IBS either post infections or without previous infection. The data of the present study supports that PI IBS may be more common than it is realized.[26]

The principal limitation of the present study was the lack of psychological measures in combination with the measurement of the serological biomarkers as these measures are a valuable tool in the diagnosis of IBS compared to healthy controls as reported previously.[27]

Conclusion

The present findings support the hypothesis that IBS may results from post-infectious bacterial gastroenteritis. Moreover, this hypothesis can be applied to all IBS subgroups as both anti-CdtB and anti-vinculin biomarkers were significantly elevated in IBS-C and IBS-D subgroups, with only anti-vinculin being elevated in IBS-M when compared to healthy control. These may signify the role of infection in such subgroup of IBS patients. These findings need further extensive longitudinal studies in patients with IBS.

Consent

All participants provided written informed consent and the study was conducted according to the principles outlined in the Declaration of Helsinki. Confidentiality and privacy were considered regarding personal, clinical and laboratory data.

Ethical approval

Mansoura Faculty of Medicine Institutional Research Board approved the research (R.21.01.1141).

Data availability

Figshare: "Study of antibodies to cytolethal distending toxin B (CdtB) and antibodies to vinculin in patients with irritable bowel syndrome" https://doi.org/10.6084/m9.figshare.14178908.v1.[28]

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Version 4

Reviewer Report 25 October 2021

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✔ **Gabriela Leite**
Medically Associated Science and Technology (MAST) Program, Cedars-Sinai Medical Center, Los Angeles, CA, USA

Minor change:

- Authors did not correct the cdtB and vinculin units in the results section as requested previously. - "The OD of the anti-vinculin and anti-CdtB levels were significantly elevated in IBS patients (1.58 ± 0.496ng/ml and 2.47 ± 0.60 ng/ml) respectively compared to the control subjects (1.13 ± 0.249 ng/ml and 2.1 ± 0.24 ng/ml) respectively with *P* = 0.001 for both (Table 2)."

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug development, test development for diagnosis of antibodies and antigens associated with bacterial diseases.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 3

Reviewer Report 29 September 2021

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Simon Keely
College of Health, Medicine and Wellbeing, University of Newcastle, Callaghan, NSW, Australia

I have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Gastroenterology, mucosal inflammation, immunology, microbiome, functional GI disorders.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 29 Sep 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

Thanks Sir for your great efforts

Competing Interests: No competing interests were disclosed.

Reviewer Report 31 August 2021

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Gabriela Leite
Medically Associated Science and Technology (MAST) Program, Cedars-Sinai Medical Center, Los Angeles, CA, USA

The authors did not correct the units for antibodies anti-vinculin and anti-cdtB as suggested previously. Neither Results or Abstract were corrected on version 3 of the manuscript. The authors need to clarify the choice of reporting the values as ng/mL, considering that analysis were performed with OD values as stated in Methods.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug development, test development for diagnosis of antibodies and antigens associated with bacterial diseases.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have

significant reservations, as outlined above.

Author Response 01 Sep 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

The results of units for antibodies anti-vinculin and anti-cdtB was expressed as OD values as mentioned in the method section. I will add the OD to the abstract and in the result section.

Competing Interests: No competing interests were disclosed.

Author Response 21 Sep 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

The OD was added to all sections of the study as it was the correct used unit.

Competing Interests: No competing interests were disclosed.

Version 2

Reviewer Report 09 July 2021

https://doi.org/10.5256/f1000research.57371.r88732

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Gabriela Leite
Medically Associated Science and Technology (MAST) Program, Cedars-Sinai Medical Center, Los Angeles, CA, USA

The authors addressed and clarified all comments. Thank you for that. One last adjustment:

On the paragraph below from Results, the units for cdtB and vinculin antibodies levels should be double checked. The levels were measured considering the OD results. If authors included a standard curve on the test, the details should be included in Methods. If no standard curve was included, the test can not be reported as ng/mL Thank you.

"Anti-vinculin and anti-CdtB levels were significantly elevated in IBS patients (1.58 ± 0.496ng/ml and 2.47 ± 0.60 ng/ml) respectively compared to the control subjects (1.13 ± 0.249 ng/ml and 2.1 ± 0.24 ng/ml) respectively with *P* = 0.001 for both (Table 2).Anti-vinculin and anti-CdtB levels were significantly elevated in IBS patients (1.58 ± 0.496ng/ml and 2.47 ± 0.60 ng/ml) respectively compared to the control subjects (1.13 ± 0.249 ng/ml and 2.1 ± 0.24 ng/ml) respectively with *P* =

0.001 for both (Table 2)."

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug development, test development for diagnosis of antibodies and antigens associated with bacterial diseases.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 13 Jul 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

Thanks for your accurate review, I will respond

Competing Interests: No competing interests were disclosed.

Author Response 13 Jul 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

- *On the paragraph below from Results, the units for cdtB and vinculin antibodies levels should be double checked. The levels were measured considering the OD results. If authors included a standard curve on the test, the details should be included in Methods. If no standard curve was included, the test can not be reported as ng/mL Thank you.*
- **Response:** Corrected

Competing Interests: No competing interests were disclosed.

Reviewer Report 07 July 2021

https://doi.org/10.5256/f1000research.57371.r88731

?

Simon Keely
College of Health, Medicine and Wellbeing, University of Newcastle, Callaghan, NSW, Australia

It is very disappointing to see the author's responses to my comments. The comments have largely been dismissed, ignored or superficially addressed and there are errors and discrepancies in the article that have not been amended.

Referring to my previous comments:

1. The response is satisfactory.

2. The authors amended text "the following was added: 'In the present study there was no history of previous infection with *C. jejuni*. However, the elevated levels of antiCdtB and antivinculin can be used as biomarkers for diagnosis of IBS either post infections or without previous infection" is not supported by these results. The authors do not know if the patients were previously infected and thus there is no evidence to suggest that the test can detect or discern post-infectious and idiopathic IBS. The authors have no way of confirming the individuals in their cohort are truly without previous infection. If anything, the data suggest that PI IBS is more common than is realised.

3. The authors still refer to the Rome IV criteria in their Abstract and Methods (and keyword). But their results (paragraph 1) figure legends (Figure 1) suggest that they have classified patients on Rome III criteria. The fact that the authors ignored or misunderstood this critique calls into question the validity of the methodology.

4. The authors have removed the sample size calculation, largely in line with the second reviewer's suggestion. However, without the sample size calculation the conclusion "The principal limitation of the present study was the small sample size. This necessitates the extension of the study to include large sample size." is unfounded. How can you conclude you are underpowered if you have not provided a sample size calculation?

5. The authors choose not to discuss this limitation.

6. With larger studies published and no sample size, the conclusion that a larger study is required is not valid and should be removed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Gastroenterology, mucosal inflammation, immunology, microbiome, functional GI disorders.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 07 Jul 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

The response will be performed in more detail.

Competing Interests: No competing interests were disclosed.

Author Response 13 Jul 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

- *The authors amended text "the following was added: 'In the present study there was no history of previous infection with C. jejuni. However, the elevated levels of antiCdtB and antivinculin can be used as biomarkers for diagnosis of IBS either post infections or without previous infection" is not supported by these results. The authors do not know if the patients were previously infected and thus there is no evidence to suggest that the test can detect or discern post-infectious and idiopathic IBS. The authors have no way of confirming the individuals in their cohort are truly without previous infection. If anything, the data suggest that PI IBS is more common than is realised.*
- **Response:** The following was added: 'The data of the present study support that PI IBS may be more common than it is realized'.

- *The authors still refer to the Rome IV criteria in their Abstract and Methods (and keyword). But their results (paragraph 1) figure legends (Figure 1) suggest that they have classified patients on Rome III criteria. The fact that the authors ignored or misunderstood this critique calls into question the validity of the methodology.*
- **Response:** Corrected

- *The authors have removed the sample size calculation, largely in line with the second reviewer's suggestion. However, without the sample size calculation the conclusion "The principal limitation of the present study was the small sample size. This necessitates the extension of the study to include large sample size." is unfounded. How can you conclude you are underpowered if you have not provided a sample size calculation?*
- **Response:** The recommendation of larger sample size was removed

- *Were any additional data taken on patients that may impact results, BMI or anxiety/depression, for instance? There are previous studies that suggest that HADS can improve the sensitivity of blood based biomarkers for FGIDs (Jones et al. (2014)).*
- **Response:** Added as a limitation as there was no psychological studies performed for those patients

- *With larger studies published and no sample size, the conclusion that a larger study is required is not valid and should be removed.*
- **Response:** Removed

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 11 June 2021

https://doi.org/10.5256/f1000research.55313.r85381

?

Simon Keely
College of Health, Medicine and Wellbeing, University of Newcastle, Callaghan, NSW, Australia

This is an interesting study validating previous work on the utility of CdtB and vinculin as potential biomarkers for functional GI disorders, specifically IBS.

The study is clearly reported although there are a number of inconsistencies that need to be addressed.

There are also a number of statements that would benefit from revision with the more current literature in mind.

1. The introduction speculates on microbiome alterations in IBS however it should be noted that the idea of a specific IBS microbiome is someone controversial with larger studies analysing mucosal microbiomes showing now distinct signature (Hugerth *et al*. (2021[1])).

2. The introduction discusses the association between CdtB and vinculin antibodies and post-infectious IBS and *C. jejuni*, however there is no mention of whether the patients have a post infectious history (reading the manuscript, one assumes they are idiopathic IBS cases) and the increases in these antibodies in a potentially non PI cohort is not addressed in the discussion. This warrants revision.

3. Methodology - the abstract and methods state that these are Rome IV diagnosed patients but the results state that they are Rome III. Please explain this discrepancy.

4. The power calculation is very concerning. The line " the minimum number of the patients was 16585 cirrhotic patients and 830 for the control group" appears to be copied from a different power calculation for a different study. There should be no reason to do this as the power calculation should be independent. Please show the detailed power calculation for this study, including the data used to estimate the sample size.

5. Were any additional data taken on patients that may impact results, BMI or anxiety/depression, for instance? There are previous studies that suggest that HADS can improve the sensitivity of blood based biomarkers for FGIDs (Jones *et al.* (2014[2])).

6. The conclusion is that a larger sample cohort is warranted, but this study validates previous, larger studies.

References

1. Hugerth LW, Andreasson A, Talley NJ, Forsberg AM, et al.: No distinct microbiome signature of irritable bowel syndrome found in a Swedish random population.*Gut*. **69** (6): 1076-1084 PubMed Abstract | Publisher Full Text
2. Jones MP, Chey WD, Singh S, Gong H, et al.: A biomarker panel and psychological morbidity

differentiates the irritable bowel syndrome from health and provides novel pathophysiological leads.*Aliment Pharmacol Ther*. 2014; **39** (4): 426-37 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Gastroenterology, mucosal inflammation, immunology, microbiome, functional GI disorders.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 11 Jun 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

Thanks for reviewing the article. The response will be followed.

Competing Interests: There is no any competing interest

Author Response 15 Jun 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

Response to reviewers:

We would like to thank the reviewer the insightful comments on the paper, as these comments led us to an improvement of the work. Our revisions reflect all reviewers suggestions. Detailed responses to the reviewers are given below.

- *This is an interesting study validating previous work on the utility of CdtB and vinculin as*

potential biomarkers for functional GI disorders, specifically IBS.

- *The study is clearly reported although there are a number of inconsistencies that need to be addressed.*
- *There are also a number of statements that would benefit from revision with the more current literature in mind.*
- **Response:** Thanks, we really appreciate that.

- *The introduction speculates on microbiome alterations in IBS however it should be noted that the idea of a specific IBS microbiome is someone controversial with larger studies analysing mucosal microbiomes showing now distinct signature (Hugerth et al. (2021)).*
- **Response:** Thanks for your nice comment, we mentioned this observation in the introduction.

- *The introduction discusses the association between CdtB and vinculin antibodies and post-infectious IBS and C. jejuni, however there is no mention of whether the patients have a post infectious history (reading the manuscript, one assumes they are idiopathic IBS cases) and the increases in these antibodies in a potentially non PI cohort is not addressed in the discussion. This warrants revision.*
- **Response:** The following was added: 'In the present study there was no history of previous infection with *C. jejuni.* However, the elevated levels of antiCdtB and antivinculin can be used as biomarkers for diagnosis of IBS either post infections or without previous infection'.

- *Methodology - the abstract and methods state that these are Rome IV diagnosed patients but the results state that they are Rome III. Please explain this discrepancy.*
- **Response:** Thanks for your meticulous observation but we depend on Rome IV and we divided patients according to the results of the questionnaire into three groups as there were no patients unclassified.

- *The power calculation is very concerning. The line " the minimum number of the patients was 16585 cirrhotic patients and 830 for the control group" appears to be copied from a different power calculation for a different study. There should be no reason to do this as the power calculation should be independent. Please show the detailed power calculation for this study, including the data used to estimate the sample size.*
- **Response:** Thanks for your comments, it was corrected.

- *Were any additional data taken on patients that may impact results, BMI or anxiety/depression, for instance? There are previous studies that suggest that HADS can improve the sensitivity of blood based biomarkers for FGIDs (Jones et al. (2014)).*
- **Response**: Thanks for your observation but actually we did not have full data about these points.

- *The conclusion is that a larger sample cohort is warranted, but this study validates previous, larger studies.*
- **Response:** Thanks, we agree with you our study validates previous larger studies but to the best of our knowledge this was the first study which discussed this point in Egypt. Unfortunately, due to lack of financial support it was difficult to do the study on large scale of patients.

Competing Interests: No competing interests were disclosed.

Reviewer Report 27 May 2021

https://doi.org/10.5256/f1000research.55313.r86122

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Gabriela Leite
Medically Associated Science and Technology (MAST) Program, Cedars-Sinai Medical Center, Los Angeles, CA, USA

In this study, Zaki and collaborators observed increased levels of plasma CdtB and vinculin antibodies in IBS subjects when compared to controls (non-IBS subjects) and analysis also revealed differences between IBS subtypes. The study has an important clinical relevance, but to be published, several corrections and clarifications should be addressed. The major concern is about the cdtB antibody test. It is not clear which kit was used for this test, authors need to clarify and double-check if the clostridium difficile toxin B (CDTB) kit was used. CdtB from *C. difficile* is a binary toxin termed the *C. difficile* toxin (CDT) and it has pore-forming or delivery subunit termed CDTb. This toxin is not the same of that observed in *Campylobacter jejuni*, the cytolethal distending toxin B. These are two completely different toxins. All publications with cdtB in IBS subjects were based on cdtB from *C. jejuni*. I couldn't find the ELISA kit the authors mentioned on Creative Diagnostics webpage.

Suggestions of writing are marked in italic.

Abstract

- Authors need to restructure the abstract considering suggestions on the manuscript.
- Overall suggestion: the word "patients" should be replaced by "subjects".

Introduction

Paragraph 01:

- "IBS mainly manifests in *subjects with* abdominal pain and bowel habit changes in the

absence…"

- In between Paragraph 01 and 02, authors should present info about IBS subtypes.

Paragraph 02:

- "Irritable bowel syndrome" needs to be substituted by IBS.
- "In these studies, progression to IBS was accompanied by the detection of *circulating levels* of a specific bacterial toxin named cytolethal distending toxin B (CdtB), a potential factor attributing to the pathogenesis of PI-IBS."
- "This was supported by the low incidence of IBS in patients infected with a mutant strain of *C. jejuni* that lacks CdtB." These studies were based on results in rats, not patients.

Paragraph 03:

- "These findings *were linked* to the ability of anti-CdtB to cross-react *with vinculin, a host cell adhesion protein* present in interstitial cells of Cajal and the myenteric ganglia that control the normal activity of the intestinal tract, including phase III of inter-digestive motor activity."
- "Based on these data, it has been suggested that loss of vinculin in the neuromuscular system of the GIT may be associated with the affection of the gut in animal models of post-infection *C. jejuni*." This statement needs a reference. GIT needs to be spelled out.

Paragraph 04"

- "The present study aims to detect *and to quantify* anti-CdtB and anti-vinculin levels *in subjects* with IBS and their possible role in the diagnosis of different IBS subtypes."

Methods

- In the abstract, authors mentioned this study was based in a retrospective study, but in Methods, a prospective study was mentioned
- The authors provided information about samples size calculation (which in my opinion is not needed for this study), but authors mentioned the need of 16585 cirrhotic patients (Paragraph 01 in Methods). Not sure why cirrhotic subjects.
- "According to the actual calculated sample size, we needed to enroll 385 with IBS and 193 for control group at a power of 80% and type I error = 0.05, while at a power of 99% and type I error = 0.01, the minimum number of the patients was 16585 cirrhotic patients and 830 for the control group. This is very hard to achieve in lower economy countries like Egypt. We cannot afford to measure all the parameters in these patients. We had to design the study depending on self-funding without any further support." I don't think this info is needed. Since this is a retrospective study, 100 subjects with matched control group (gender and age) should give you enough power to check differences in quantities/levels. If you are planning to define a cutoff for clinical/diagnosis purpose, then you would need a sample size calculation.
- "Patients were recruited, *and IBS was* determined by a questionnaire-based upon the Rome

IV criteria"

- "The third aliquot was overlaid on heparin for plasma separation, and the remaining sera were stored at -20°C to be used for evaluation of anti-vinculin antibodies by laboratory prepared ELISA and anti-CdtB antibodies by commercial ELISA (Creative Diagnostics. 45-16 Ramsey Road Shirley, NY 11967, USA)." If heparin was used on third aliquot, everything should be plasma, not serum. What was the kit catalog number used for this assay? I only could find the kit "Human Anti-Clostridium Difficile Toxin B (CDTB) ELISA" on Creative Diagnostics webpage. CdtB from this kit refers to *clostridium difficile* toxin B, but cdtB from IBS studies refers to cytolethal distending toxin B, mostly from *C. jejuni*. This information should be clarified as a major priority. The toxin B from *Clostridium* is not the same as cdtB from *C. jejuni.*

- "Anti-vinculin levels were measured in separated plasma using human vinculin protein in a concentration of 1.21.2 µg/ml (Novoprotein Scientific, Summit, New Jersey, USA) as an antigen." Vinculin concentration should be fixed.

- Authors said that "Optical densities (ODs) were read at 370nm, and the results were interpreted as OD", but vinculin antibodies were reported as ng/mL. Please explain. If a calculation was performed, this need to be described.

Results

- "This study included 100 patients with IBS (49 males and 51 females) aged 46.6 ± 6.8 years and 100 healthy controls *(please add sex distribution and mean age/SD here). No differences were observed between IBS subjects and control* regarding age and sex (P = 0.8 and P = 0.6, respectively)." I added a suggestion.

- "Patients were classified according to Rome III criteria into 40 patients with IBS-C, 26 patients with IBS-D, and 34 patients with IBS-M (Figure 1)." Authors mentioned Rome IV criteria in Methods. Figure 1 is not needed.

- "Anti-vinculin and anti-CdtB levels were significantly elevated in IBS patients (1.58 ± 0.496 ng/ml and 2.47 ± 0.60 ng/ml) respectively compared to the control subjects (1.13 ± 0.249 ng/ml and 2.1 ± 0.24 ng/ml) respectively with P = 0.001 for both (Table 2)." Authors need to double check vinculin antibodies unit.

- In table 3, the first P-value of the anti-cdtB results was calculated based on what? All the other P-values (P1 to P6) are explained on the text below the table.

Discussion

- Overall suggestion: the discussion can be better expanded.

- "There is an extreme necessity for the utilization of accessible and reliable, low-cost biomarkers in identifying IBS for the low-risk population to reduce the use of colonoscopy". Low-risk population? What do the authors mean with low risk? Please expand a little bit more why the need of development of a test for IBS.

- "Previous studies have recognized anti-CdtB and anti-vinculin for use as valuable

biomarkers in different IBS patients from healthy controls". Please rephrase it. It is confusing. I encourage the authors to perform a language review of the manuscript. Some suggestions were already included in my review, but I strongly recommend the authors to perform a in deep language review of the entire manuscript.

- "This discrepancy in findings may be attributed to the difference in etiology of different IBS subtypes, as it is hypothesized that most cases of post-infectious IBS manifest as IBS-D or IBS-M, with a minority of patients manifesting as IBS-C. Another factor may make the microbiome profile difference between IBS patient subgroups; bacterial species producing methane are decreased in IBS-D and IBS-M and increased in IBS-C. Patients included in the present study, particularly those in the IBS-C subgroup, may represent patients who develop IBS following infections associated with their microbiota profile changes. These findings need extensive longitudinal studies to be confirmed." These statements are very confusing, not sure if that can be used to explain why IBS-C also had increased cdtB and vinculin antibodies. This needs to be better clarified. In addition, methane producers are mostly Archaea, not bacteria. Increases in methanogens also represents microbiome changes.

- "Anti-vinculin and anti-CdtB levels in this study were significantly elevated in patients with IBS-D, a concordance finding with Pimentel *et al.*, who reported that anti-CdtB and anti-vinculin distinguished IBS-D *from IBD, other* organic GI *diseases* and healthy control. *In addition,* Bayoumy *et al.* reported that anti-vinculin could be an important biomarker for IBS-D diagnosis among Egyptian patients."

- "Cytolethal distending toxin *(use abbreviation) is* a virulence factor for bacterial pathogens such as *Escherichia coli, Salmonella, Shigella*, and *Campylobacter jejuni*, by causing epithelial barrier breakdown and suppression of the acquired immune response to invading pathogens, resulting in an amplified pro-inflammatory response with consequent persistence of bacterial infection. Development of anti-CdtB antibodies occurs in response to secretion of cytolethal distending toxin *(use abbreviation)* following infection with bacterial pathogens. Molecular mimicry accounts for the potential cross-reaction between anti-CdtB and vinculin with resultant anti-vinculin autoantibody production leading to injury to interstitial cells of Cajal (ICC) with the development of IBS. Based on the suggestion of an association between the metabolic syndrome and liver affection and IBS, this study group performed liver function tests as a simple evaluation of liver affection. However, liver enzymes were normal in IBS patients' studied group, in contrast to reports by Lee *et al*." This entire paragraph should be rewritten, it is confusing and not well constructed.

Conclusion

- "The *present findings* support the hypothesis that IBS *may* results from *post-infectious bacterial gastroenteritis.* Moreover, this hypothesis can be applied to all IBS subgroups as both anti-CdtB and anti-vinculin biomarkers were significantly elevated in IBS-C and IBS-D subgroups, with only anti-vinculin being elevated in IBS-M when compared to healthy control."

- "These biomarkers were significantly elevated in IBS-D compared to IBS-C and IBS-M, possibly reflecting the post-infectious state's severity. These findings need further extensive longitudinal studies in patients with IBS." How was severity of the disease accessed? There is

no mention of that in any part of the manuscript, so no conclusion can be made based on severity.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug development, test development for diagnosis of antibodies and antigens associated with bacterial diseases.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 May 2021

Maysaa El Zaki, Mansoura University, Mansoura, Egypt

Response to reviewers:

We would like to thank the reviewer the insightful comments on the paper, as these comments led us to an improvement of the work. Our revisions reflect all reviewers suggestions. Detailed responses to the reviewers are given below.

- *In this study, Zaki and collaborators observed increased levels of plasma CdtB and vinculin antibodies in IBS subjects when compared to controls (non-IBS subjects) and analysis also revealed differences between IBS subtypes. The study has an important clinical relevance, but to be published, several corrections and clarifications should be addressed.*
- **Response**: Many thanks, we really appreciate that.

- *The major concern is about the cdtB antibody test. It is not clear which kit was used for this test, authors need to clarify and double-check if the clostridium difficile toxin B (CDTB) kit*

was used. CdtB from C. difficile is a binary toxin termed the C. difficile toxin (CDT) and it has pore-forming or delivery subunit termed CDTb. This toxin is not the same of that observed in Campylobacter jejuni, the cytolethal distending toxin B. These are two completely different toxins. All publications with cdtB in IBS subjects were based on cdtB from C. jejuni. I couldn't find the ELISA kit the authors mentioned on Creative Diagnostics webpage.

- **Response:** Thanks for your meticulous comment and observation and we already clarify this point in the methodology as the measured was anticdtB from C.jejuni.

Abstract

- *Authors need to restructure the abstract considering suggestions on the manuscript. Overall suggestion: the word "patients" should be replaced by "subjects".*
- **Response:** Thanks, we corrected it.

Introduction

Paragraph 01:

- *"IBS mainly manifests in subjects with abdominal pain and bowel habit changes in the absence…"*
- **Response:** Thanks, corrected.

- *In between Paragraph 01 and 02, authors should present info about IBS subtypes.*
- **Response:** Thanks for your suggestion, we added it.

Paragraph 02:

- *"Irritable bowel syndrome" needs to be substituted by IBS.*
- **Response:** Thanks, corrected.
- *"In these studies, progression to IBS was accompanied by the detection of circulating levels of a specific bacterial toxin named cytolethal distending toxin B (CdtB), a potential factor attributing to the pathogenesis of PI-IBS."*
- **Response:** Thanks for your advise, corrected.
- *"This was supported by the low incidence of IBS <u>in patients</u> infected with a mutant strain of C. jejuni that lacks CdtB." These studies were based on results in rats, not patients.*
- **Response:** Thanks for meticulous observation, modified.

Paragraph 03:

- *"These findings were linked to the ability of anti-CdtB to cross-react with vinculin, a host cell adhesion protein present in interstitial cells of Cajal and the myenteric ganglia that control the normal activity of the intestinal tract, including phase III of inter-digestive motor activity."*
- **Response**: Thanks, corrected.

- *"Based on these data, it has been suggested that loss of vinculin in the neuromuscular system of the GIT may be associated with the affection of the gut in animal models of post-infection C. jejuni." This statement needs a reference. GIT needs to be spelled out.*

- **Response:** Thanks, corrected.

Paragraph 04:

- *"The present study aims to detect and to quantify anti-CdtB and anti-vinculin levels in subjects with IBS and their possible role in the diagnosis of different IBS subtypes."*
- **Response:** Thanks, corrected.

Methods

- *In the abstract, authors mentioned this study was based in a retrospective study, but in Methods, a prospective study was mentione*d
- **Response:** Thanks for your comment, corrected

- The authors provided information about samples size calculation (which in my opinion is not needed for this study), but authors mentioned the need of 16585 cirrhotic patients (Paragraph 01 in Methods). Not sure why cirrhotic subjects.
- *"According to the actual calculated sample size, we needed to enroll 385 with IBS and 193 for control group at a power of 80% and type I error = 0.05, while at a power of 99% and type I error = 0.01, the minimum number of the patients was 16585 cirrhotic patients and 830 for the control group. This is very hard to achieve in lower economy countries like Egypt. We cannot afford to measure all the parameters in these patients. We had to design the study depending on self-funding without any further support." I don't think this info is needed. Since this is a retrospective study, 100 subjects with matched control group (gender and age) should give you enough power to check differences in quantities/levels. If you are planning to define a cutoff for clinical/diagnosis purpose, then you would need a sample size calculation*
- **Response:** Thanks for your advise and suggestion. This calculation was added as a response to the editor of the journal and now it is removed as your suggestion.

- *"Patients were recruited, and IBS was determined by a questionnaire-based upon the Rome IV criteria"*
- **Response:** Thanks, corrected.

- *"The third aliquot was overlaid on heparin for plasma separation, and the remaining sera were stored at -20°C to be used for evaluation of anti-vinculin antibodies by laboratory prepared ELISA and anti-CdtB antibodies by commercial ELISA (Creative Diagnostics. 45-16 Ramsey Road Shirley, NY 11967, USA)." If heparin was used on third aliquot, everything should be plasma, not serum. What was the kit catalog number used for this assay? I only could find the kit "Human Anti-Clostridium Difficile Toxin B (CDTB) ELISA" on Creative Diagnostics webpage. CdtB from this kit refers to clostridium difficile toxin B, but cdtB from IBS studies refers to cytolethal distending toxin B, mostly from C. jejuni. This information should be clarified as a major priority. The toxin B from Clostridium is not the same as cdtB from C. jejuni.*
- **Response:** Thanks for your comments, the study of antibodies for anti cdtB was performed on serum samples as clarified in the methods and the commercial refers to the used components of the ELISA method used. The plasma was used for anti-vinculin antibodies.
- *"Anti-vinculin levels were measured in separated plasma using human vinculin protein in a*

concentration of 1.21.2 µg/ml (Novoprotein Scientific, Summit, New Jersey, USA) as an antigen." Vinculin concentration should be fixed.

- **Response**: Thanks for your observation, corrected.
- *Authors said that "Optical densities (ODs) were read at 370nm, and the results were interpreted as OD", but vinculin antibodies were reported as ng/mL. Please explain. If a calculation was performed, this need to be described.*
- **Response:** Thanks for your insightful advise, we corrected it.
- *"This study included 100 patients with IBS (49 males and 51 females) aged 46.6 ± 6.8 years and 100 healthy controls (please add sex distribution and mean age/SD here). No differences were observed between IBS subjects and control regarding age and sex (P = 0.8 and P = 0.6, respectively)." I added a suggestion.*
- **Response**: Thanks, corrected.
- *"Patients were classified according to Rome III criteria into 40 patients with IBS-C, 26 patients with IBS-D, and 34 patients with IBS-M (Figure 1)." Authors mentioned Rome IV criteria in Methods. Figure 1 is not needed.*
- **Response:** Thanks for your suggestion, we removed it.
- *"Anti-vinculin and anti-CdtB levels were significantly elevated in IBS patients (1.58 ± 0.496 ng/ml and 2.47 ± 0.60 ng/ml) respectively compared to the control subjects (1.13 ± 0.249 ng/ml and 2.1 ± 0.24 ng/ml) respectively with P = 0.001 for both (Table 2)." Authors need to double check vinculin antibodies unit.*
- **Response:** Thanks for your comments, we check it and methodology was modified.
- *In table 3, the first P-value of the anti-cdtB results was calculated based on what? All the other P-values (P1 to P6) are explained on the text below the table.*
- **Response:** Thanks for your comments, we added it and it was already present in the primary submission.

Discussion

- *Overall suggestion: the discussion can be better expanded.*
- **Response:** Thanks, done.
- *"There is an extreme necessity for the utilization of accessible and reliable, low-cost biomarkers in identifying IBS for the low-risk population to reduce the use of colonoscopy". Low-risk population? What do the authors mean with low risk? Please expand a little bit more why the need of development of a test for IBS.*
- **Response:** Thanks for your comment, we clarified this point.
- *"Previous studies have recognized anti-CdtB and anti-vinculin for use as valuable biomarkers in different IBS patients from healthy controls". Please rephrase it. It is confusing. I encourage the authors to perform a language review of the manuscript. Some suggestions were already included in my review, but I strongly recommend the authors to perform a in deep language review of the entire manuscript.*
- **Response:** Thanks, Paraphrasing was done
- *"Anti-vinculin and anti-CdtB levels in this study were significantly elevated in patients with*

IBS-D, a concordance finding with Pimentel et al., who reported that anti-CdtB and anti-vinculin distinguished IBS-D from IBD, other organic GI diseases and healthy control. In addition, Bayoumy et al. reported that anti-vinculin could be an important biomarker for IBS-D diagnosis among Egyptian patients."

- **Response:** Thanks, corrected
- *"Cytolethal distending toxin (use abbreviation) is a virulence factor for bacterial pathogens such as Escherichia coli, Salmonella, Shigella, and Campylobacter jejuni, by causing epithelial barrier breakdown and suppression of the acquired immune response to invading pathogens, resulting in an amplified pro-inflammatory response with consequent persistence of bacterial infection. Development of anti-CdtB antibodies occurs in response to secretion of cytolethal distending toxin (use abbreviation) following infection with bacterial pathogens. Molecular mimicry accounts for the potential cross-reaction between anti-CdtB and vinculin with resultant anti-vinculin autoantibody production leading to injury to interstitial cells of Cajal (ICC) with the development of IBS. Based on the suggestion of an association between the metabolic syndrome and liver affection and IBS, this study group performed liver function tests as a simple evaluation of liver affection. However, liver enzymes were normal in IBS patients' studied group, in contrast to reports by Lee et al." This entire paragraph should be rewritten, it is confusing and not well constructed.*
- **Response:** Thanks, paraphrasing was done

- *The present findings support the hypothesis that IBS may results from post-infectious bacterial gastroenteritis. Moreover, this hypothesis can be applied to all IBS subgroups as both anti-CdtB and anti-vinculin biomarkers were significantly elevated in IBS-C and IBS-D subgroups, with only anti-vinculin being elevated in IBS-M when compared to healthy control*
- **Response:** Many thanks, corrected

- *"This discrepancy in findings may be attributed to the difference in etiology of different IBS subtypes, as it is hypothesized that most cases of post-infectious IBS manifest as IBS-D or IBS-M, with a minority of patients manifesting as IBS-C. Another factor may make the microbiome profile difference between IBS patient subgroups; bacterial species producing methane are decreased in IBS-D and IBS-M and increased in IBS-C. Patients included in the present study, particularly those in the IBS-C subgroup, may represent patients who develop IBS following infections associated with their microbiota profile changes. These findings need extensive longitudinal studies to be confirmed." These statements are very confusing, not sure if that can be used to explain why IBS-C also had increased cdtB and vinculin antibodies. This needs to be better clarified. In addition, methane producers are mostly Archaea, not bacteria. Increases in methanogens also represents microbiome changes.*
- **Response:** Many thanks, deleted.

Competing Interests: No any competing interests