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Anti-CdtB and anti-vinculin antibodies to diagnose irritable bowel syndrome in inflammatory bowel disease patients

Luisa Leite Barros[1†], Gabriela Leite[2†], Walter Morales[2], Gillian M. Barlow[2], Matheus Freitas Cardoso de Azevedo[1], Alexandre de Sousa Carlos[1], Adérson Omar Mourão Cintra Damião[1], Mark Pimentel[2,3] and Alberto Queiroz Farias[1*]

Abstract

Background Despite adequate treatment, a subgroup of patients with inflammatory bowel disease (IBD), including Crohn`s disease and ulcerative colitis, have persistent gastrointestinal symptoms that are not always related to mucosal damage. Recently, two autoantibodies, anti-CdtB and anti-vinculin, were validated as post-infectious IBS (PI-IBS) markers, however there is limited evidence of its diagnostic role in IBD population.

Methods Patients with more than 3 bowel movements/day and indication of colonoscopy were enrolled. Samples were collected at the time of colonoscopy for assessment of serum levels of anti-CdtB and anti-vinculin antibodies.

Results A total of 160 subjects were included in 4 groups: active IBD ($n=44$); quiescent IBD and chronic diarrhea IBD-IBS ($n=25$); predominant-diarrhea IBS ($n=45$) and controls ($n=46$). The mean value of the optical density for anti-CdtB was 1.2 ± 0.65 in group 1, 1.27 ± 0.64 in group 2, 1.49 ± 0.47 in the group 3 and 1.6 ± 0.68 in group 4, $p=0.012$. For anti-vinculin, optical densities were: 1.34 ± 0.78 in group 1, 1.46 ± 0.92 in group 2, 1.31 ± 0.79 in group 3 and 1.41 ± 0.86 for controls ($p=0.875$). Using a cut-off of 1.56 for anti-CdtB, the positivity between groups was $n=10$ (22.7%) in group 1, $n=9$ (34.6%) in group 2, 19 (43.2%) in group 3, 21 (45.7%) in group 4 ($p=0.106$). The positivity of anti-vinculin using a cut-off of 1.6 was $n=18$ (40.9%) in group 1, $n=11$ (42.3%), $n=15$ (34.1%), $n=22$ (47.8%) ($p=0.622$).

Conclusions Our findings show that anti-CdtB and anti-vinculin could not identify IBD-IBS patients or discriminate IBS-D from healthy controls.

Keywords Inflammatory bowel disease, Irritable bowel syndrome, Anti-CdtB, Anti-vinculin

Introduction

Inflammatory bowel disease (IBD) comprises both Crohn's disease (CD) and ulcerative colitis (UC), which are chronic immune-mediated disorders characterized by a relapsing and remitting course [1, 2]. They frequently affect young adults, demand continuous surveillance, and significantly impact the healthcare system [3]. The pathophysiology of IBD is a result of genetic inheritance, dysrupted epithelial barrier, decreased microbial diversity, and inappropriate activation of the immune system directed to commensals or

†Luisa Leite Barros and Gabriela Leite contributed equally to this work.

*Correspondence:
Alberto Queiroz Farias
alberto.farias@hc.fm.usp.br
[1] Department of Gastroenterology, University of Sao Paulo School of Medicine, Av. Dr. Eneas C Aguiar 255, Sao Paulo-SP 9117, Brazil
[2] Medically Associated Science and Technology (MAST) Program, Cedars-Sinai Medical Center, Los Angeles, CA, USA
[3] Karsh Division of Gastroenterology and Hepatology, Cedars-Sinai Medical Center, Los Angeles, CA, USA

external pathogens [4]. Over the past decade, epidemiologic studies have shown an increasing incidence and prevalence of IBD in developing countries from Latin America, suggesting that environmental factors may play a role as triggers of these conditions [5].

An important part of IBD prognosis lies in maintaining histo-endoscopic healing to prevent long-term bowel damage [6]. Yet, a great number of patients experience persistent gastrointestinal symptoms such as watery diarrhea and abdominal pain that are not always related to the inflammatory burden, but to other conditions these patients often develop, such as irritable bowel syndrome (IBS) [7].

IBS is recognized as microbiome-gut-brain axis disorder [8]. Recent studies have demonstrated that superimposed IBD-IBS patients have impaired neuronal response to chronic inflammation, visceral hypersensitivity, hyperalgesia, allodynia, dysmotility, and abnormal intestinal secretion. Moreover, there is increased intestinal permeability and leucocyte activation as demonstrated in IBD pathogenesis [9, 10]. In clinical practice, IBS is the most common superimposed diagnosis, especially in CD, and a recent meta-analysis demonstrated that one-in-three patients with IBD report symptoms compatible with IBS [7]. Although the ROME IV criteria are the standard tool to diagnose IBS in the general population, they have not been validated in the IBD population, with high false-positive results [11].

Recent data have shown promising results for IBS biomarkers, including the validation of specific antibodies related to the pathophysiology of post-infectious IBS (PI-IBS) and diarrhea-predominant IBS (IBS-D) [12]. A possible causal factor in PI-IBS/IBS-D is the development of autoimmunity to a host protein, vinculin, triggered by exposure to cytolethal distending toxin B (CdtB), a toxin produced by *Campylobacter jejuni*, and a few specific strains of *Escherichia coli, Salmonella* and *Shigella,* which also encode a slightly modified CdtB in their genome. In a large cohort of approximately 3,000 patients from the United States, higher levels of antibodies to CdtB and vinculin were detected in IBS-D subjects as compared to IBD subjects and healthy controls [13]. However, there is limited evidence regarding the utility of anti-CdtB and anti-vinculin in confirming IBS in quiescent IBD, or in diagnosing IBS-D subjects from countries where the incidence of gastroenteritis caused by *C. jejuni* and specific strains of CdtB-encoding bacteria is very low, such as Brazil. Thus, the aim of this study was to determine the clinical utility of an antibody-based strategy to diagnose IBS/IBS-D in IBD patients that present with chronic diarrhea despite endoscopic remission.

Materials and methods

Study design and population

In this prospective observational single-center study of diagnostic accuracy, the STARD guidelines (Standards for Reporting of Diagnostic Accuracy Studies) were followed to explore the utility of anti-vinculin and anti-CdtB antibodies to diagnose superimposed IBD-IBS [14]. The study was approved by the Institutional Ethics Review Board of Clinics Hospital, University of São Paulo School of Medicine, number 2.727.928, and all subjects provided informed written consent prior to inclusion in the study.

This study was conducted between 2019 and 2020 at the Department of Gastroenterology, University of São Paulo in collaboration with the Karsh Division of Gastroenterology and Hepatology, Cedars-Sinai Medical Center, Los Angeles.

Inclusion criteria for all groups were three or more bowel movements a day for more than one month and clinical indication for ileocolonoscopy. All subjects for the study were older than 18 years of age. Exclusion criteria were current or previous malignancy, HIV infection, other concomitant intestinal diseases, previous bowel surgery, and corticosteroids use 6 months prior to inclusion.

IBD was diagnosed based on symptoms, evidence of inflammatory activity (C-reactive protein, fecal calprotectin), ileocolonoscopy with biopsies and radiographic exams, and subjects with IBS-D based on the ROME IV criteria. All IBS-D patients were routinely screened for stool ova and parasites testing, and stool culture. In addition, IBD groups were also tested for *Clostridoides difficile* toxins A and B to rule out *C. diff* infection. All subjects underwent a stringent protocol that included thorough medical history, physical examination and chart review including laboratory, imaging, and biopsy data. IBD activity scores of Harvey-Bradswaw index and partial Mayo scores were calculated as appropriate [15, 16].

Groups and diagnostic criteria

Subjects were recruited into 4 different groups (Fig. 1):

- Group 1: Active IBD defined by the presence of diarrhea, inflammatory markers (increased C-reactive protein or fecal calprotectin) and endoscopic activity (presence of ulcers).
- Group 2: Quiescent IBD with persistent diarrhea (IBD-IBS). For Crohn's disease subjects, remission was defined by Harvey-Bradshaw index < 5, CRP < 5 mg/dL, fecal calprotectin < 250 µg/g and simple endoscopic score for Crohn's disease (SES-CD)

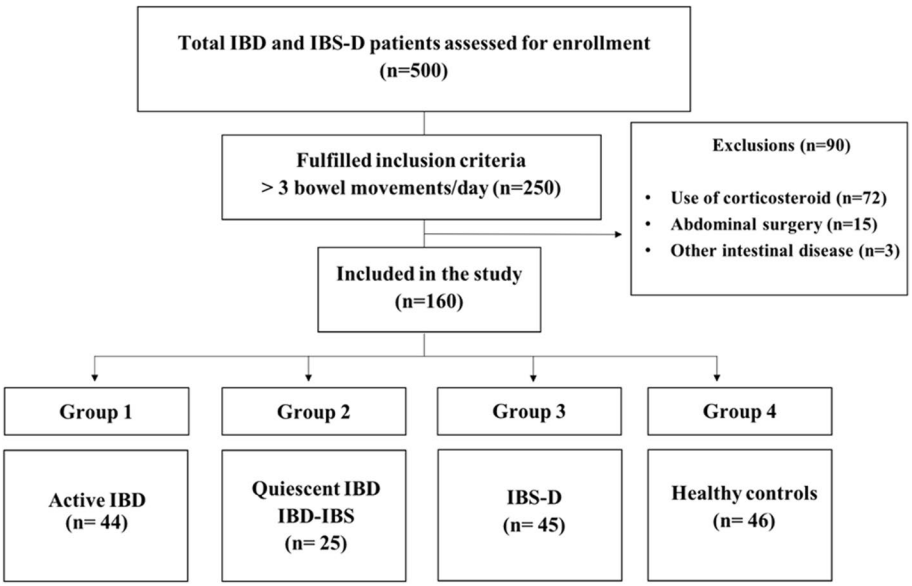

Fig. 1 Flowchart of the study population

score < 2. For UC subjects, remission was defined by clinical assessment, simplified Mayo index of 0 or 1, CRP < 5 mg/dL, fecal calprotectin < 250 µg/g and Mayo score of 0 or 1 in the ileocolonoscopy.

– Group 3: Diarrhea-predominant irritable bowel syndrome (IBS-D) diagnosed based on the ROME IV criteria. All subjects had normal CRP values, negative IgA anti-transglutaminase and fecal calprotectin < 50 mcg/g.
– Group 4: Healthy controls. Asymptomatic individuals recruited from the colorectal cancer screening program. All healthy controls were screened for prior history of gastrointestinal disease and for active gastrointestinal symptoms based on history and a bowel symptom questionnaire.

Plasma collection, anti-CdtB and anti-vinculin testing

Blood was collected from each subject (approximately 20 mL) by venipuncture into a lavender-top tube on the day of inclusion or immediately before colonoscopy, centrifuged at 1300xg for 10 min, and the plasma was stored at—80 °C.

Levels of anti-CdtB and anti-vinculin antibodies were analyzed by indirect ELISA, as described previously [17]. Briefly, synthetic CdtB and vinculin were immobilized overnight at 4 °C onto high-binding 96-well plates (Grenier Bio-One, Monroe, NC) in Borate Buffered Saline (BBS) (Medicago, Uppsala, Sweden) at a pH of 8.2. Wells were alternately coated with antigens or left uncoated in BBS to allow determination of non-specific binding of antibodies present in the plasma. Bovine albumin 3% in a phosphate buffered solution was used to block non-binding sites for one hour. Coated and uncoated wells were then incubated with a 1:512 dilution of plasma for anti-CdtB detection and a 1:32 dilution of plasma for anti-vinculin detection for 1 h at room temperature. Isolated antibodies to CdtB and vinculin were used as positive controls. Plates were washed five times with 0.05% PBS-Tween 20 and incubated for 1 h with HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Finally, a 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution (Pierce, Rockford, IL) was used for visualization and immediately read on a BioTek Synergy HT plate reader (Winooski, VT). The optical densities were read after 70 min at a wavelength of 370 nm.

Statistical analysis

The qualitative variables were expressed by absolute (n) and relative (%) frequency. Numerical variables were summarized by mean ± standard deviation. Comparisons between groups were made using t-tests, Mann–Whitney tests, Chi-square, or Fisher exact tests, as dictated by data type and distribution. A p-value < 0.05 was considered significant for all statistical analyses in this study.

Results

Subject demographics

In total, 160 subjects were recruited into 4 groups, group 1 (n = 44, mean age: 39.7 ± 14.1, female (F) = 45.5%), group 2 (n = 25, mean age: 45.7 ± 14.6, F = 69.2%), group

3 ($n=45$, mean age: 40 ± 14.5, F = 77.3%), group 4 ($n=46$, mean age: 56.6 ± 11.6, F = 71.7%).

UC was diagnosed in 54.5% and 72% of the subjects from groups 1 and 2, respectively. The majority of subjects in group 1 had pancolitis (66.6%), as opposed to 33.3% of UC patients from group 2. Crohn's disease was ileocolonic in 75% and 57.1% of subjects in groups 1 and 2, respectively. In CD subjects, 78% had mild clinical activity, 16% had moderate activity and 7% exhibited severe active disease. In UC subjects, 25% had mild activity and 75% moderate activity, respectively, and none had severe UC (Table 1). The mean disease duration was 10.7 years (SD 14.4) in group 1 and 9 years (SD 9.8) in group 2.

Anti-CdtB and anti-vinculin are not elevated in superimposed IBD-IBS subjects

The mean optical density (OD) of circulating anti-CdtB antibodies was 1.2 ± 0.65 in group 1, 1.27 ± 0.64 in group 2, 1.49 ± 0.47 in group 3 and 1.6 ± 0.68 in group 4 (Fig. 2A). Levels of anti-CdtB did not differ between any group pair or across all groups ($p=0.017$, Fig. 2A). Using an OD cutoff of 1.56 for anti-CdtB positivity, the frequency of positive cases was 22.7% ($n=10$) in group 1, 34.3% ($n=9$) in group 2, 43.2% ($n=19$) in group 3, and 45.7% ($n=21$) in group 4 (Table 2).

In contrast, group 2 subjects demonstrated a trend of higher levels of anti-vinculin (OD mean = 1.46 ± 0.92) when compared to group 1 (OD mean = 1.34 ± 0.78), group 3 (OD 1.31 ± 0.79) and group 4 (OD mean = 1.41 ± 0.86), but no statistically significanct differences were observed ($p=0.875$, Fig. 2B). Using an OD cutoff of 1.6 for anti-vinculin, the frequency of positity was 40.9% ($n=18$) in group 1, and 42.3% ($n=11$), 34.1% ($n=15$) and 47.8% ($n=22$) in groups 2, 3 and 4, respectively (Table 3).

Discussion

In the present study, we assessed the clinical utility of anti-CdtB and anti-vinculin antibodies to diagnose superimposed IBS-D in IBD subjects, and to diagnose IBS-D in Brazil. Circulating levels of anti-CdtB and anti-vinculin antibodies did not differ between groups when compared to healthy controls [17].

IBS prevalence in IBD is three times greater than that in the general population [18]. A previous metanalysis have shown a pooled prevalence of IBS-type symptoms in 32.5% of IBD subjects. The prevalence was lower

Table 1 Group demographics

	Group 1 (Active IBD)	Group 2 (IBD-IBS)	Group 3 (IBS-D)	Group 4 (Healthy controls)	*P*-value (Across all groups)
Number of subjects	44	25	45	46	NA
Age	39.7 ± 14.1	45.7 ± 14.6	40 ± 14.5	56.6 ± 11.6	< 0.0001
Female (%)	45.5	69.2	77.3	71.7	0.0124
BMI	22.9 ± 4.9	24.7 ± 3.5	23.9 ± 4.6	26.9 ± 3.8	0.0001
Smoking (%)	4.5	3.8	4.5	8.7	0.8152
CD[a] (%)	47.7	28	NA	NA	0.2022
Location					
Ileal only (n)	0	2			
Ileocolonic (n)	15	4	NA	NA	0.0834
Colonic (n)	5	1			
Behaviour					
Inflammatory (n)	14	5	NA	NA	0.3366
Stricturing (n)	4	0			
Penetrating (n)	2	2			
UC[b] (%)	54.5	72	NA	NA	0.2022
Proctitis (n)	1	4			
Left-side colitis (n)	7	8			0.0568
Pancolitis (n)	16	6			
CRP[c] (NR < 5 mg/dL)	13.4 ± 18.4	2.8 ± 3.6	NA	NA	0.0014

Values are given as mean ± standard deviation

[a] Crohn`s disease

[b] Ulcerative colitis

[c] C-reactive protein

[d] Normal range

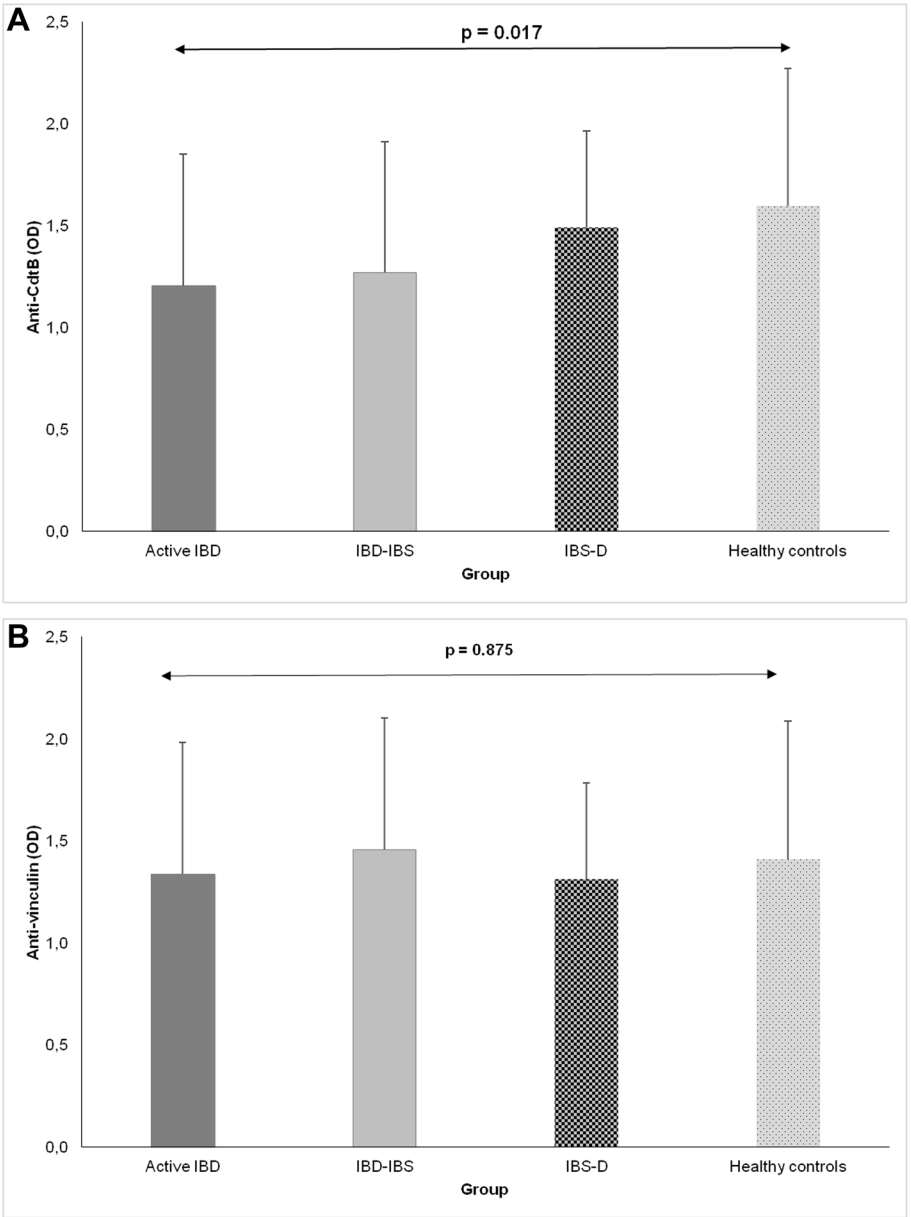

Fig. 2 **A, B** Anti-CdtB (**A**) and anti-vinculin (**B**) optical densities in subjects from group 1 (active IBD), group 2 (IBD-IBS), group 3 (IBS-D), and group 4 (healthy controls). Group pairs were compared using unpaired t-test

Table 2 Anti-CdtB positivity between groups

Anti-CdtB cutoff	Group 1 (Active IBD)		Group 2 (IBD-IBS)		Group 3 (IBS-D)		Group 4 (Healthy controls)		*p*
≤ 1.56	34	77.3%	17	65.4%	25	56.8%	25	54.3%	0.106
> 1.56	10	22.7%	9	34.6%	19	43.2%	21	45.7%	
Total	44	100%	25	100%	45	100%	46	100%	

Table 3 Anti-vinculin positivity between groups

Anti-vinculin cutoff	Group 1 (Active IBD)		Group 2 (IBD-IBS)		Group 3 (IBS-D)		Group 4 (Healthy controls)		*p*
≤ 1.6	26	59.1%	15	57.7%	29	65.9%	24	52.2%	0.622
> 1.6	18	40.9%	11	42.3%	15	34.1%	22	47.8%	
Total	44	100%	25	100%	45	100%	46	100%	

when remission was defined by endoscopic evaluation compared with clinical assessment and was higher in Crohn's disease than in ulcerative colitis (OR 1.58; 95% CI 1.27–1.98) [7]. Recently, various biomarkers have been developed with the aim of making IBS a diagnosis of inclusion. Considering that the anti-CdtB and anti-vinculin antibodies are directed against microbial epitopes, we hypothesized that the accuracy of these biomarkers could be influenced by the high prevalence of intestinal pathogens in developing countries. As far as we know, this is the first study that analyzed an antibody-based approach in IBD-IBS subjects and the first one to compare antibodies levels with IBD endoscopic activity.

The results of the present study diverge from those observed by Pimentel et al. [13]. They analyzed anti-CdtB and anti-vinculin antibodies in a cohort of 2,681 patients from 180 hospitals in the United States. The primary endpoint was to assess the accuracy of these biomarkers in a group of individuals diagnosed with IBS-D according to the ROME IV criteria, and to compare them to IBD subjects, subjects with celiac disease, and healthy controls [13]. More recently, the same authors developed a second-generation test that incorporated epitope stabilization for CdtB and vinculin [17]. They retrospectively analyzed samples from 100 patients with IBS-D and 31 patients with IBD, and found the sensitivity and specificity of anti-CdtB and anti-vinculin were 43% and 52.2%, and 93.5% and 90.9%, respectively [17]. In the present study, we used second generation tests. However, our groups were divided between active IBD, IBD-IBS, IBS-D and controls. No difference was observed between UC or CD patients. The present study was single-centered and included subjects with a severe disease phenotype from a quaternary hospital. Therefore, the majority of IBD subjects were treated with immunosuppressive drugs or biologics. We can argue whether the levels of both antibodies were lower due to the effect of immunosuppressants. Supporting this argument, it is known that these agents can reduce the development of anti-drug antibodies and, theoretically, other antibodies [19].

The second difference was the IBS duration between the two studies. In our study, both the active and quiescent IBD groups had a long-standing disease. Klem et al. published a meta-analysis that included 45 prospective studies of PI-IBS [20]. The authors calculated an incidence of 10.1% at 3 months and 14.5% at more than 1 year after infectious enteritis. The risk of PI-IBS was 4.2 times higher in subjects who had intestinal infection compared to controls [20]. We can further speculate as to whether anti-CdtB and anti-vinculin antibodies would perform differently if a previous gastrointestinal infection was identified prior to subject inclusion.

Scallan Walter et al. showed the burden of Campylobacter infection in the development PI-IBS [21]. They calculated a 1-year incidence rate of IBS following *Campylobacter jejuni* infection of 16.7 and 3.9 per 1,000 among cases and non-cases, respectively, with an unadjusted risk ratio of 4.3 (95% CI: 3.0–6.2) [21]. However, in Brazil, data on the prevalence of PI-IBS and its most frequent pathogens are limited.

C. jejuni infection is one of the most common causes of bacterial gastroenteritis in the world, with different epidemiological profiles between developed and underdeveloped countries. In developed countries, prevalence is mainly related to outbreaks, but is endemic, asymptomatic, and rarely seasonal in low-income countries. Veras et al. analyzed the virulence genes of *C. jejuni* and inflammatory biomarkers in a case–control study with 340 children in Brazil [22]. Fecal DNA samples were extracted and exposure to *C. jejuni* was evaluated using a polymerase chain reaction assay. The prevalence of *C. jejuni* in this population was 9.7% and the most expressed genes were CadF, IamA, CheW and SodB. The CdtB gene was found to be expressed in 15.2% of the population. In addition, the authors observed that malnourished children had higher rates of infection with strains of *C. jejuni* lacking the CdtB gene [22]. This might explain the lower titers of anti-CdtB in our study. In Brazil, 85% of *Campylobacter* strains did not express the CdtB antigen and this may have negatively impacted our results [22].

Antibodies raised against CdtB are thought to cross-react with vinculin due to molecular mimicry [23]. An experimental study by Narcisi et al. demonstrated the presence of vinculin in the cytoskeleton of *Giardia duodenalis* [24]. Giardiasis affects 280 million individuals a year and its estimated prevalence in Brazil is around 30% [25]. Therefore, the positivity of anti-vinculin amongst

the four groups could be explained by the high prevalence of giardiasis and could possibly represent cross-reactivity.

Schmulson et al. analyzed anti-CdtB and anti-vinculin antibodies in Mexican subjects with chronic diarrhea. Thirty patients with IBS-D, IBS-M, functional diarrhea, microscopic colitis, and tropical sprue were included. Antibody positivity was 55% in IBS subjects, similar to that in the original study by Pimentel et al. (58.6%) [26].

Talley et al. evaluated the diagnostic utility of anti-CdtB and anti-vinculin antibodies among individuals with IBS, functional dyspepsia, and healthy controls [27]. The authors found no statistically significant difference in anti-CdtB and anti-vinculin levels between the groups. However, anti-CdtB results were numerically higher in IBS patients compared to controls (2.36 vs 2.14, $p=0.06$). IBS diagnosis was based on clinical assessment, rather than the ROME IV criteria, and IBD subjects were not included. Furthermore, Talley et al. assessed serum levels of the antibodies rather than plasma, which differed from our work as well as that of Pimentel et al.

Our study has some limitations. The number of CD were not similar to the UC patients. We could.

IBS-D subjects were not classified as PI-IBS. They were included if they had 3 or more bowel movements a day and met ROME IV criteria. According to the ROME IV, the diagnosis of PI-IBS requires the onset of symptoms following a resolution of an acute gastroenterocolitis, defined by positive stool culture in a symptomatic patient or by the presence of two of the symptoms, such as fever, vomiting or diarrhea [28]. Since the anti-CdtB antibody results from the pathophysiology of PI-IBS, negative results should be analyzed with caution. Furthermore, the antibodies were not assessed at the time of the IBS-D diagnosis. Some authors have demonstrated that IBS symptoms, such as abdominal pain and diarrhea, begin in the first 6 months following infectious enteritis [29, 30]. Clinical improvement occurs in 25% of patients in the first year and in 50% in 6 to 8 years.

The lack of statistical significance of the present study does not weaken the antibody-based approach to diagnose IBS-D. The antibodies were developed in light of the current knowledge of IBS pathophysiology: altered microbiota composition, dysmotility, increased intestinal permeability, immune dysregulation, and visceral hypersensitivity [23, 31, 32]. However, in the IBD setting, they have not contributed to the diagnosis of superimposed IBS-D.

Our results cannot be generalized to the wider population. This study included complex subjects from a quaternary hospital, with long-term conditions treated with immunosuppressive drugs. IBS-D is a prevalent disease and non-invasive diagnosis reduces costs to the health system, avoids futile tests and guides proper treatment, as previously demonstrated in the literature [33]. Future studies are needed for the external validation of anti-CdtB and anti-vinculin antibodies and to establish ideal cutoff values in different populations.

Authors' contributions

L.L.B., A.Q.F. and M.P. conceived and designed the study. M.F.C.A., A.S.C. and A.O.M.C.D. recruited subjects and performed clinical assessment. W.M. performed laboratory experiments. L.L.B. and A.Q.F. analysed the data. L.L.B. and G.L. prepared the original draft. A.Q.F., G.M.B. and M.P. reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Ethics Review Board of Clinics Hospital, University of São Paulo School of Medicine. Written informed consent was obtained from patients before enrollment.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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