

ORIGINAL ARTICLE

# No difference in serum levels of B-cell activating receptor and antibodies against cytolethal distending toxin B and flagellin in post-infectious irritable bowel syndrome and chronic fatigue syndrome after *Giardia* infection

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## Key words

B-cell activating factor, biomarker, chronic fatigue syndrome, cytolethal distending toxin B, flagellin, irritable bowel syndrome, myalgic encephalitis.

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## Abstract

**Background and Aim:** Functional gastrointestinal disorders (FGIDs) and chronic fatigue syndrome (CFS) frequently occur as comorbid conditions to each other. A shared etiology of these syndromes has been proposed because of their shared symptomatology and triggering by infections. Antibodies against the bacterial antigens cytolethal distending toxin B (CdtB) and flagellin have been proposed to be biomarkers of irritable bowel syndrome (IBS), especially diarrhea-predominant IBS (IBS-D). It is unknown if they may also be associated with comorbid conditions such as CFS. On the other hand, elevated level of B-cell activating factor (BAFF) has been associated with CFS and inflammatory bowel disease (IBD) and subjective food intolerance.

**Methods:** We evaluated serum levels of anti-flagellin and anti-CdtB using an in-house enzyme-linked immunosorbent assay (ELISA) and BAFF with a commercially available ELISA kit in a cohort of patients who developed fatigue syndromes and/or FGIDs after *Giardia* infection, by comparing them with healthy controls without these conditions.

**Results:** We did not find significant differences in circulating BAFF, anti-CdtB, or anti-flagellin antibody levels in these patient groups compared to healthy controls. Therefore, our results do not support a role for BAFF, anti-CdtB, or anti-flagellin antibodies as universal biomarkers for IBS or CFS.

**Conclusion:** BAFF, anti-CdtB, or anti-flagellin antibodies cannot be considered as universal biomarkers for IBS or CFS.

## Introduction

Functional gastrointestinal disorders (FGID) and chronic fatigue syndrome (CFS) are based on self-reported symptoms and the exclusion of other causal etiology of symptoms. Patients with FGID, among which irritable bowel syndrome (IBS) is one of the most well-known disorders, often report fatigue symptoms. Sometimes these are severe enough to fulfill the criteria for CFS, making these two enigmatic conditions comorbidities to each other.<sup>1</sup> In fact, there is a large body of literature supporting a shared etiology of these syndromes because of their shared symptomatology and triggering by infections.<sup>2–4</sup>

There is a need for measurable biomarkers for both conditions for diagnostic purposes and to help discern the poorly understood underlying pathophysiology. B-cell activating factor (BAFF) and antibodies against cytolethal distending toxin B (CdtB) and flagellin have been suggested to be such markers.

BAFF is a cytokine that is important for B-cell survival, maturation, and function. Significantly, though moderately, elevated serum levels of BAFF have been found in CFS patients ( $n = 70$ ) compared to healthy controls ( $n = 56$ ).<sup>5</sup> This may possibly reflect a more activated B-cell compartment in these patients. Higher serum and fecal BAFF levels have also been found in patients with IBD<sup>6</sup> and also in the serum in a small study of patients with self-reported food intolerance,<sup>7</sup> a condition that overlaps considerably with IBS.

CdtB is the active subunit of Cdt and is produced by several Gram-negative pathogenic enteric bacteria such as enteropathogenic *Escherichia coli*, *Shigella*, *Salmonella*, and *Campylobacter*. Antibodies against CdtB (anti-CdtB) were found to be elevated in 2375 cases of diarrhea-predominant IBS (IBS-D) compared to 43 healthy controls.<sup>8</sup> It could be potentially useful in the differential diagnosis of IBD and IBS, as it has been found to be elevated in IBS compared to IBD.<sup>8,9</sup> Recently an

Australian study found elevated anti-CdtB in functional dyspepsia, a sub-category of FGID, compared to healthy controls, but it could not confirm that anti-CdtB discriminated IBS from the organic disease.<sup>10</sup>

Flagellin is an important structural component of bacterial flagella. Antibodies against flagellins are commonly found in Crohn's disease, and have also been found to be more frequent in IBS patients than healthy controls.<sup>11</sup> Antibodies to flagellin have also been reported to be elevated in IBS patients compared to controls, especially in the fraction with IBS-D.<sup>12</sup> Elevated levels of flagellin and anti-CdtB have been suggested because many IBS cases are post-infectious in nature, elicited by bacteria expressing one or both these antigens.<sup>9</sup> Anti-CdtB and anti-flagellin levels have not been evaluated in patients with CFS until now.

After a waterborne outbreak of giardiasis in Bergen, Norway, in 2004, we have followed a cohort of patients with post-infectious FGID.<sup>13</sup> Some patients were also diagnosed with CFS and the related condition idiopathic chronic fatigue (ICF).<sup>1</sup> ICF fulfills the primary criterion but less than four of the eight additional Fukuda CFS criteria.<sup>14</sup> Based on the above-mentioned studies, we hypothesized that there would be elevated serum levels of BAFF in post-giardiasis ICF/CFS and FGID as an indication of low-grade immune activation, or possibly elevated levels of anti-CdtB and anti-flagellin due to a potentially compromised barrier function.

The aim of the study was to examine the potential of BAFF, anti-CdtB, and anti-flagellin as diagnostic markers for CFS and IBS in a cohort of patients developing one or both these disorders after *Giardia lamblia* gastroenteritis,<sup>1</sup> a nonbacterial pathogen-induced disease.

## Methods

*Giardia*-exposed cases were recruited via mail, based on their replies to a fatigue questionnaire in an epidemiological study in 2007,<sup>13</sup> 3 years after the outbreak. Sera as well as data from Rome II FGID and clinical evaluation ( $n = 102$ ) were collected in 2009, 5 years after the *Giardia* outbreak among persons who had lab-confirmed giardiasis. Clinical data from this study was published in 2013. Fifty-three cases were evaluated 5 years post infection, and clinical work-up and categorization were based on each patient being evaluated by a neurologist, psychiatrist, and internist, as well as completed case report forms as described previously.<sup>1</sup> One patient with known ulcerous colitis was excluded. Eighteen patients were found to have CFS and four had ICF according to the Fukuda criteria. Based on Rome II criteria, 46 cases had FGID and 31 of these had IBS, including 14 with IBS-D. Five of the cases had partially recovered from fatigue or were found to have concomitant diseases, and were excluded from further analysis.

Thirty healthy controls without known previous giardiasis, who neither had fatigue nor FGID, were recruited by advertisements. Twenty control persons with lab-confirmed giardiasis during the outbreak were recruited by mail. The latter group had recovered from their *Giardia* infection after metronidazole treatment, in the sense that they had not experienced prolonged fatigue, and were recruited into the study based on normal Chalder fatigue score in the 2007 questionnaire. All controls

completed the questionnaires in 2009, and sera were collected in the same way. Nine controls in the *Giardia*-exposed group still had various FGIDs other than IBS (Table 1). Thus, for analysis of the association between the three markers and FGID, these 9 persons were included in the FGID group, resulting in a total of 55 participants in the FGID group, which was compared with the remaining subgroup of 41 controls without any FGID.

Serum samples obtained at the same time as the clinical work-up 5 years after the onset of symptoms as well as concurrent control samples were included. The three serum markers were measured in duplicates by enzyme-linked immunosorbent assay (ELISA), with samples from each group stratified across plates. A commercially available kit, Quantikine ELISA Human BAFF Immunoassay (R&D Systems, Minneapolis, MN, USA), was used to obtain serum concentrations of BAFF. For anti-CdtB and anti-flagellin, the optical density (OD) values from an in-house ELISA were recorded. ELISA plates were coated with antigens from *Campylobacter jejuni* CdtB (Creative BioMart, Shirley, NY, USA) at the concentration  $1.2 \times 10^{-3}$  mg/mL and FLA-ST Ultrapure, purified flagellin from *Salmonella typhimurium*—TLR5 ligand at a concentration  $2.5 \times 10^{-4}$  mg/mL (InvivoGen, Toulouse, France) in boric acid buffer. After washing and blocking with 2% bovine serum albumin and washing, wells were incubated with serum at 1:500 dilution. After washing and the addition of horseradish peroxidase (IgG; 1:5000), wells were washed again and tetramethylbenzidine was added. Absorbance at 450 nm was recorded after subtracting the background values of wells without the serum.

The Mann–Whitney *U*-test was used to compare the concentrations of BAFF and OD values for anti-CdtB and anti-flagellin antibodies between groups. Values where duplicates showed a coefficient of variation above 10% were discarded.

The Western Norway Committee for Medical and Health Research Ethics approved the study.

## Results

The mean age of all participants was 41.0 years (SD 11.8), and 68.6% were females. There were no significant age or gender differences between cases, or case subgroups, and controls (Table 2).

When comparing serum-BAFF concentrations in FGID or ICF/CFS subgroups *versus* their respective control groups, we did not find any significant differences. Also, when comparing the serum levels of anti-CdtB and anti-flagellin, we found these to be similar in cases and controls (Table 3).

We also did not find any differences when comparing the more specific FGID subgroups, namely IBS and IBS-D. Thus, there were no significant differences in the three markers for either CFS or FGID or any of its subgroups compared to the healthy controls (Table 3). As can be seen from Table 1, all except one of the ICF/CFS cases had accompanying FGID, and thus this group largely reflects patients with comorbid severe fatigue and FGID.

Because no differences were found for either FGID or ICF/CFS, multinomial regression analysis to discern a potential modifying effect of one condition on the other was not done.

**Table 1** Overview of functional gastrointestinal disorder (FGID) subgroups in cases and control groups

	No FGID	Non-IBS FGIDs				IBS, non-D	IBS-D	Total
		Functional dyspepsia	Functional diarrhea	Functional bloating	Functional constipation			
Unexposed controls	30	0	0	0	0	0	0	30
Exposed controls	11	1	0	5	3	0	0	20
Cases with ICF/CFS	1	2	0	4	1	7	7	22
Cases without ICF/CFS	5	3	1	4	0	10	7	30
Total	47	6	1	13	4	17	14	102

IBS, irritable bowel disorder; IBS-D, diarrhea-predominant IBS; ICF/CFS, idiopathic chronic fatigue/chronic fatigue syndrome.

**Table 2** Participant characteristics by groups defined and compared in this study

	<i>n</i>	Age (mean [SD])	Females (%)
All participants	102	41.0 (11.8)	68.6
ICF/CFS	22	42.4 (9.5)	81.8
FGID	55	41.7 (10.8)	70.9
IBS	31	41.3 (11.2)	77.4
IBS-D	14	36.6 (9.7)	71.2
Controls, no ICF/CFS w/wo FGID	50	40.4 (12.4)	60.0
Controls, no ICF/CFS, no FGID	41	40.4 (12.4)	61.0

Note that some cases and controls are part of several groups depending on which disorders were compared, because FGIDs were present in some ICF/CFS patients and also in some controls.

FGID, functional gastrointestinal disorder; IBS, irritable bowel disorder; IBS-D, diarrhea-predominant IBS; ICF/CFS, idiopathic chronic fatigue/chronic fatigue syndrome.

**Table 3** Serum concentrations of B-cell activating factor (BAFF) (pg/mL) and anti-CdtB and anti-flagellin optical density (OD) values in the patient groups and subgroups compared with their respective control groups

Marker	Cases			Controls			Total	<i>P</i> -value†
	Condition	<i>n</i>	Mean (±SD)	Condition	<i>n</i>	Mean (±SD)	<i>n</i>	
BAFF (pg/mL)	ICF/CFS	20	1223 (250)	No ICF/CFS, w/wo FGID	49	1236 (241)	69	0.76
	FGID	52	1207 (217)	No ICF/CFS, no FGID	40	1210 (243)	68	0.72
	IBS	28	1212 (181)				92	0.97
	IBS-D	14	1240 (168)				54	0.46
Anti-CdtB (OD)	ICF/CFS	22	0.763 (0.402)	No ICF/CFS, w/wo FGID	48	0.833 (0.449)	70	0.47
	FGID	53	0.811 (0.476)	No CFS, no FGID	39	0.807 (0.352)	68	0.55
	IBS	29	0.795 (0.414)				92	0.47
	IBS-D	13	0.731 (0.476)				52	0.22
Anti-flagellin (OD)	ICF/CFS	21	0.523 (0.224)	No ICF/CFS, w/wo FGID	48	0.666 (0.473)	69	0.53
	FGID	52	0.567 (0.341)	No ICF/CFS, no FGID	39	0.697 (0.516)	67	0.73
	IBS	28	0.580 (0.413)				91	0.68
	IBS-D	13	0.639 (0.567)				52	0.80

†Mann-Whitney *U*-test.

Four BAFF measurements and five measurements each of anti-CdtB and anti-flagellin were excluded due to a CV >10% between duplicates.

FGID, functional gastrointestinal disorder; IBS, irritable bowel disorder; IBS-D = diarrhea-predominant IBS; ICF/CFS, idiopathic chronic fatigue/chronic fatigue syndrome; w/wo, with or without.

## Discussion

There is a clear association between fatigue and FGIDs.<sup>2,13</sup> The post-giardiasis cohort described here, with shared symptomatology of fatigue and abdominal complaints, constituted an

opportunity to examine the three potential biomarkers reported for one or both these conditions. These biomarker candidates have so far only been examined in patients characterized along either the fatigue or the gastrointestinal axis, and no significant differences have been found.

A recent noncontrolled study has advocated the use of CdtB, in conjunction with anti-vinculin, to differentiate between IBD and IBS,<sup>9</sup> which was based on the finding of significantly higher levels of these two antibodies in IBS patients. The authors suggested the possible explanation that many IBS cases may be post-infectious and that CdtB is a toxin expressed by many microbes commonly causing gastroenteritis. An alternative explanation could be that such bacteria, even if they do not cause overt disease, are brought into closer contact with the immune system in IBS patients due to loss of mucosal integrity. Antibodies against CdtB may cross-react with host vinculin, and this may lead to a leaky gut.<sup>9</sup>

While previous studies have found increased anti-CdtB levels in IBS-D,<sup>8</sup> anti-CdtB in our population of post-giardiasis IBS-D patients tended to be lower than in controls. The variability in levels of anti-CdtB is quite large and overlapping between the groups. In the present study, we did not find the anti-CdtB levels to be higher in post-giardiasis FGID patients or in the IBS or the IBS-D subgroups. A possible explanation could be that the post-infectious FGIDs in our cohort were associated with a microbe not expressing CdtB and therefore do not seem to be linked to CdtB exposure. However, our results are in line with a report by Talley *et al.*, who did not find a significant difference between 63 IBS patients and 245 healthy controls.<sup>10</sup>

The association between anti-CdtB and anti-flagellin with CFS has not been examined before, and we did not find a difference between CFS patients compared to non-fatigued controls. Contrary to Dlugosz *et al.*,<sup>12</sup> we did not find elevated levels of anti-flagellin in our patient population with post-giardiasis IBS or IBS-D.

Lunde *et al.* have found elevated BAFF concentrations in CFS, with rather high overlap between CFS cases and controls.<sup>5</sup> In the present study, using the same analysis method, we found a lower mean serum concentration of BAFF in ICF/CFS, although the difference was not significant. Elevated BAFF has been found in self-reported food intolerance,<sup>7</sup> a condition overlapping with FGID. However, we did not find BAFF levels to be different between FGID and healthy controls.

Strengths of the present study include the lab-confirmed common eliciting pathogen and the very thorough clinical assessment of the cases to be able to group them into the ICF/CFS category. Still, we did not find even trends for differences in the three markers examined, implying limited diagnostic value in our clinical setting.

However, the study included a rather low number of participants, limiting the power to find small differences. We have not recorded potential episodes of bacterial gastroenteritis in participants and do not know whether this may have biased the results. However, we have no reason to believe there are systematic differences in their exposure to other pathogens. The Rome II criteria have been superseded by the Rome III and the stricter Rome IV criteria. Many of the FGID patients in our study had mild FGIDs. Possibly, a Rome III criteria-based selection of generally more affected patients, or the Rome IV criteria, which does not include abdominal discomfort, would have shown differences in the markers. In our ICF/CFS patient group, only one patient did not have FGID, and we could therefore have missed the differences between healthy controls and that group, potentially being masked by concomitant abdominal disorders.

In conclusion, in a cohort of patients with post-giardiasis FGID with or without concomitant ICF/CFS, we did not find significant differences in the levels of circulating BAFF, anti-CdtB, or anti-flagellin antibody compared to healthy controls without these disorders. The results do not support use of these analytes as universal biomarkers for diagnosis of IBS or CFS.

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