Functional Gastrointestinal Disorders following Giardia infection

Visceral hypersensitivity and low-grade inflammation

Vernesa Dizdar

Thesis for the degree of Philosophiae Doctor (PhD)
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ABSTRACT

Background

Giardia lamblia is a non-invasive protozoan parasite, infecting mainly the upper small intestine. It is the most common cause of waterborne outbreaks of diarrhea in an endemic country.

The first registered large outbreak of giardiasis in Bergen occurred Autumn 2004, where about 1262 subjects were diagnosed with *Giardia lamblia* and 139 continued to have abdominal symptoms, despite several treatments with metronidazole. A long-term follow-up study from our group showed that a subgroup of these patients developed symptoms consistent with *Giardia* induced Post-Infectious Functional Gastrointestinal Disorder (PI-FGID).

Aims

This thesis aimed to study visceral sensitivity and low-grade inflammation, assessed by duodenal EC cells, serotonin (5-HT), cholecystokinin (CCK), T- and B-lymphocytes in patients with long-term abdominal symptoms after *Giardia* infection. The most of PI-FGID patients had an overlap of FD and IBS.

Methods

In Paper I, gastric emptying and visceral sensitivity were assessed in PI-FD/IBS patients and recovered controls (RC) by using 3D ultrasonography (3D US) in combination with a low caloric soup meal. A subgroup of PI-FD/IBS patients underwent a double-blind, randomized, placebo-controlled study with 5-HT3 antagonist (Ondansetron®) in combination with a low-caloric soup meal and 3D US.

In Paper II, fasting and postprandial changes in plasma 5-HT and CCK after the carbohydrate-rich meal were assessed in PI-FD/IBS patients and RC. In addition,

duodenal EC cells, 5-HT, CCK, mast cells and CgA were stained immunohistochemically (IHC) and quantified.

In paper III, duodenal mucosal intraepithelial lymphocytes (IELs), lamina propria villus (Lpv) and lamina propria crypt (Lpc) for CD3+, CD4+, CD8+ and CD20+ lymphocytes were stained immunohistochemically (IHC) and quantified in chronic giardiasis (CG) patients, PI-FGID patients, recovered controls (RC) and healthy (non-Giardia exposed) controls (HC).

Results

In Paper I, PI-IBS/FD patients had more abdominal symptoms as well as lower drinking capacity and increased visceral sensitivity.

5-HT3 antagonist (Ondansetron®) had effect on postprandial nausea, otherwise there was no effect on other symptoms, drinking capacity or gastric emptying.

In Paper II, only a few PI-FD/IBS patients had a sign of microscopical duodenal inflammation. When compared to recovered controls, patients had lower duodenal 5-HIAA/5-HT ratio and reduced duodenal 5-HT containing EC cell counts. There was a positive correlation between duodenal EC cell numbers and mucosal 5-HT content in the PI-FD/IBS patients, who also had increased number of duodenal CCK containing cells with a greater CCK/5-HT ratio compared to recovered controls.

After the carbohydrate-rich meal, PI-FD/IBS patients had lower fasting and postprandial plasma 5-HIAA, with a higher abdominal symptom score compared to RC. Plasma 5-HIAA showed no correlation to abdominal symptoms. CCK plasma levels did not differ between PI-IBS/FD and recovered group but showed positive correlation to fullness and bloating in PI-IBS/FD patients.

In Paper III, duodenal IEL CD4 cells was significantly elevated in chronic giardiasis and then decreased, followed by an upwards trend after one year in both the PI-FGID

and recovered control group. Duodenal lamina propria crypt CD4 T cells were lower in chronic giardiasis patients and remained low for about 14 months before normalizing. Duodenal lamina propria crypt (Lpc) CD20+ cells were persistently elevated, longer than 19 months, in all three *Giardia* exposed groups, compared to healthy controls.

Conclusions

Patients with persistent abdominal symptoms after cured *Giardia* infection, in whom most had overlap IBS and FD, had increased visceral sensitivity with lower drinking capacity and delayed gastric emptying assessed by the low-caloric soup test combined with 3D ultrasound.

The 5-HT3 antagonist (Ondansetron®) improved only nausea without influencing other symptoms, drinking capacity or gastric emptying (Paper I).

These patients had decreased number of 5-HT containing EC cells and increased number of duodenal CCK containing cells, with a greater CCK/5-HT ratio compared to recovered controls (Paper II). After intake of the carbohydrate-rich meal, PI-FGID patients had lower plasma 5-HT, before and after a meal, with more dyspepsia symptoms without significant differences in plasma CCK.

The decreased number of duodenal CD4 cells in the lamina propria crypt, were found in chronic giardiasis and PI-FGID patients, but also recovered controls, and it normalized approximately 14 months after the acute illness, while elevated B cells in the crypts appeared to last longer than 19 months in all three *Giardia* exposed groups (Paper III).

In PI-FGID group, duodenal EC cell counts (Paper II) were positively correlated to persisting low duodenal Lpv CD4 and Lpc CD4 cells (Paper III).

List of publications

Paper I

Dizdar V, Gilja O.H, Hausken T. ”Increased visceral sensitivity in *Giardia*-induced postinfectious irritable bowel syndrome and functional dyspepsia. Effect of the 5 HT3-antagonist ondansetron”. Neurogastroenterol Motil (2007) 19, 977–982.

Paper II

Dizdar V, Spiller R, Singh G, Hanevik K, Gilja O.H, El-Salhy M, Hausken T. “Relative importance of abnormalities of CCK and 5-HT (serotonin) in *Giardia*-induced post-infectious irritable bowel syndrome and functional dyspepsia” Aliment Pharmacol Ther (2010) 31, 883–891.

Paper III

Dizdar V, Hausken T, Lærum OD, Gilja OH, Langeland N, Hanevik K “Prolonged duodenal mucosal lymphocyte alterations in patients with and without post-infectious functional gastrointestinal disorders after *Giardia* infection”, Journal of Infectious Diseases J Infect Dis. 2019 Jun 19;220(2):321-329.

List of abbreviations

5-HT = 5-Hydroxytryptamine, serotonin

CCK = Cholecystokinin

FD = Functional Dyspepsia

EEC = Enteroendocrine cell

EC = Enterochromaffin cell

FGID = Functional Gastrointestinal Disorders

GE = Gastric emptying

GI = Gastrointestinal

HC= Health Controls (non-Giardia exposed)

IBS = Irritable Bowel Syndrome

IBS-A = Alternating bowel pattern - Irritable Bowel Syndrome

IBS-C = Constipation predominant - Irritable Bowel Syndrome

IBS-D = Diarrhea-predominant - Irritable Bowel Syndrome

IBS-M = Mixed bowel pattern - Irritable Bowel Syndrome

IEL(s) = Intraepithelial Lymphocyte(s)

IHC= Immunohistochemistry

Lpv = Lamina propria villus

Lpc = Lamina propria crypt

PI = Post-Infectious

PI-FD = Post-Infectious Dyspepsia

PI-IBS = Post-Infectious Irritable Bowel Syndrome

RC= Recovered Control

1. INTRODUCTION

1.1. Giardia outbreak in Bergen and persistent abdominal symptoms after giardiasis

A large waterborne outbreak of giardiasis, the first recognized parasite outbreak caused by drinking water occurred in Bergen, Norway in Autumn 2004. The patients

were probably infected late August and early October (1). It took almost two months before Giardia outbreak was recognized (2). The retrospective analysis carried out by the Bergen Legevakt (emergency health center) concluded that patients, who visited the emergency with diarrhea, and abdominal *Giardia* infection, had few characteristic, alarming symptoms and findings.

During this period, approximately 2500 surplus treatments of metronidazole were prescribed. About 1262 subjects were diagnosed with *Giardia lamblia* and many of the infected continued to have abdominal symptoms, despite several treatments with metronidazole.

These patients were thoroughly investigated without obvious cause of persistent symptoms. After our best knowledge, our group (3) were first to conclude that eradicated *Giardia* infection may elicit Functional Gastrointestinal Disorders (FGID).

Abdominal symptoms in patients referred to our outpatient clinic (82 of totally 124) were evaluated 12-30 months after the onset of *Giardia* infection and at least 6 months after *Giardia* eradication by Hanevik et al (3) and concluded that 68/82 patients (80.5%) had symptoms consistent with IBS and 17/82 patients (24.3 %) had functional dyspepsia. Abdominal problems prior to *Giardia* infection were not associated with post-infectious abdominal symptoms.

The follow-up study of our cohort, performed by Hanevik et al (4) found persisting *Giardia* duodenal infection in about 32.3 %, with signs of duodenal inflammation, especially in those with illness duration less than 7 months, but these findings decreased over time. Moreover, about 28 % of *Giardia* negative patients, had microscopical duodenal inflammation. Two years after *Giardia* infection, these patients had still a high prevalence of persistent abdominal symptoms (38%) and fatigue (41 %) (5).

The following risk factors have been identifying: More than one treatment course, treatment refractory infection, delayed education, bloating and female gender (6). Mesalazine® did not improve abdominal symptoms in our PI-FGID patients (7).

In patients with persisting symptoms, *Giardia* infection was found to be associated with a high prevalence of IBS and chronic fatigue, three (8) and five years later (9), the perceived food intolerance (10), the long term complications with IBS, CFS (chronic fatigue syndrome) and reduced quality of life (11, 12).

Previous study showed that bacterial overgrowth is implicated in the pathogenesis of IBS (13). Morken et al (14) showed that the persisting symptoms after *Giardia* infection in Bergen, Norway, cannot be explained with intestinal bacterial overgrowth. Moreover, neither antibiotics nor bacterio-therapy had effect on symptoms in these patients, but they had a high fecal excretion of fat and SCFAs suggesting intestinal malabsorption(15). The genetic characterization of patients from our cohort showed that assemblage B gradually predominated over time (16). Five years after acute giardiasis, the analysis of peripheral blood showed that the long term cellular immune response mainly occurs in CD4 T-cells (17) with increased CD8 T-cells (18).

1.2. Giardiasis

Giardia lamblia (syn. *G. intestinalis*, *G. duodenalis)* is a protozoan parasite, infective to humans, with variable clinical manifestations ranging from the absence of symptoms to acute or chronic diarrhea (19). The WHO has estimated that more than 280 million humans are infected every year with giardiasis and the disease was, in 2004, included in the "neglected disease" category (20).
As a non-invasive pathogen, *Giardia* attaches to the intestinal epithelium (21)mainly of the upper small intestine without invading tissues (22), although there is a case report of invasive intraepithelial giardiasis (23). The parasite can also be found in the

stomach, ileal and colonic mucosa (24). The histological changes induced by *Giardia* are non-specific (25).

The laboratory diagnosis of *Giardia* species is mainly based on demonstration of microscopic cyst or trophozoite in stool samples by light microscopy, but immunofluorescence assay (IFA), enzyme immunoassay (EIA) or polymerase chain reaction (PCR) methods are also used for diagnostic or research (26). Analyses of serum or duodenal aspirates have also been used.

Fecal direct microscopy examination, performed to detect cysts and trophozoites, is a gold, standard method, economical and rapid for the diagnosis of giardiasis. Sensitivity of direct microscopy increases with increased number of exanimated stool sample (one stool sample-allow the diagnosis of 60 to 80% of infections, two stool samples 80-90%, three stool samples over 90%). In addition to number of examined fecal samples, the sensitivity of fecal microscopy is also dependent on using direct or concentration methods as well as trained laboratory persons (27).

PCR is a sensitive and specific method also when there are a few cysts, but there is a risk of false positive results if used as a single test (28).

There are eight *Giardia* genotypes, named A-H, where A and B genotypes infect humans and B genotype is most frequent worldwide (29).

The pathophysiology of giardiasis is multifactorial (30, 31). The tight attachment between *Giardia* trophozoite and intestinal epithelial cells is followed by enterocyte apoptosis with disrupting of the epithelial tight junctions (32-34), with diffuse shortening of mucosal microvilli, disturbed epithelial-barrier dysfunction hypersecretion of Cl^- and inhibition of brush-border enzymes (22, 35).

Both innate and adaptive immune mechanisms are involved in giardiasis (29) with activation of mast cells, T- and B-cells, dendritic cells, immunoglobulin A and nitric oxide.

In a recent novel neonatal rat model, Halliez et al (36) found that during the acute stage of infection, *Giardia* caused the translocation of commensal bacteria in rats, primarily paracellular with disruption of the tight junction proteins occludin and claudin-4. Fifty days after parasite eradication, *Giardia duodenalis* (assemblage A or B) caused visceral hypersensitivity in the jejunum and rectum that was associated with villous atrophy, crypt hyperplasia, increased IEL and mast cells.

The presence of microbiota in the gastrointestinal tract, as an innate defense mechanism against pathogens may have an anti-*Giardia* effect but also protect and preserve gut integrity during infection. Therefore, variability in pathology and susceptibility to infection could be explained by differences in microbiota composition between individuals (37). Extra-intestinal a long-term complications after *Giardia* infection such as ocular changes, arthritis, allergies, impaired cognitive function and failure to thrive have also been described (38).

1.3. Functional Gastrointestinal Disorders (FGID)

Functional gastrointestinal disorders (FGIDs) are common, unexplained gastrointestinal (GI) symptom complexes, without known underlying pathophysiology. Functional dyspepsia (FD) and the irritable bowel syndrome (IBS) are the two most recognized disorders.
Functional dyspepsia (FD) is a chronic, recurrent symptom complex referred to the upper gastroduodenal region, characterized by postprandial fullness, early satiety or epigastric pain/burning (39-41).

Irritable bowel syndrome (IBS) is a functional bowel disorder, characterized by recurrent abdominal pain or discomfort associated with changes in stool form and frequency without structural or biochemical abnormalities (42, 43). Based on symptoms, we can discern between the following subtypes: IBS with predominant

constipation (IBS-C), IBS with predominant diarrhea (IBS-D) or mixed IBS (IBS-M) (43).

The first symptom based criteria were made by Manning et al in 1978 (44), followed by ROME I (1989), ROME II (45) and ROME III criteria (42, 46). In ROME IV criteria are disorders classified by GI symptoms related to any combination of visceral hypersensitivity, motility disturbance, altered mucosal and immune function, gut microbiota and CNS dysfunction (47).

Infectious enteritis, reported for the first time in 1963 (48), has been described as one of the most important risk factor for development of postinfectious dyspepsia (PI-FD)(49) and PI-IBS (13).
Post-infectious IBS (PI-IBS) is defined as IBS developed after an episode of acute infectious gastroenteritis (43), characterized by an acute illness with ≥ 2 of the following clinical features; fever, vomiting, diarrhea and a positive stool culture (50). The site of the infection in the GI tract can determine symptom type (51). Several studies have shown an association between symptoms and viral (52), bacterial (53, 54) and parasitic (55) GI infection. The persisting post-infectious GI symptoms may last for months or years, as previous demonstrated in both animal (36, 56) and human studies (57-61).
Salmonella gastroenteritis were found to be a significant risk factor, not only for IBS but also for dyspepsia and at the 1 year follow up, 1 in 7 and 1 in 10 developed dyspepsia or IBS, respectively (62).

Klem et al (63) showed that the rate of PI-IBS was higher after a protozoan or parasitic infection (42%) rather than bacterial infection (14%), whereas overall risk of IBS was 4-fold higher in those with infectious enteritis in the prior 12 months compared to controls. Grazioli et al. (64) showed that *Giardia* infection accounted for 6.5% of patients with IBS and dyspepsia.

The longitudinal cohort analysis, performed by Nakao et al (65) using a large health insurance database, showed that one-year incidence of IBS was higher in persons with giardiasis.

Study from our group showed that *Giardia* may cause chronic sequelae that can persist for a long time after parasite eradication such as post-infectious irritable bowel syndrome and chronic fatigue (3, 8, 11, 66). The mechanisms remain unknown, but recent studies suggest that variability in *Giardia* strains, host mucosal immune responses, immune modulation by *Giardia,* the composition of host microbiota, co-infection and host nutritional status may be important in the development of disease manifestations after *Giardia* infection (67).

1.3.1 Epidemiology of FGID

There is a varying prevalence of FGID depending on criteria used for classification and it is reported to be higher in western countries.
The global prevalence of uninvestigated dyspepsia occurred in up to 21% (68). A moderate, but significant higher prevalence was found in women, smokers, NSAID users and Helicobacter pylori-positive individuals. It is challenging to discriminate between organic and functional dyspepsia since symptoms do not necessarily distinguish between these two forms of disease (40).

The meta-analysis covered epidemiological population-based data across 90 studies in 33 countries worldwide, reported the prevalence rates of IBS between 1.1% and 45% (69). In the western countries, IBS affects up to 18% of adults (70). Only 8 % of Norwegian adults reported IBS symptoms with frequent somatic and psychiatric comorbidity, reduced health, working disability and increased use of health services (71).

The prevalence of post infectious FD (PI-FD) varies between 2.8 % and 42.4% but most studies on adult populations have shown the prevalence of PI-FD to be around 10% (49) with persisting symptoms for many years (60).

PI-IBS occurs in over 10 % of IBS patients after an episode with GI infection, with a 4-6 fold higher risk of developing IBS than in individuals who did not have infectious enteritis (63, 72-74). The risk factors are women, especially those with severe enteritis, as well as psychological distress and users of antibiotics during the enteritis (13, 63).

1.3.2. Association between FD and IBS with other disorders

Depending on the criteria used, the degree of overlap between IBS and FD varies between 15% and 42%. Dyspeptic patients should be considered also for IBS, as there is an 8-fold increase of IBS in patients with dyspepsia compared with those without dyspepsia (75).

In one of the longest, population-based, follow-up study of FGID subjects, Halder et al (76) found that patients with FD may have had IBS in the past and vice versa. IBS and FD patients had a high turnover in symptom status, since many episodes of symptom disappearance were due to subjects changing symptoms rather than total symptom resolution. This transition between different FGIDs suggests a common etiopathogenesis.

FD and IBS may overlap with other functional syndromes as a gastroesophageal reflux **(77)**, temporomandibular joint disorder, interstitial cystitis/painful bladder syndrome and chronic fatigue syndrome (78) as well as fibromyalgia, migraine and depression (79). Of GI diseases, IBS-like symptoms can occur in IBD patients in remission (80), coeliac disease (81) and microscopic colitis (82).

1.3.3. Pathophysiology of FGID

The pathophysiology of FGID is a complex and multifactorial. For several years ago, underlining mechanism of FGID have been based mainly on the GI motility and visceral hypersensitivity, but in the recent years, there had been more focus on infections, genetic factors, effect of diet, alteration in the intestinal microbiota,

immune activation, altered intestinal permeability low-grade inflammation with abnormalities in 5-hydroxytryptamine (5-HT) metabolism as well as disorders in brain- gut axis (83).
The important risk factor in the pathophysiology of functional dyspepsia are visceral hypersensitivity, impaired gastric accommodation after a meal, delayed gastric emptying, abnormal duodenojejunal motility, acid hypersensitivity, *Helicobacter pylori* (84) and stress, particularly anxiety (85). Sometimes, gut symptoms occur before anxiety, which could be can explain by brain-gut driven axis (86), but sometimes it is uncertain if symptoms first started in the gut or CNS (87).

As FD and IBS often overlap each other, they can have common pathophysiology (51, 88) which involves visceral hypersensitivity with GI dysmotility, increased intestinal permeability (89, 90), acute GI infection (13, 49), abnormalities in serotonin signaling and genetic predisposition (91, 92), dysregulation of the brain-gut axis (86, 93, 94) as well changes in the intestinal microbiome (74).

1.3.3.1. Visceral hypersensitivity

Visceral hypersensitivity, defined as increased sensitivity to distention and/or sensitivity to chemical food contents, is considered as a key mechanism in the pain perception (95) and an important contributor for symptom generation (96). The possible pathophysiological mechanisms have been described in details before (97, 98). Visceral hypersensitivity is present in a subset of patients with functional dyspepsia (99-102) and IBS (103, 104).

The stepwise balloon distention of different parts of the GI tract is a gold standard for assessing of visceral sensitivity, but this method is bothersome and non-physiological (105).Increased gastric visceral sensitivity to distention is associated with symptoms in FD patients (99) and can be abolished by CCK-A receptor antagonist (106). One group (107) showed a poor correlation to abdominal symptoms.

Between 20%-90% of IBS patients have increased visceral sensitivity (108). In general, IBS patients have a lower threshold for pain tolerance along the entire gastrointestinal tract (94). IBS patients had an increased psychological tendency to report pain, which in turn, is associated with psychological distress (109). In an fMRI study combined with barostat, IBS patients experienced markedly more pain and overall discomfort upon repeated distensions in the scanner, despite unaltered rectal sensory thresholds (110).

In addition to mechanical distention or chemical stimuli, visceral sensitivity can be tested by either the low or high caloric nutrient drink or water (111), chemical stimulation, e.g. acid provocation, electrical or thermal stimulation (112). Meal induced symptoms are a recognized feature in FD patients. Between 60-70 % of FD patients showed hypersensitivity to nutrients (113, 114) with more symptoms after a high-fat meal than an isocaloric high carbohydrate meal (115). Mucosal afferents in the stomach sense the presence of the luminal contents via mechanical and chemical receptors. Ingested food is exposed to vagal afferents causing distention of the stomach wall which activates mechanoreceptors. There are two types of mechanosensitive vagal afferents ending in the stomach, mucosal receptors and tensions receptors. Tension receptors send signals to the CNS about the level of stomach distention, which is important for regulating food intake, generating the sensation of satiety and fullness as well control of gastric emptying. Both chemo- and mechanosensitive receptors have an important role in the generation of satiety sensations, nausea and vomiting via chemical and osmotic stimuli.

A study using gastric barostat combined with a liquid meal showed that FD patients had a significantly greater postprandial sensitivity to balloon distention compared to fasting sensitivity. Only postprandial sensitivity was correlated to the severity of symptoms of meal-related FD (116). FD patients may also have abnormal central processing of visceral perception (117).

In the animal model of post-infective bowel dysfunction with *Trichinella spiralis,* transient infection lead to persistent gut dysfunction with increased sensitivity(118). In the new rat model of post-giardiasis IBS, Halliez et al (36) showed that rats expressed visceral hypersensitivity to luminal balloon distension in the jejunum and rectum, 50 days after infection with *Giardia duodenalis* (assemblage A or B), long after the parasite was cleared.

1.3.3.2 Gastric accommodation

Gastric accommodation is one of the most important pathophysiological mechanisms in FD (119).

It consists of receptive- and adaptive relaxation, an intrinsic vagovagal reflex that dilates the proximal part of the stomach in response to a meal without following an increase in pressure (120). About 40 % of FD patients have impaired proximal gastric accommodation (121) with altered meal distribution and impaired relaxation of the proximal stomach.

The barostat has been considered as a gold standard for measuring gastric accommodation, but the procedure is invasive, time-consuming and uncomfortable. Other methods for assessing accommodation (122) are imaging with magnetic resonance (MRI) (123), single photon emission computed tomography (SPECT) (124) or ultrasonography (US) (120, 125, 126).

The intragastric volume and thus indirect relaxation of the stomach can be evaluated by measuring the size of the stomach with ultrasound. Gilja et al (127) were first to develop a ultrasonographic methods to assess gastric accommodation.

As a non-invasive test, drink tests with water or nutrients are used to assess the sensation of fullness and early satiety after meal ingestion. A drink test may be used as a surrogate marker of proximal stomach function, predicting impaired gastric

accommodation (128, 129). These methods showed that 40-60 % of FD patients had impaired accommodation to a meal (130, 131).

The impaired relaxation of the proximal stomach, reported in a high proportion of FD patients, can be restored by the 5-hydroxytryptamine (5-HT1) receptor agonist sumatriptan, which induces a relaxation of the proximal stomach in humans (121, 132). Patients with presumed PI-FD had more prevalent impaired accommodation compared with unspecified-onset dyspepsia. 5-HT1 agonist sumatriptan relaxed the stomach in controls and patients with unspecified-onset dyspepsia but not in patients with presumed post-infectious dyspepsia, whereas the NO donor amyl nitrite relaxed the stomach in all subjects (133).

1.3.3.3 Gastric emptying (GE)

After a meal, ingested nutrients enter the stomach and the intestine, inducing the relaxation of the proximal stomach (enteral-gastric reflex). This reflex is vagus mediated and depends on the type and amount of nutrients. Infusion of duodenal nutrients increases gastric sensitivity to distention (113).Transport of nutrients to the duodenum depends on gastric emptying.

Delayed gastric emptying for either solids or liquids, occurs in a subset of FD patients, ranging from 25 % to 50 % (134). Scintigraphy, a non-invasive test of gastric emptying, is considered to be the gold standard, but it is an expensive, time-consuming and not widely available procedure.

Ultrasonography (US) is an inexpensive and widely distributed real-time technique, without radiation, that can be applied repeatedly and at the bedside (135). Since gravity affects the propulsion of gastric content, accommodation should be measured in a sitting or standing position (120). In combination with a low caloric meal, US has been used to study gastric motility (136), accommodation (127, 137) and gastric emptying (138) in FD patients.

In FD patients, by using breath test, delayed GE of solids was associated with postprandial fullness and vomiting, whilst delayed GE for liquids was related to postprandial fullness and early satiety (134).

Delayed gastric emptying has also been reported in a subset of IBS patients. IBS patients with overlapping dyspepsia had significantly delayed gastric emptying, tested by scintigraphy, that was associated with postprandial fullness and nausea, while IBS patients without overlapping dyspepsia had normal gastric emptying of solids (139).

The CCK-A antagonist, Loxiglumide, has a prokinetic effect and is effective in the treatment of FD symptoms (140). The CCK-1 receptor antagonist dexloxiglumide, accelerates gastric emptying and delays proximal but not overall colonic transit in patients with C-IBS (141).

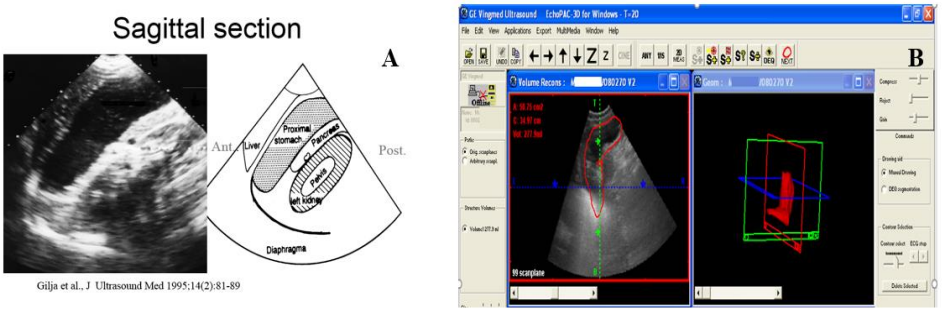

Fig. 1 Assessment of the accommodation of the proximal stomach by using 2 D (A) and 3D (B) ultrasound.

1.3.3.4 Low-grade inflammation

The recent studies emphasized the importance of low-grade inflammation in both FD (101), (49, 142), IBS (143) and PI-FGID (13, 92).

Altered mucosal immune cells, including impaired number of EC cells (144) with alterations in 5-HT and increased IEL and T lymphocytes (50, 145, 146), changes in the peripheral blood T cells (147, 148), increased mast cell activation (149-151), eosinophils (150, 152) as well as food intolerance and gut-microbial interactions (92) have been implicated in the disease process.

Increased number of colonic EC cell counts in IBS patients correlate with visceral hypersensitivity (153). Increased 5-HT release, assessed with quantitative immunohistochemistry on colonic biopsies, in IBS patients correlates with mast cell counts and the severity of abdominal pain, suggesting that 5-HT contributes to the development of abdominal pain (154). PI-IBS patients, after *Campylobacter jejuni* enteritis, have persistent low-grade inflammation within rectal mucosa, with an increased number of lamina propria T lymphocytes and enterochromaffin (EC) cells, serotonin bioavailability as well as increased small intestinal permeability (53). Several studies reported evidence of low-grade inflammation which may persist up to 4 years (53, 58).

1.4. Gut endocrine cells

Neuroendocrine system of GI tract consists of the endocrine cells and the enteric nervous system (ENS). Enteroendocrine cells (EEC), as one of the largest endocrine organ in the body representing 1 % of the intestinal epithelium, are dispersed among the epithelial cells in the gut mucosa except for esophagus (155). From the base of intestinal crypts, EEC cells differentiate from stem cells and migrate up the crypt-villus axis. They had a key role in the regulation of food intake, GI secretions, and motility (156) releasing their secretory product by exocytosis at the basolateral membrane upon mechanical, chemical or neural stimulation. They are called “the gut sensors” as they have specialized microvilli that project into the gut lumen, sensing luminal nutrients and then secreting more than 20 peptides, including cholecystokinin

(CCK), glucagon-like peptide 1 and 2 (GLP-1, GLP-2), glucose-dependent insulinotropic peptide (GIP), peptide YY (PYY), somatostatin and ghrelin, as well as bioactive amines such as serotonin (5-HT). The release of CCK is triggered by protein and fat.

There is an inetraction between neuroendocrine and immune systems in addition to gut microbiota. The alterations in the number of EEC during infection have been reported in both animal (56, 157, 158) and human studies (58, 159).

1.4.1. 5-HT (5-hydroxytryptamine)

Serotonin (5-hydroxytryptamine, 5-HT) is an important enteric mucosal signaling molecule, produced by enterochromaffin (EC cells). The serotonin secreting cells are found throughout the GI tract. Approximately 95 % of the 5-HT is synthesized from EC cells (160) as well as in serotonergic neurons of the ENS (161).

5-HT is synthesized from amino acid tryptophan (5-HTP) by the rate limiting enzyme tryptophan hydroxylase (TPH). There are 2 types of TPH, TPH1 (predominantly found in EC cells) and TPH2 (in all serotonergic neurons)(162) . Serotonin is inactivated by the serotonin reuptake transporter (SERT). 5-HT has been implicated in a number of GI diseases (163). It plays a major role in promoting of intestinal motility through a combination of neuronal and mucosal mechanisms. Serotonin (5-HT) has also an important role in visceral hypersensitivity (164).

1.4.1.1. 5-HT3 antagonist

There are 7 major groups of 5-HT receptors which have an important role in the regulation of inflammatory and immune responses (165). It is expressed in the numerous cells of GI tract, as EC cells, Goblet cells and interstitial cells of Cajal (ICC)

(166). After ingestion of meal, EC cells secrete serotonin into the gut wall and stimulate primary afferent neurons, which start the peristaltic reflex (160).

In animal study, intestinal perfusion with carbohydrates inhibits gastric emptying via vagal and spinal capsaicin-sensitive afferent pathways and pretreatment with an 5-HT3 antagonist can abolish carbohydrate induced inhibition of gastric emptying (167).

5-HT3 receptors are involved in acid-induced duodenogastric sensitization in healthy volunteers (168), where 5-HT3 receptor antagonist ondansetron decreased gastric sensitivity during duodenal acid infusion and gastric distension and improve nausea and vomiting (169).

In IBS-D patients (170), 5-HT3 receptor antagonist ondansetron induced rectal relaxation with increased rectal compliance but did not alter gastric compliance or visceral perception. 5-HT3 receptor antagonist alosetron (171) modulated abdominal pain and discomfort in IBS patients.

1.4.2 Cholecystokinin (CCK)

Cholecystokinin (CCK) is a brain-gut peptide released from the I cells of the duodenum and jejunum in response to luminal nutrients, especially lipids and proteins, inducing satiety via vagal afferents (172). CCK has an important role in the ingestion, absorption and digestion of food. It inhibits gastric motility and emptying of the proximal stomach and pylorus via a capsaicin-sensitive vagal pathway (173).

A high fat meal provokes more symptoms in FD patients, with a greater concentration of fasting and postprandial plasma CCK compared to healthy subjects (115). One study showed that IBS patients have increased levels of CCK in plasma and rectal mucosa (174). El-Salhy et al (175) found that IBS-D had significantly reduced the density of duodenal CCK-immunoreactive cells.

In acute giardiasis in humans, plasma CCK levels were found to be elevated (159). An animal model with *Giardia* infection showed increase CCK levels, which trigger mast cell degranulation and contractions of longitudinal smooth muscle (176).

CCK-A and 5-HT3 receptors mediate the nutrient-induced reduction of food intake and gastric emptying (167). 5-HT3 receptors mediate CCK induced satiation through indirect mechanisms involving gastric emptying and gastric distention.

1.5 T- and B- lymphocytes

The immune system consists of innate and adaptive immunity and the mucosal immune system provides a first defense line of the inner body surface (177). More than 80 % of the body`s activated B-cells are terminally differentiated to plasmablasts and plasma cells (178, 179). The mucosa of the gut can be divided into the inductive site and effector site (180). The inductive site consists of gut-associated lymphoid tissue (GALT) and Peyer's patches (with B-cell follicles and M cell -containing follicle-associated epithelium). The effector site consists of lamina propria (with B-cells, Ig-producing plasma cells, and T- cells) and epithelium (mainly T-cells) (177).

Intraepithelial lymphocytes (IELs) are located in direct contact with the enterocytes, in the close relation to the antigens in the gut lumen and thus form the first defense against pathogens (181). Intraepithelial lymphocytes (IELs) belong to the T- cell population and are interspersed between epithelial cells of both the small and large intestine (182).

The upper limit of IEL number in the proximal small intestine is around 25 IELs/100 epithelial cells (183, 184). The majority of IEL in the human intestine are CD8+ with a few CD4+ cells, especially in the small intestine (180). T- cells in lamina propria are scattered throughout the lamina propria of the small bowel and colon and consist mainly of CD4+cells.

CD4+ T cells have an important role in immune protection. They help B-cells to make antibodies and they recruit neutrophils, eosinophils, and basophils to the site of infection by secretion of various cytokines and chemokines.

The mucosal immune response against *Giardia* starts with an early response, with intestinal barrier function, secretion of pro-inflammatory molecules such as IL-6 derived from dendritic cells and mast cells, and CD4+ and CD8+ T cells. The late response starts with activation of CD4+ T memory cell and B- cells.

It has also been shown that people who were infected by *Giardia* in the Bergen 2004 outbreak still had elevated *Giardia* specific CD4 T cell responses 5 years later (17).

B-cells and plasma cells are effector cells of humoral immunity and a part of the adaptive immune system. Through antigen presentation, cytokine secretion and antibody production, they have an important role in the initiation and the termination of immune responses. B-cells play important role in protection against *Giardia* infection. The animal study showed that B-cell deficient mice failed to produce antibodies against *Giardia* and developed chronic infection (185).

In the waterborne outbreak of giardiasis in Vermont (186) serology taken in the convalescent phase showed higher levels of parasite-specific antibody IgG and IgA but not IgM. During a second outbreak of giardiasis in the same area (187), previously infected cases were less likely to be re-infected suggesting an acquired, protective immunity lasting at least 5 years.

Accordingly, we hypothesized the following:

1. PI-FGID patients may have increased visceral sensitivity in upper GI tract.

2. 5-HT and EC cells may be increased in plasma and duodenum. In addition, we assess the role of CCK.

3. To study alterations in duodenal intraepithelial (IEL) and in lamina propria (Lpv/Lpc) T cells (CD3+, CD4+ and CD8+) and B (CD20+) cells.

2. AIMS OF THE STUDY

The aim of this dissertation was to study visceral sensitivity and low-grade duodenal mucosal inflammation, assessed by EC cells, serotonin (5-HT) and cholecystokinin (CCK) in patients with functional gastrointestinal disorders after cleared *Giardia* infection. To our knowledge, this has not been described before.

The specific aims in this study were:

Paper I

-to investigate visceral hypersensitivity of the upper GI tract in patients with post infectious *Giardia*-induced abdominal symptoms using a low-caloric soup meal combined with non- invasive 3D ultrasonography and to assess the effect of the 5-HT3 antagonist ondansetron in these patients.

Paper II

-to determine the importance of duodenal serotonin (5-HT) and CCK containing EC cells as well as plasma 5-HT and CCK in patients with prolonged abdominal symptoms after successful treatment of *Giardia* infection. We hypothesized that altered 5-HT and CCK signaling could be associated with the development of *Giardia*-induced PI-FGID.

Paper III

- to describe quantitatively morphological changes in duodenal mucosal T- and B-lymphocytes in patients with persisting abdominal symptoms (PI-FGID) (average 11 months) after resolution of *Giardia* infection and compare these with chronic giardiasis patients, post-giardiasis recovered controls and healthy controls.

3. MATERIALS AND METHODS

3.1 Study population

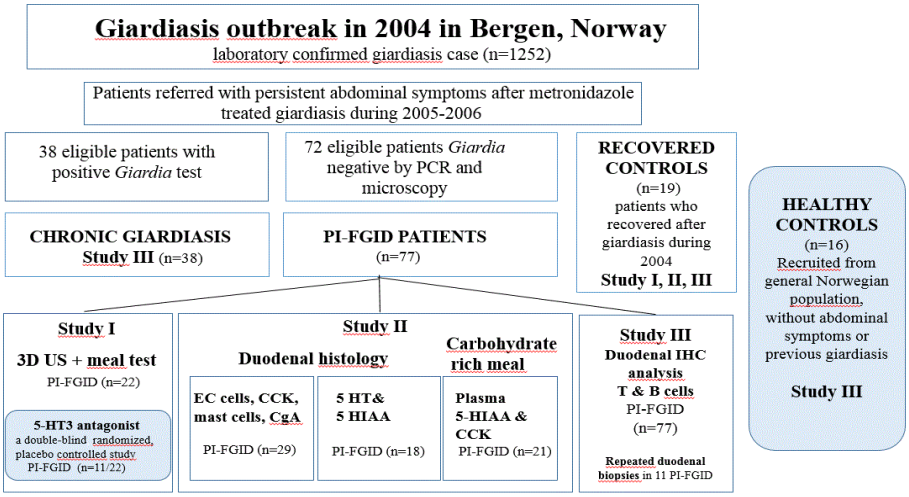

Fig 2. *Giardia* cohort populations included in all three papers of this thesis.

Patients were referred to our out-patient clinic due to persisting abdominal symptoms after *Giardia* outbreak in Bergen in 2004. In these patients, *Giardia* had been successfully eradicated, confirmed by at least three negative microscopy samples and later verified with negative PCR. Our cohort patients went through structured interview 12–30 months after the onset of *Giardia* infection, and at least 6 months after *Giardia* eradication, as described in detail previously by Hanevik et al (4).

Due to persistent symptoms, gastroduodenoscopy with duodenal biopsies were available from 28 patients with chronic giardiasis (CG) patients and 72 PI-FGID patients (66 of these were randomly selected for this study). Chronic giardiasis patients were including in Paper III for comparison of histological changes with other groups.

As a control group in Paper I and II, we used 19 previously young, healthy subjects, who had *Giardia* infection at the same time as PI-FGID patients, with laboratory-confirmed giardiasis during the outbreak and recovered rapidly after treatment with metronidazole. The recovered controls were recruited by phone from primary care, examined 12-19 months after onset of the gastroenteritis, and were asymptomatic at the time of inclusion.

In Paper III, we recruited by advertisements, 16 healthy, previous non-*Giardia* exposed controls, without persisting GI or other symptoms. They went through the same investigations as the cases and served as healthy controls (HC).

3.2. Diagnostic criteria and symptom scores

From the start of the outbreak, all subjects fulfilled abdominal symptom score, determined by an irritable bowel syndrome symptom questionnaire (188), including nausea, bloating, abdominal pain, diarrhea, constipation. These symptoms were assessed using an ordinal scale from 0 – 10, with 0 = no symptoms and 10 = severe symptoms.

In addition, shortly after the start of *Giardia* outbreak, we used a validated Norwegian version of the ROME II questionnaires for IBS (189) and FD (39).

In Paper II, abdominal symptoms (abdominal pain/discomfort, nausea, fullness and bloating) were assessed before (time) 30 min and every 30 min after a carbohydrate rich meal test, up to 4 h postprandially, by using the ROME II dyspepsia questionnaire (39).The severity of symptoms was scored using the scale 0 = none; 1 = mild; 2 = moderate; 3 = severe. The overall postprandial dyspepsia score was calculated by adding the total for each of the four symptoms over the 4-h postprandial period (0–96).

In Paper II, PI-FGID patients and recovered controls completed the Hospital Anxiety and Depression Scale (HAD) (190), Eysenck Personality Questionnaire, Short 12-item scales (EPQ-N) (191) and Short Form Nepean Dyspepsia Index (SF-NDI) (192).

3.3 METHODS

3.3.1. Gastroduodenoscopy with duodenal biopsy specimens

Following an overnight fast, gastroduodenoscopy was performed in patients, recovered controls and healthy controls. A total of six biopsy specimens were taken from the distal part of the duodenum. Three biopsy specimens were placed in 4% buffered formalin for routine histological analysis and delivered to the Department of Pathology at the same day. Three biopsies were snap frozen in liquid nitrogen and then stored at -80°C, later analyzed for 5-HT and 5-HIAA content.

Repeated duodenal biopsies were available in 11 PI-FGID patients, examined first 3-7 months after onset of symptoms (when eight were still *Giardia* positive and three were *Giardia* negative) and then 16-19 months after onset of symptoms (when all 11 were Giardia negative).

3.3.2. Diagnostic methods of Giardia spp.

In the beginning of the *Giardia* outbreak, conventional microscopy following standard formalin-ether concentration was used, but this procedure is time-consuming and requires experienced personnel (193). Therefore, the ImmunoCard STAT! Cryptosporidium／Giardia rapid assay (Meridian Bioscience, Inc., Cincinnati, OH, USA) faecal antigen test were used, which has been reported to have high sensitivity (81–93.5%) and specificity (>99%) (194, 195). However, in this thesis, diagnostic of giardiasis has not been the aim of the study, therefore it is not described in detail here.

3.3.3. Ultrasonography

In Paper I, three-dimensional ultrasound imaging was performed with a Logic 9 scanner (GE Medical Systems, Milwaukee, WI, USA) with a 3,5 MHz transducer interfaced to a magnetic position and orientation measurement system. The Bird system (Ascension Technology Corp., Burlington, VT, USA) was calibrated before

each 3D acquisition that was performed at maximal satiety. The recording was stored on a PC workstation for later analysis using dedicated software (Echopac3D, Horten, Norway). This acquisition procedure and software have demonstrated very good accuracy in volume estimation (125, 137, 196). All examinations were performed by the experienced doctor to avoid differences in interobserver variation. All gastric volume measurements were performed blinded after study completion. Gastric emptying (GE %), measured once - after a meal, was defined as the fraction of the meal emptied from the stomach immediately after a meal [(drinking capacity) intragastric volume)/drinking capacity · 100%)].

3.3.4. Drink test (low-caloric soup meal)

In Paper I, we used the low-caloric soup meal (Toro® clear meat soup; Rieber & Søn A/S, Bergen, Norway) in combination with 3D ultrasound. The subjects ingested low caloric soup meal; 100 mL every minute, until maximal drinking capacity and the volume of the stomach was then assessed using 3D ultrasound. The soup was first boiled and then cooled to 37° C. It contained 1.8 g protein, 0.9 g fat, 1.1 g carbohydrate and non-soluble seasoning (0.2 g) per 500 mL (4 kcal 100 mL)1). The pH of the soup varied between 5.4 and 5.7, and the osmolarity was350 mOsm $kg)^{-1}$ H2O.

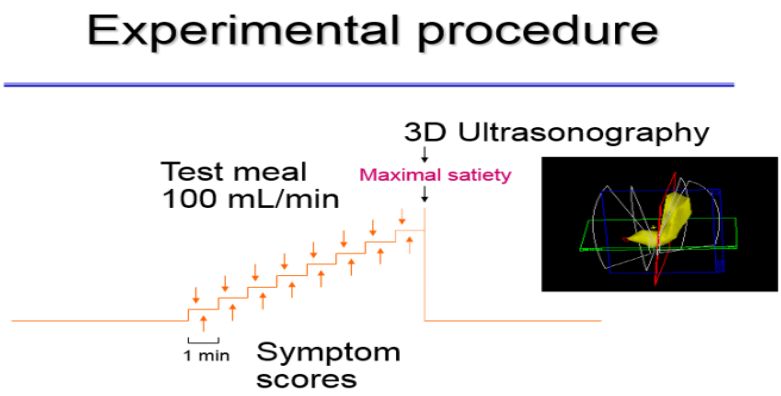

Fig. 3 Three-dimensional ultrasound (3D US) in combination with the low caloric soup meal.

3.3.5 A double-blind, randomized, placebo-controlled study with 5-HT3 antagonist Ondansetron®

Patients receiving 5-HT3 antagonist Ondansetron® were studied on two separate occasions, with 7-14 days interval between examinations. They received oral treatment with either Ondansetron®, 8 mg or placebo, 20 p.m. the day before and the next day 1 h 30 min before the drink test. Placebo and study medication (Zofran, Glaxo Smith Kline) were identical in appearance. Study subjects and the clinical investigators were blinded to the treatment assignment until the data analysis was completed.

3.3.6. Carbohydrate-rich test meal

In Paper II, we used a similar carbohydrate-rich meal as described previously (197), which consists of 100 g boiled pasta in 100 mL tomato soup (Toro, Rieber& Søn, A⁄S Bergen, Norway), 50 g white bread (two slices), 8 g soft margarine, one wheat bun,10

g jam (500 g sugar／kg) and 300 mL tap-water (calorie content of 557 kcal-carbohydrates, 63%, fat, 27%, protein,10%).

3.3.7 Extraction of 5-HT from biopsy sample and analysis of 5-HT and 5-HIAA by HPLC and blood samples for serotonin (5-HT) and cholecystokinin analysis

These analysis were performed by Spiller RC et al. and are described in detail in Paper II (198).

3.3.8. Immunohistochemistry

Endocrine cells distribution in humans was previously examined by immunohistochemistry (199).

In Paper II, the standard technique was used to prepare each biopsy for immunohistochemistry for serotonin-producing enterochromaffin (EC) cells, as described previously (200), 5-HT(56), CCK producing enteroendocrine cells (201), mast cells and the nonspecific marker for all enteroendocrine cells, Chromogranin A (CgA).

In Paper III, we performed immunohistochemistry of T- and B-lymphocytes. Formalin-fixed paraffin-embedded duodenal specimens were cut into 4 μm sections, de-paraffinized in xylene and rehydrated through graded ethanol series and distilled water.

After heat-induced epitope retrieval (HIER) in Tris-EDTA buffer, pH 9.0 for 15 minutes at 350W, endogen peroxidase activity was blocked with 0.3% peroxide (Dako) for 5 minutes. Tissue was then incubated with primary antibodies: CD3 (Polyclonal Rabbit Anti-Human CD3), CD4 (Monoclonal Antibody NCL-CD4-IF6, clone IF6), CD8 (Monoclonal Mouse Anti-Human CD8α, clone C8/144B) and CD20 (Monoclonal Mouse Anti-Human CD20, clone L26). EnVision (DAKO 5007) was used as a secondary antibody for 30 minutes, with DAB as chromogen. Sections were counterstained with Haematoxylin (Dako S3301).

3.3.9. Computerized image analysis

In Paper II, 5-HT cells and mucosal mast cells were counted using the Weibel 2 graticule method as previously described (56, 202) and results expressed as cells per mm^2.

Quantifications method for CCK cells in Paper II has been described elsewhere (203). Morphometric analysis for CCK immunoreactive cells was performed using the Olympus program 'Cell P', with an x 40 objective and in a frame representing an area of 0.13 mm^2 of the tissue. The number of nucleated CCK cells in the crypts was counted in coded slides from 20 randomly chosen fields from three different sections from each individual. All measurements were performed by the same person and double-checked by an experienced person.

In Paper III, quantification of duodenal T- and B-lymphocytes has been described in detail.

The numbers of intraepithelial lymphocytes (IEL), located above the basal membrane, per 100 epithelial cells were counted on five, well-orientated villi with longitudinal sections and expressed as the number of IELs per 100 epithelial cells (204). Lamina propria villous (Lpv) lymphocytes located underneath epithelial basal membrane, assessed in five villi and then expressed as average cell counts per area (cell count/mm^2). Lamina propria crypt lymphocytes (Lpc) were counted per area within five consecutive, non-overlapping 200x-fields of crypt lamina propria and the results averaged (cell count/mm^2). Positive cells in the vicinity of lymphoid follicles or clusters were not taken into consideration. Only cells with a visible nucleus were considered as positive and counted.

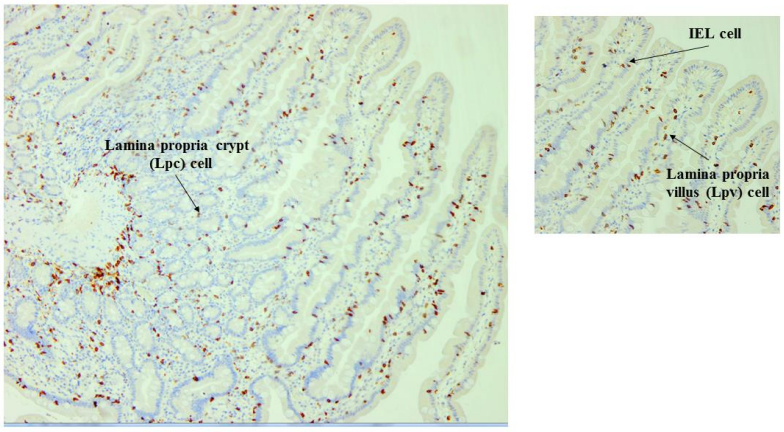

Fig. 4 Duodenal IEL, Lamina propria villus (Lpv) and Lamina propria crypt (Lpc) cells in PI-FGID

3.4 Ethics

All studies were approved by the Regional Committee for Medical Research Ethics and conducted according to the Declaration of Helsinki. All data collection and protection were approved by the Norwegian Social Science Data Services.

3.5 Statistical analysis

Differences between groups were measured using the parametric (paired- and unpaired Students *t*-test) or non-parametric (the Wilcoxon's or Mann-Whitney test) test. The area under the postprandial concentration curve for used for CCK plasma concentrations. The relationship between symptoms score and CCK or 5-HIAA was calculated using regression analysis (Paper II). Correlations were assessed using Pearson (parametric data) or Spearman rank test (non-parametric data). Kruskal Wallis

was used in Paper III for assessing of differences between groups for age, illness duration, and CD cell counts. Chi-square (Fisher exact) test was used in Paper III for categorical values. The Wilcoxon paired test was used in paper II to compare lymphocytes in repeated biopsies.

Data were analyzed using Graph Pad Prism 4 (San Diego California, USA) and SPSS 14.0 (Chicago, Illinois) for Windows.

4. BRIEF SUMMARY OF RESULTS

PAPER I

Increased visceral sensitivity in *Giardia* induced post-infectious irritable bowel syndrome and functional dyspepsia. Effect of the 5-HT3 antagonist ondansetron.

Twenty-two patients with IBS and FD after *Giardia* infection showed increased visceral hypersensitivity with lower drinking capacity and reduced gastric emptying compared to 19 recovered controls. They had more symptoms both fasting and postprandially than recovered controls. The subset of IBS/FD patients (n=15) underwent double-blind, randomized, placebo-controlled study with the 5-HT3 antagonist ondansetron, that had no effect on symptoms except of less nausea postprandially. For the interpretation of results, we refer to Discussion chapter.

PAPER II

The relative importance of abnormalities of CCK and 5-HT (serotonin) in *Giardia*-induced post-infectious irritable bowel syndrome and functional dyspepsia.

PI-FGID patients (n=32) had increased numbers of CCK cells and lower numbers of EC cells, compared to recovered controls (n=19). After consuming of the high-carbohydrate meal, the subgroup of PI-FGID patients (n=21) had significantly lower

plasma 5-HIAA, before and after a meal, as well as more dyspepsia compared with recovered controls. Plasma CCK correlated significantly with postprandial dyspepsia scores.

PAPER III

Prolonged duodenal mucosal lymphocyte alterations in patients with and without post-infectious functional gastrointestinal disorders after Giardia infection.

Duodenal mucosal intraepithelial lymphocytes (IELs) and lamina propria CD3, CD4, CD8, and CD20 lymphocytes were quantified in 28 chronic giardiasis (CG) patients, 66 PI-FGID, 19 recovered controls (RC) and 16 healthy volunteers (HC). There was assessed the associations with illness duration, abdominal symptoms, and histology grade. Duodenal CD4 IEL were significantly elevated in CG, and then decreased, followed by an upward trend after one year in both the PI-FGID and RC groups. Duodenal lamina propria crypt CD4 T cells were decreased in CG and stayed low for about 14 months before normalizing in both PI-FGID and RC group. Lamina propria CD20 cells were persistently elevated in all three *Giardia* exposed groups. Biopsies with microscopic inflammation showed increased lamina propria CD20 levels.

5. DISCUSSION

Due to persistent symptoms, our cohort patients were thorough an extensive follow up (3, 4, 8, 11). We found no organic cause that could explain the long-term symptoms and concluded that patients had symptoms consistent with *Giardia* induced Post-Infectious Functional Gastrointestinal Disorders (PI-FGIDs).

In this thesis, we found that patients with persisting abdominal symptoms, after eradicated *Giardia,* most of them with overlapping FD and IBS, had increased

visceral sensitivity with delayed gastric emptying, as assessed by ultrasound (US), in combination with a low-caloric soup meal. 5-HT 3 antagonist had effect on the postprandial nausea only (Paper I).

In Paper II, after intake of carbohydrate-rich meal, PI-FD/IBS patients had lower plasma serotonin (5-HT), assessed as 5-HIAA, with more dyspepsia symptoms, lower number of duodenal 5-HT containing EC cells and increased number of duodenal CCK cells compared to recovered controls.

In Paper III, all three *Giardia* exposed groups had decreased duodenal lamina propria crypt (Lpc) CD4 cells, that normalized approximately 14 months after the acute illness, while elevated dduodenal Lpc CD20 cells appeared to last longer than 19 months in all three *Giardia* exposed groups.

Visceral sensitivity

In general, FGID patients often experience worsening of symptoms after meal intake with subsequent increased visceral sensitivity (205). Increased visceral sensitivity has been shown to correlate with symptoms in a subset of FD patients (99, 102) and impaired accommodation in the acute onset of FD (133). IBS patients also expressed visceral hypersensitivity with the higher scores for all symptoms, except for nausea, both fasting and after intake of meal with 540 kcal (36% fat, 15% proteins, 49% carbohydrates; 8.9 g fiber) (206).

In Paper I, we hypotesised that increased gastric sensitivity could be important for symptom generation in PI-FGID patients after cured *Giardia* infection. We used the low-caloric meat soup test combined with ultrasound, as an non-invasive, inexpensive, and effective method to assess visceral sensitivity and gastric emptying (120).

Clinical overlap of FD and IBS is very common (207). Of all PI-FGID patients included in the Paper I, approximately over 2/3 had overlapping FD and IBS.

In fasting state, PI-FGID *Giardia* patients had significantly higher scores of abdominal pain, discomfort, nausea, and fullness compared to recovered controls. Postprandially, they expressed significantly more pain, discomfort as well as pooled symptom score than recovered controls.

After subgroup analysis, we found that patients with overlapping FD/IBS (n=15) had more fullness in fasting state compared to only IBS (n=7), otherwise there was no difference in symptoms between these two subgroups. Similar findings, with more epigastric pain, nausea, and upper GI discomfort both before and after the soup meal in both FD and IBS patients were reported by Steinsvik et al (208). Bisschops et al (114) reported that postprandial fullness in FD patients, is the most severe symptom aggravated by a meal and aproximately 50% of subjects with dyspeptic complaints in the general population, have postprandial symptoms that are often correlated to fasting symptoms. Other reported that FD patients with coexisting IBS had a greater overall symptom severity with hypersensitivity to distention tested by gastric barostat (209) and only postprandial, not fasting, gastric distention thresholds are related to the severity of meal-related symptoms (116) .

Our PI-FD/IBS patients showed delayed gastric emptying for low-caloric soup as measured by 3D US. Other studies show different findings, depending on the methods used. By using scintigraphy, Stanghellini et al (139) found that IBS patients with overlapping FD had significant slower emptying of solids compared to IBS without dyspepsia. Kindt et al (144) showed that PI-FD patients had delayed gastric emptying for solids, without an observable difference for liquids when tested by breath test for liquids and solids. Sarnelli et al (134) showed that delayed gastric emptying of solids or liquids in a subset of FD patients was associated with postprandial fullness and early satiety.

In Paper I, we did not measure gastric specific accommodation, but overall volume gives an indirect measure about it.

We found that fullness and satiety were positively correlated to the total gastric volume at maximal satiety in the PI-FGID patient group. It could be possible that distention of the stomach and subsequent stretch of mechanoreceptors may explain the symptoms.

Effect of 5-HT3 (Ondansetron®)

Previous study (210) showed improvement of nausea after treatment with a 5-HT3 antagonist without changing of gastric motility. Moreover, the ingested meal at maximum satiation was significantly increased by ondansetron (211).

Therefore, we hypothesized that 5-HT3 antagonist can improve symptoms in our PI-FGID patients. In a subgroup of PI-FGID patients (Paper I), with overlapping FD and IBS, the effect of 5-HT3 antagonist (Ondansetron®) on symptoms was tested by using ultrasound combined with the low caloric soup meal in addition to symptom questionnaires. They received oral treatment with either ondansetron , 8 mg or placebo, 20 p.m. the day before and the next day 1 h and 30 min before the drink test. 5-HT3 antagonist resulted with had less postprandial nausea in the treatment group compared to placebo. Otherwise, there was no effect on other symptoms, drinking capacity, 3D volumes, or gastric emptying.

The possible lack of effect on other symptoms could be explained by the small patient size or a low dose of 5-HT3 antagonist.

Previous studies showed that 5-HT3 antagonist (Alosetron®) relieved symptoms in FD (212) and upper GI symptoms in IBS patients (213). Similar findings were shown in healthy subjects by Kuo et al (214) using SPECT with assessing fasting or postprandial gastric volumes after a liquid meal (nutrient drink). They found that 5

HT-3 antagonist reduce nausea and bloating, but not pain or fullness, without increase in gastric maximal tolerable volumes, suggesting a role for 5-HT3 in a visceral afferent function.

We found also that nausea was negative correlated to maximal drinking volumes, indicating that it inhibits maximal drinking capacity. Postprandial symptoms after low-caloric meal soup in our PI-FD/ IBS patients may arise from mechanical stimulation of afferent nerves.

Feinle et al (169) found out that the gastric relaxation is not always necessary for reduction of postprandial symptoms, since they observed a reduction in symptom scores without a significant increase in the postprandial relaxation of the stomach, so the effect may be well be related to drug effect on chemo-sensitive afferents.

Another explanation with possible activation of duodenal chemoreceptors in our cohort patients may be of less importance since our PI-FGID patients had decreased gastric emptying.

Anyway, an interaction between mechanical and chemical stimuli in the upper gastrointestinal tract is important in the induction of postprandial sensations and symptoms in humans(215).

Plasma serotonin and cholecystokinin in post-Giardia FGID

After our best knowledge, there is no human study on duodenal 5-HT and CCK in patient with persisting symptoms after cured *Giardia* infection.

Therefore, the aim of Paper II was study plasma and duodenal 5-HT and CCK levels in PI-FGID patients. We chose to use carbohydrate rich meal, as it is previously showed that 5-HT levels increased after carbohydrate meal, compared to fat and protein rich meal (216). The meal ingestion is often associated with gastrointestinal symptoms in

FGID patients (99, 114, 116, 134). 5-HT has important role in postprandial symptoms, in both healthy (217), FD (218) and IBS patients (219). Cholecystokinin (CCK) release, from enteroendocrine cell, is stimulated by luminal releasing factors, secreted after meal ingestion. IBS patients have increased fasting and postprandial plasma levels of CCK (220).

Analysis of plasma 5-HT and CCK as well duodenal mucosal 5- HT in Paper II, were performed in England by Spiller RC and co, who has a long experience with serotonin and PI-IBS(197, 221).

The fasting 5-HT levels (Paper II), in plasma rich platelet, did not different between PI-FGID and recovered controls. After a carbohydrate meal challenge, PI-FD/IBS patients had a significantly lower plasma 5-HIAA, at all time points up to four hours after a carbohydrate reach meal, compared with recovered controls.

This is in a line with study done by Cheung 2013 (218), where FD patients had decreased basal ad postprandial plasma levels of 5-HT after intake of carbohydrate rich meal. Dunlop et al (221) found lower platelet poor plasma 5-HT, from 0 to 180 min after carbohydrate rich meal, in IBS-C patients, compared with PI-IBS and healthy controls. PI-IBS patients showed significantly higher peak of postprandial plasma 5-HT. Atkinsons al (222) reported lower concentrations of plasma 5-HT, following meal ingestion in only IBS-C, not IBS-D patients. Other group (197) found that IBS-D, after a standard carbohydrate meal (457 kcal), had a significant higher postprandial plasma 5-HT compared to healthy subjects.

We found no correlation between symptom scores and 5-HIAA, neither fasting nor postprandially.

In addition to 5-HT (Paper II), we assessed plasma CCK before and after carbohydrate rich meal, without finding significant differences between the patients and recovered controls.

The presence of lipid in the small intestine stimulates secretion of CCK from enteroendocrine cells into the circulation. As shown previously, a high-fat meal induces more symptoms in FD patients, than an isocaloric high carbohydrate meal with a greater concentration of fasting and postprandial plasma CCK compared to healthy subjects (115). Therefore, it is possible that lower fat content in the carbohydrate rich meal in our study may have caused insufficient CCK excretion.

Another possible explanation may be that asymptomatic recovered controls had similar findings as PI-IBS/FD patients without expressing symptoms. For that reason, a healthy control group will be preferable for comparison rather than recovered controls. The CCK plasma levels in our PI-FGID patients did correlate with postprandial fullness (Paper II), that probably may be mediated by delayed gastric emptying in these patients (Paper I).

Long-term alteration of duodenal EC cells, serotonin (5-HT) and cholecystokinin (CCK)

The long-term alteration of duodenal EC cells, 5-HT and CCK in patients with persistent abdominal symptoms, after eradicated *Giardia* infection, were not described before.

The type of infection and the site of maximal injury is important factor in developing of clinical picture of PI-IBS. As *Giardia* trophozoite adhere to the epithelial cells of proximal intestine and replicate without invading of intestinal mucosa, we supposed that possible changes may taking place in upper GI tract.

In Paper II, the main outcome was to study duodenal mucosal and plasma serotonin (5-HT). In addition, we assessed duodenal mucosal and plasma cholecystokinin (CCK).

We found that PI-IBS/FD patients had significantly decreased number of duodenal 5-HT containing EC cells, with unaltered 5-HT content, compared to recovered controls.

In addition, they had decreased duodenal mucosal 5-HT turnover, assessed by mucosal 5-HIAA ratio. These findings are consistent with IBS-C and PI-IBS patients in study performed by Dunlop et al (200). Similar to our findings, an decreased number of endocrine duodenal cells, without significant difference in 5-HT content compared to controls, was reported in FD patients by Witte et al (223). Other study (224) reported significantly higher number of EC cells in post-infectious FD patients compared to nonspecific FD or healthy controls.

Spiller et al (50, 53, 58) showed that PI-IBS patients had increased rectal 5-HT containing EC cell counts with increased 5-HT content Although infection resolved, the persistent number of elevated EC cells were found in rectal biopsies 3 months after *Campylobacter* infection (53) and in colon biopsies, 3 years after acute *Shigella* infection (2010).

We found the opposite findings from what we expected. It is possible that decreased number of duodenal 5-HT containing EC cells with decreased duodenal mucosal 5-HT turnover, assessed by mucosal 5-HIAA ratio after eradicated *Giardia* infection, could be explained by the impaired release of 5-HT or impaired SERT levels.

Previous studies suggest that inflammation may depress SERT levels. The platelet SERT could be a more convenient biomarker for increased serotonin availability. Foley et al (225) hypothesized that the changes in SERT induced by low-grade inflammation in the duodenum might also alter SERT in platelets. They found that IBS-D patients with duodenal immune activation had increased IEL, however normal villous had reduced platelet SERT. Dunlop et al (221) found decreased rectal 5 HIAA/ 5 HT ratio, despite increased 5 HT release, that could be explained by either defective SERT or disturbed mono amino oxidase activity. It will be desirable to study SERT role in post *Giardia* FGID patients.

Increase numbers of mast cells in gastric and duodenal mucosa of FD patients were suggested to be the histologic markers of FD (226). Activated colonic mast cells found

in proximity to colonic nerves may contribute to abdominal pain perception in IBS patients (151). We found no significant difference in duodenal mast cell counts between PI-FGID patients and recovered controls (Paper II). Dunlop et al (50) found no difference in rectal mast cells in PI-IBS patients when compared to non PI-IBS.

The further analysis of colonic mast cells could provide more knowledge about their role in patients with persisting symptoms after cured *Giardia* infection.

Long-term alteration of duodenal mucosal T- and B- cells

In Paper III, duodenal microscopic inflammation was found in a surprisingly high proportion (85.7%) of chronic giardiasis patients, 28.8% of PI-FGID and in only a few recovered controls. Previous study (227) showed that dyspeptic postprandial symptoms and epigastric pain have been associated with duodenal microscopic inflammation. The histological changes induced by *Giardia lamblia* are non-specific (25). The colonic biopsies in chronic giardiasis patients had either normal or only mild inflamed ileal mucosa with an increased number of plasma cells and lymphocytes (228). As reported previously (4), *Giardia* positivity was significantly associated with pathological histology with the strongest association occurring early after the acute infection.

In our cohort of patients (4), *Giardia* trophozoite were visible in duodenal biopsies in only in 4 (10%) of the 40 cases with *Giardia* positive fecal samples. In a large study of 567 giardiasis patients (24), the majority had normal duodenal histology with visible *Giardia* trophozoite in 82.5% of duodenal, but also in antral, jejunal, ileal and colonic mucosa.

When compared histologically normal (H0) with inflamed (H1) biopsies (Paper II), we found increased CD20 cell counts, in lamina propria villus and lamina propria crypt in PI-FGID patients with microscopic inflammation in an early post infectious period (3-

10 months). Otherwise there were no significant differences between H0 and H1 for duodenal CD4 or CD8 cell counts.

PI-FGID patients from our cohort (Paper III) had predominant IEL CD8+ T cells in duodenum as described previously (180). We found no difference in duodenal IEL cell count between groups. Similar findings were reported in duodenal mucosa of FD (145) and PI-FD (144) patients as well as in rectal mucosa of PI-IBS after *Campylobacter* gastroenteritis (50, 58).

Our chronic giardiasis patients (Paper III) had decreased lamina propria crypt CD4 + T cells, which could be explained by the fact that CD4+ T cells are necessary for clearance of the *Giardia* parasite, as shown in animal studies (229). Surprisingly, the persistent decrease in duodenal Lpc CD4 cells counts over time was found in PI-FGID but also in recovered controls, gradually increasing to normal levels more than one year after the start of giardiasis symptoms. Similar findings were reported by Kindt et al (144), who found a reduced number of intravillar CD4 cells in PI-FD without a difference in the numbers of IEL when compared to unspecified FD patients. They concluded that PI-FD patients showed impaired ability of the immune system to terminate the inflammatory response after the acute insult.

Including recovered control subjects, who were exposed to the *Giardia* infection at the same time, allows a better interpretation of the findings. As lower CD4 cell numbers were present in both PI-FGID and RC groups, this finding seems to be a prolonged effect of the inflammatory response to gastroenteritis, rather than associated with the presence of PI-FGID symptoms. A study from our group (230) showed that *Giardia*-infected individuals in Norway who had presumably acquired the infection while traveling abroad had increases in IL-17A, producing CD4 + and as well increased TNF production, indicative of a memory T cell response.

In Paper III, duodenal lamina propria crypt CD20+ cells were elevated in chronic giardiasis, FGID and surprisingly in recovered controls up to 19 months after *Giardia*

eradication, compared with healthy controls. Similar findings with increased activation of mucosal B-lymphocytes and plasma cells were shown in jejunal biopsies in IBS-D patients (231). In Belgium, one year after an outbreak with norovirus, *Giardia lamblia* and *Campylobacter jejuni* (55), PI-IBS patients had only increased rectal B-cell numbers without evidence of persistent immune activation in blood or rectal biopsies. The same group showed that rectal biopsies in these patients (232), taken 2 years after outbreak showed direct evidence of aberrant neuronal signaling in PI-IBS. Surprisingly, this sensitization of gut was not mediated by persistent low-grade inflammation but appears to be mediated by other pro-nociceptive changes in the mucosal microenvironment.

As previously described, there is a cross-talk between enteroendocrine cells and the immune system (233). An animal study showed that secretory products from CD4+ T-cells interact with EC to enhance the production of 5-HT in the gut via Th2-based mechanisms (158). PI-FGID patients from our cohort had a lower number of duodenal EC cells (Paper II) with the decreased counts of duodenal CD4 cells (Paper III) that may indicate a temporarily lower 5-HT production. In addition, Lpc CD20 cells (Paper III) in PI-FGID patients were positive correlated to EC cells (Paper II) (data not shown).

In Paper III, due to persistent symptoms, repeated duodenal biopsies were taken at two time points taken approximately one year apart in 11 *Giardia* patients. They showed increased duodenal lamina propria crypt CD4+ cells and lamina propria crypt CD 8+ cells, while Lpc CD20+ B cells showed a declining trend after one year without reaching significant difference.

Persistent symptoms after treated Giardia infection

After *Giardia* outbreak in Bergen many patients developed abdominal symptoms, that persist for severeal years, although parasite was eradicated. After an detailed work-up and repeated follow-up during the first two years after *Giardia* infection, we could not

find out any organic cause that can explain the persisting abdominal symptoms (3). A long-term follow-up studies performed in our cohort patients 2, 3, 6 years and 10 years after *Giardia* outbreak (3, 5, 8, 11) confirmed that *Giardia* infection was associated with increased risk of both IBS and chronic fatigue. Therefore, we concluded that *Giardia* may elicit functional gastrointestinal diseases (FGID). A such persisting symptoms after cured *Giardia* infection had not been described before. Searching the literature, we found only two studies that reported increased risk of IBS after *Giardia* infection (234) (65).

The persistent abdominal symptoms may have different causes. The nature of the infection has been reported as the strongest predictor of abdominal symptoms (235).There is an association between abdominal symptoms with initial severity of illness (236)

The late detection of *Giardia* infection in Bergen may have contributed that many patients developed persistent abdominal symptoms. According to study from our cohort (1), it took on average 17 days from onset of illness to the first physician contact, and 33 days from illness onset to the treatment start. Only a few patients were hospitalized.

On the other side, many FGID patients did not seek medical help, as the symptoms were too weak and unspecific. Sometimes, the stool samples were not sent for analysis, since patients had not been abroad, and of those submitted for analysis, many samples were not analyzed for *Giardia*, unless the doctor specifically asked for it. The doctors should be aware of such postinfectious sequela following all types of GI infection (237).

Nygaard et al (1) reported that those of our PI-FGID patients, who drank a lot of water had a much higher risk for development of symptoms. There was a predominance of women (aged 20–29 year), consistent with the previous study report where strongest risk factor for developing of FGID was female sex (238). In generally, many patients

report symptoms aggravation by certain ingested food (239, 240) and the perceived food intolerance is a common problem in IBS as reported in cross-sectional study performed in Norway (241).

In our cohort, PI-FGID patients reported symptom worsening due to food intolerance (3), that persisted three years after *Giardia* infection (10).

The symptoms elicited by *Giardia* are not specific and could mimic other disease. Therefore, it is important to consider coeliac serology since *Giardia* can elicit symptoms that resemble coeliac disease (242, 243). Our group (244) described eight cases with elevated coeliac serological markers where six of these later normalized after *Giardia* treatment. Temporary lactose intolerance may also be implicated in persisting symptoms after treated *Giardia* infection (245), although one study (246) showed no relationship between bacterial gastroenteritis and persistent lactose intolerance. In a small group of PI-FGID patients from our cohort (3), duodenal lactase activity was performed without confirming of lactase deficiency. Other possible causes of persistent symptoms after *Giardia* infection can be bile acid disturbance and small intestinal bacterial overgrowth (SIBO)(13), but in our cohort patients, symptoms were not associated with hydrogen breath excretion after lactulose challenge and cannot be related to intestinal bacterial overgrowth (14).

GI infection and psychological disorders appear to be distinct risk factors, contributing additively to the risk of developing both IBS and chronic fatigue syndrome(237). Previous studies (247, 248) showed that PI-IBS patients had the higher score for anxiety, depression, somatization and neuroticism compared to those who returned to normal bowel habits.

Our PI-FGID patients (Paper II), is a subgroup of 124 referred patients thoroughly investigated after outbreak (3, 4), reported significantly higher score for depression (measured by HADs (190), the lower quality of life (measured by The short form Nepean Dyspepsia Index-SF-NDI) (192) as well as a tendency to higher neuroticism

score (measured by Eysenck Personality Questionnaire)(191) compared to post-*Giardia* recovered controls. Increased neuroticism score has previously been reported in both FD and IBS (208) and PI-IBS patients (249) compared to healthy controls. Ten years after *Giardia* outbreak in Bergen, Litleskare et al (12) that exposure to *Giardia* infection was associated to a lower quality of life, mainly due to the development of IBS and fatigue.

The increasing levels of anxiety, depression, and somatization are associated with higher pre-prandial and/or postprandial GI symptom levels in IBS patients (250) .

The acute mental stress has been showed to modify visceral perception in both healthy controls and IBS patients, but only IBS patients exhibited both altered visceral and an exaggerated neuroendocrine response (251). The pre-existing anxiety and somatization, investigated in a cohort of over 18 000 people exposed to contaminated drinking water (*Norovirus, Giardia lamblia, Campylobacter jejuni*) (55) were linked to a lower immune response and a greater risk of developing GI infection. Of 124 patients with severe abdominal symptoms, referred to our outpatient clinic 2–18 months after the *Giardia* outbreak, approximately 15% reported pre-outbreak slight IBS-like symptoms (4)

In the prospective study in subjects without previous IBS history who developed PI-IBS after *Campylobacter* gastroenteritis, Spence et al (252) found significantly higher levels of perceived stress, anxiety, somatization and negative illness beliefs at the time of infection compared to those who did not develop IBS. This study supports cognitive-behavioral model of IBS, supported that gastroenteritis may trigger the symptoms, but cognitions, behavior and emotions may help to prolong and maintain them over time.

It cannot be ruled out that persistent symptoms in our PI-FGID patients caused prolonged stress and health worry which in turn cause prolonged symptoms.

5.6. Strengths and limitations

One of the main strengths in this study was a well-defined cohort of PI-FGID patients who developed persisting abdominal symptoms after a large *Giardia* outbreak in Bergen in 2004. All of them had confirmed *Giardia* parasite in the stool under outbreak, without co-infection, and were successfully treated with metronidazole. The inclusion of the relatively large number of patients allowed a description of duodenal mucosal lymphocytes kinetics. Repeated biopsies, even in a small number of PI-FGID patients, allowed us to follow up these patients over time. One of the limitations in this thesis, is that we did not assess ROMA scores in some PI-FGID patients at the start of initially work up. Healthy controls were not included at the same time as PI-FGID patients and recovered controls.

5.7. Conclusions and future perspectives

The patients with persisting abdominal symptoms after cured Giardia infection, showed increased visceral sensitivity with delayed gastric emptying assessed by ultrasound in combination with low-caloric meal soup. The 5-HT3 antagonist (Ondasentron®) influenced only postprandial nausea without improving of drinking capacity, 3D volumes, or gastric emptying.

PI-FGID patients had lower fasting and postprandial plasma 5-HIAA and CCK plasma levels in PI-FGID group correlated to fullness and bloating. PI-FD/IBS patients had reduced duodenal 5-HT containing EC cell count. There was a correlation between duodenal EC cell numbers and mucosal 5-HT content.

Only a few patients had a sign of microscopical duodenal inflammation. Duodenal lamina propria crypt (Lpc) CD20+ cells were persistently elevated, longer than 19 months, in all three *Giardia* exposed groups, compared to healthy controls.

Duodenal EC cell counts did correlate to persisting decrease duodenal Lpv CD4 and Lpc CD4 cells. This may indicate a temporarily lower 5-HT production that could be associated with ongoing immune activation.

We believe that interactions involving Giardia and gut microbiota may cause persistent dysbiosis that may have predisposed for persistent visceral hypersensitivity.

Further research highlighting the emerging immune-endocrine axis interaction with intestinal microbial dysbiosis caused by *Giardia*, as a potential target for therapeutic strategies in FGID, is warranted.

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Increased visceral sensitivity in *Giardia*-induced postinfectious irritable bowel syndrome and functional dyspepsia. Effect of the $5HT_3$-antagonist ondansetron

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Abstract *In an outbreak of waterborne giardiasis where 1300 subjects were diagnosed, with Giardia lamblia, 139 continued to have abdominal symptoms of whom two of three had negative stool culture and microscopy. These were considered to have a postinfectious functional gastrointestinal disorder. We investigated visceral hypersensitivity in patients with persisting abdominal symptoms after Giardia infection and assessed the effect of $5HT_3$-antagonist ondansetron. Twenty-two patients with Giardia negative stools and 19 controls were included. A subset of patients (n = 15) had both irritable bowel syndrome (IBS) and functional dyspepsia (FD). All subjects underwent a satiety test with a soup combined with three-dimensional ultrasound. Fifteen of 22 patients underwent double-blind, randomized, placebo-controlled study with the 5-HT_3 antagonist ondansetron given orally. Drinking capacity was lower in patients than in controls ($P < 0.01$) and gastric emptying was reduced ($P < 0.05$). Patients had more symptoms both fasting and postprandially ($P < 0.001$) compared to controls. Ondansetron had no effect on these parameters except from less nausea postprandially ($P < 0.05$). In conclusion, patients with Giardia-induced gastrointestinal symptoms developed both IBS and FD. They exhibited gastric hypersensitivity with lower drinking capacity and delayed gastric emptying. The 5-HT_3 antagonist ondansetron did not improve drinking capacity, gastric emptying or symptoms except nausea.*

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INTRODUCTION

In 2004 an extensive outbreak of waterborne giardiasis occurred in Bergen, the first large outbreak of a waterborne parasitic disease ever recorded in Norway.[1] In this period, 1300 cases were confirmed to have *Giardia* by stool examination. The most prominent clinical signs in these patients were abdominal pain, nausea, bloating and loose stools. Most patients were treated with metronidazole by their general practitioners. However, in 139 of them, symptoms persisted even after several treatment courses with metronidazole. In two-third of patients with persisting symptoms, no *Giardia* cysts were found in stools and they were thus considered to have *Giardia*-induced postinfectious functional gastrointestinal disorders (PI-FGID). Long lasting persisting GI symptoms in post-treatment cyst-negative *Giardia* patients have to our knowledge not previously been reported in a similar scale.

The prevalence of PI-IBS (irritable bowel syndrome) after Salmonella, Shigella and Campylobacter gastroenteritis varies from 7% to 31%.[2] In PI-IBS after Campylobacter *jejuni* infection, Dunlop *et al.*[3] found increased number of serotonin (5-hydroxytriptamine, 5-HT) containing enterochromaffin (EC) cells with abnormalities in serotonin metabolism and persistent low-grade bowel wall inflammation. Three months after infection, these patients had increased the gut transit and enhanced visceral sensitivity.[4] Previous studies have shown that the 5-HT_3 antagonist could improve upper and lower GI symptoms in patients with functional dyspepsia (FD) and IBS.[5–12] As visceral hypersensitivity is proposed as biological marker of

both FD and IBS, we hypothesized that abnormal serotonin metabolism was involved in developing of postgiardia symptoms and that 5-HT_3 antagonists could improve visceral hypersensitivity and symptoms.

Ultrasonography in combination with a low caloric meal is used to study gastric motility,[13] accommodation[14–18] and gastric emptying[19] in FD patients. Drink tests with high or low caloric drinks, as a non-invasive test to study visceral sensitivity and gastric accommodation in upper GI tracts, has been developed.[20,21] When using a low caloric drink test in combination with three-dimensional (3D) ultrasonography of the stomach, an optimal discrimination between FD patients and controls was obtained by the combination of symptoms and intragastric volume.[22]

The aims of this study were to investigate visceral hypersensitivity of the upper GI tract in patients with postinfectious *Giardia*-induced abdominal symptoms using a drink test combined with 3D ultrasonography and to assess the effect of $5HT_3$-antagonist ondansetron in these patients.

MATERIALS AND METHODS

Study subjects

Twenty-two patients [M/F 10/12, 45.5%/54.5%, median age 33, range 20–50, mean body mass index (BMI 23 ± 2.9)], with persisting abdominal symptoms 12 months after *Giardia* infection were consecutively included from our 'Giardia outpatient clinic'. In the large group of 139 individuals, median age was 31 (range 16–79 years), 38.8% was men and 61.2% women. Accordingly, this indicates that our study population was representative of the whole population (n = 139) with continuous symptoms.

Subsequently, they were examined with blood tests, stool culture and microscopy, upper endoscopy and duodenal biopsies. None of them had *Giardia* cysts or other microorganisms in faecal samples at the time of study. None was taking any medication during the last 2 weeks before the start of the study. All patients fulfilled the ROME II criteria for the IBS.[23] Fifteen patients also had symptoms compatible with the diagnosis of FD.[24]

Before inclusion into the study, a medical history was obtained, and ultrasound of the liver, pancreas and biliary tract was performed to rule out other diseases of the upper GI system. Criteria of exclusion from the study were serious systemic or suspect malignant disease, previous surgery in the upper GI tract, previous peptic ulcer disease, alcoholism, diseases of the liver, pancreas or bile ducts, pregnant or lactating women, or subjects using any medication.

As controls served 19 young, healthy persons (M/F 10/9, median age 31, range 22–45, mean BMI 24 ± 3.8), who had *Giardia* infection at the same time but recovered rapidly from symptoms after one-to-two courses with metronidazole. Control subjects were asymptomatic at the time of inclusion. They underwent a general medical examination with blood tests, stool culture with microscopy and gastroscopi with duodenal biopsies.

Fifteen, of totally 22, consecutive patients (M/F 9/6, median age 35, range 21–51, mean BMI 23 ± 2.5) agreed to undergo a double-blind, randomized, placebo-controlled study with 5-HT_3 antagonist ondansetron. The effect of ondansetron on visceral hypersensitivity was evaluated with a drink test in combination with 3D ultrasonography.

Study design

The participants were examined between 08:00 and 10:00 hours after an overnight fast. The subjects ingested meat soup, 100 mL every minute, until maximal drinking capacity and the volume of the stomach was then assessed using 3D ultrasound. All investigations were made while the individuals were breathing normally, sitting in a chair, leaning slightly backwards at an angle of 120. Abdominal symptoms were assessed both fasting and at maximal drinking capacity.

Patients receiving ondansetron were studied on two separate occasions, with 7–14 days interval between examinations. They received oral treatment with either ondansetron, 8 mg or placebo, 20 p.m. the day before and the next day 1 h 30 min before the drink test. Placebo and study medication (Zofran®; Glaxo SmithKline) were identical in appearance. Study subjects and the clinical investigators were blinded to the treatment assignment until the data analysis was completed.

Test meals

The test meal was commercial available meat soup (Toro clear meat soup; Rieber & Søn A/S, Bergen, Norway). The soup contained 1.8 g protein, 0.9 g fat, 1.1 g carbohydrate and non-soluble seasoning (0.2 g) per 500 mL (4 kcal 100 mL^{-1}). The pH of the soup varied between 5.4 and 5.7, and the osmolarity was 350 mOsm kg^{-1} H_2O. The soup was first boiled and then cooled to 37 °C.

Ultrasonography

Three-dimensional ultrasound imaging was performed with a Loqic 9 scanner (GE Medical Systems, Milwaukee, WI, USA) with a 3,5 MHz transducer interfaced to a magnetic position and orientation measurement system. The Bird system (Ascension Technology Corp., Burlington, VT, USA) was calibrated before each 3D acquisition that was performed at maximal satiety. The recording was stored on a PC workstation for later analysis using dedicated software (Echopac3D, Horten, Norway). This acquisition procedure and software have demonstrated very good accuracy in volume estimation.[17,25] All examinations were performed by the same doctor (TH) to avoid differences in intraobserver variation. All gastric volume measurements were performed blinded (by VD) after study completion. Gastric emptying (GE %), measured once – after meal, was defined as the fraction of the meal emptied from the stomach immediately after meal [(drinking capacity – intragastric volume)/drinking capacity × 100%)].

Symptoms

Abdominal pain/discomfort, nausea, fullness and satiety were assessed using a visual analogue scale (VAS), a 100 mm unmarked line where 0 expressed 'no symptoms' and 100 expressed 'excruciating' symptoms.

The sum of the scores for nausea, fullness and pain at maximal drinking capacity was denoted as the 'pooled symptom score'.

Ethics approval

The protocol was approved by the Regional Committee for Research Ethics, Bergen, Norway. All participants gave written informed consent to participate in the study.

Statistical analysis

Data are presented as a mean values or mean ± SD. Variables were analysed using parametric (paired- and unpaired Student's *t*-test as appropriate) or non-parametric (the Wilcoxon's or Mann–Whitney test as appropriate).

Correlations were assessed using Pearson correlation's coefficient. Significance was accepted at the 5% level. We expect an improvement in drinking capacity of 200 mL soup as effect of treatment and calculated that a minimum of 15 subjects were required to have a power of 0.80 assuming *P*-value <0.05. All statistical analyses were performed with Prism 4.0 (Graph Pad Software Inc., San Diego, CA, USA) and SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

All 22 patients with persisting abdominal symptoms after *Giardia* infection fulfilled ROME-II criteria for diarrhoea-predominant IBS and 15 of 22 had overlapping FD.[23,24] None of the study subjects had abdominal symptoms prior to the onset of acute giardiasis.

In fasting state, patients had more abdominal pain ($P < 0.001$), discomfort ($P < 0.001$), nausea ($P < 0.01$) and fullness ($P < 0.001$) compared to controls. Moreover, postprandial pain ($P < 0.001$) and discomfort ($P < 0.001$) were significantly higher in patients than in controls (Fig. 1). Pooled symptom scores were significantly higher in patients compared to controls both before ($P < 0.001$) and after meal ($P < 0.001$).

Drinking capacity was lower in patients than controls (795.5 ± 370.9 mL *vs* 1295 ± 765.6 mL respectively; $P < 0.01$) (Fig. 2). Similarly, intragastric volumes were lower in patient compared to control group (509.1 ± 186.9 *vs* 683.4 ± 342.2 mL respectively; $P < 0.05$) (Fig. 3). Gastric emptying was significantly reduced in patient compared to controls (30.6 ± 18.02% *vs* 43.8 ± 12.3% respectively; $P < 0.01$) (Fig. 4).

Nausea was significantly negatively correlated to maximal ingested drinking volume ($r = -0.48$; $P < 0.05$) and total gastric volume ($r = -0.54$; $P < 0.05$). Fullness ($r = 0.47$; $P < 0.05$) and satiety

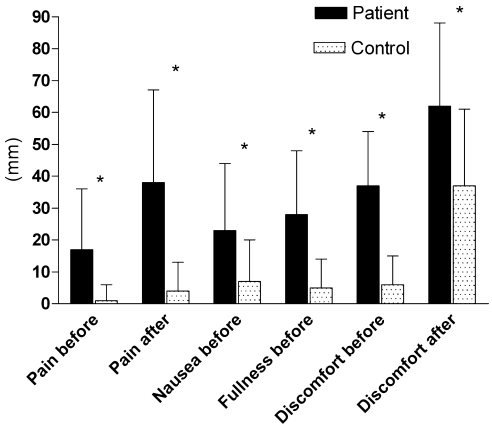

Figure 1 Dyspepsia symptoms, scored on visual analogue scale, 0–100 mm, ($P < 0.01$), before and after the soup drink test in 19 controls and 22 patients with *Giardia*-induced postinfectious irritable bowel syndrome/functional dyspepsia. Values are expressed as mean ± SD; Mann–Whitney test. The figure shows only significant symptoms score.

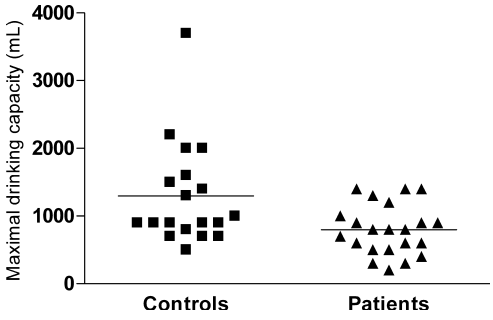

Figure 2 Maximal drinking capacity (mL) in 19 controls and 22 patients with *Giardia*-induced postinfectious irritable bowel syndrome/functional dyspepsia. Patients had significantly lower maximal ingested volume than controls ($P < 0.001$). Horizontal bar represents mean.

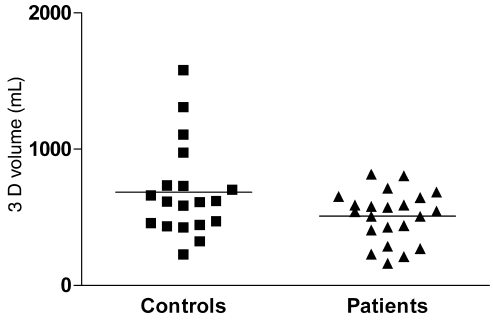

Figure 3 Intragastric (3D) volumes (mL) measured after maximal soup ingestion in 19 controls and 22 patients with *Giardia*-induced postinfectious irritable bowel syndrome/functional dyspepsia. Patients had lower 3D volume compared to controls ($P < 0.05$). Horizontal bar represents mean.

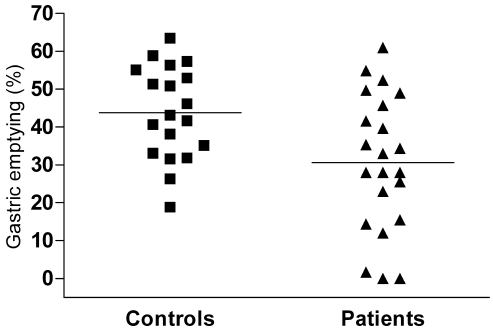

Figure 4 Gastric emptying (%) after soup ingestion was much slower in 22 patients with *Giardia*-induced postinfectious irritable bowel syndrome/functional dyspepsia compared to 19 controls ($P < 0.01$). Horizontal bar represents mean.

($r = 0.45$; $P < 0.05$) were significantly positively correlated to total gastric volume at maximal satiety.

There was no significant difference between subgroups IBS + FD *vs* IBS for drinking volumes, gastric emptying and 3D volumes. However, patients with overlapping IBS + FD experienced more fullness before ingestion of meal ($P < 0.05$) compared to those with only IBS (Fig. 5). No significant differences were found between groups for other symptoms or gastric volumes (results not shown).

Effect of ondansetron

Twelve of 15 patients had both IBS and FD and three had IBS only. There were no significant differences between the ondansetron and placebo group neither for drinking capacity (900 ± 358.6 mL *vs* 813.3 ± 264.2 mL; P = ns), 3D volumes (490 ± 191.7 mL *vs* 500 ± 216.7 mL; P = ns) and gastric emptying (43.16 ± 22.30 mL *vs* 38.07 ± 18.71 mL; P = ns). However, nausea score postprandially was significantly lower in the treatment group compared to placebo group (27.47 ± 21.89 *vs* 41.40 ± 23.04; $P < 0.05$) (Fig. 6). Otherwise, there were no significant differences for pain, nausea, fullness, satiety and sum symptoms before or after drinking test in the ondansetron group compared to placebo group (results not shown).

DISCUSSION

We found that patients with persisting abdominal symptoms after *Giardia* infection had enhanced visceral sensitivity after meal ingestion with reduced

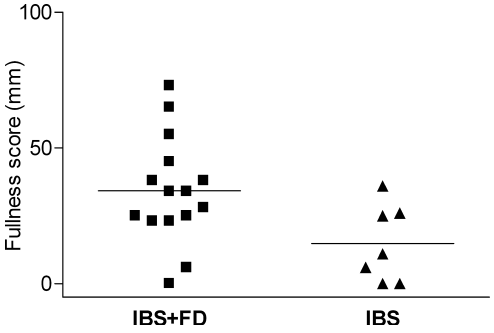

Figure 5 Fullness before drink test in 22 patients with persistent abdominal symptoms after *Giardia* infection. Subgroup analyse showed that 15 patients had overlapping irritable bowel syndrome (IBS) + functional dyspepsia (FD) and seven had only IBS. There was no significant difference in symptoms between subgroups except more fullness before meal in IBS/FD subgroup ($P < 0.03$).

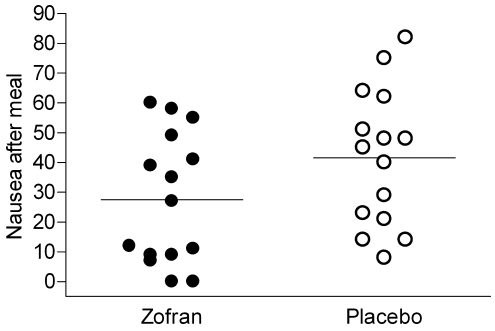

Figure 6 Influence of ondansetron on meal induced nausea in the 15 patients with *Giardia*-induced postinfectious irritable bowel syndrome/functional dyspepsia. There was significantly less nausea, after meal ingestion, in ondansetron group compared to placebo group ($P < 0.03$). Horizontal bar represents mean.

drinking capacity, delayed gastric emptying and significantly more postprandial symptoms compared to controls. This view is supported by a previous study showing that bacterial GI infection is a significant risk factor not only for IBS but also for Dyspepsia.[26] Furthermore, hypersensitivity to gastric distension, delayed gastric emptying and impaired accommodation was found in acute-onset FD.[27] Accordingly, postinfectious IBS and FD seems to be a real condition affecting a substantial number of patients, at least after infection with *Giardia lamblia.* As the major habitat of *Giardia* trophozoites is in the duodenum and 15 of 22 of our patients developed dyspepsia in addition to IBS, we hypothesized that these patients had visceral hypersensitivity in the upper GI tract.

Investigation of visceral perception in humans is based primarily on distension test where the electronic barostat remains as a gold standard.[28] However, this technique is invasive, unpleasant and influences gastric physiology.[17,29] Drink tests have been proposed as a more physiological and non-invasive way to test visceral hypersensitivity and were found to be highly reproducible and well correlated with barostat measurement.[21] Furthermore, ultrasonography is widely available, applicable for scanning of the stomach and can easily be combined with a drink test.[16,22,30] Perception of fullness and satiety was significantly positively correlated to 3D volume, which suggested that distension of the stomach induce fullness and satiety. These results suggest that triggering of mechanoreceptors may generate symptoms. The perception of postprandial nausea in our study was negatively correlated to maximal drinking volumes, which indicates that nausea inhibits maximal drinking capacity to be reached. Liquid meal appears to enter the duodenum within 1 min postprandially, before the occurrence of symptoms, which suggests that tasting of ingested material by intestinal chemo-receptors may be involved in symptom generation.[17,31] We found that patients with *Giardia*-induced persisting abdominal symptoms had a significantly lower emptying rate of liquid meal than controls. Delayed gastric emptying has been reported both in IBS and FD, although conflicting results exists. Stanghellini *et al.*[32] showed that 66% of IBS patients had overlapping dyspepsia where this group had significant lower gastric emptying of solids, evaluated by scintigraphy, compared to IBS without overlapping dyspepsia.

Postinfectious IBS is associated with increased serotonin (5-HT)-containing EC cells,[33] which are found throughout the GI tract, most in proximal duodenum and rectum. 5-HT_3 receptors are located peripherally on the parasympathetic terminals and centrally in the central nervous system (CNS). Feinle and Read[9] investigate involvement of 5-HT_3 antagonist in gastric motor and sensory response to distension, using gastric barostat and lipid infusion, and found that ondansetron reduced nausea and gastric sensitivity to distension. Moreover, 5-HT_3 antagonist was found to reduce postprandial symptoms[10–12] and gastric emptying[5] in both healthy subjects and FD patients. However, 5-HT_3 antagonists have mainly been used in IBS and showed to relax colon, delay colon transit and improve symptoms of diarrhoea-predominant IBS.[6–8,11]

In our study, however, ondansetron reduced only nausea after meal compared to placebo, but had no significant effect on other symptoms or drinking capacity, intragastric volume and gastric emptying.

Serotonin is a key mediator in signalling to the CNS, via 5-HT_3 receptors, and acts as a major cause of nausea and vomiting associated with cancer chemotherapy[34] and abdominal surgery. Although we had a small number of patients and type 2 error cannot be excluded, the results from our study argue against an important role of serotonin in generation of dyspeptic symptoms in postgiardiasis. The effect of ondansetron on postprandial nausea can possibly be explained by central mechanisms.

In conclusion, we found that most patients with *Giardia*-induced postinfectious GI symptoms developed both FD and IBS with enhanced visceral sensitivity due to lower drinking capacity and delayed gastric emptying assessed by a 3D ultrasound drink test. However, 5-HT_3 receptors did not appear to be involved in pathogenesis of dyspeptic symptoms in patients with postinfectious *Giardia*-induced IBS and FD.

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Alimentary Pharmacology & Therapeutics

Relative importance of abnormalities of CCK and 5-HT (serotonin) in *Giardia*-induced post-infectious irritable bowel syndrome and functional dyspepsia

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SUMMARY

Background
Post-infectious irritable bowel syndrome (PI-IBS) and functional dyspepsia (FD) have been described after both *Campylobacter jejuni* gastroenteritis and *Giardia* infection. After *C. jejuni*, there is increased rectal serotonin (5-HT)-containing EC cells and postprandial plasma 5-HT, while a pilot study suggested increased plasma cholecystokinin (CCK) after *Giardia* infection.

Aim
To determine changes in plasma and duodenal mucosal 5-HT and CCK in *Giardia*-induced PI-IBS.

Methods
A total of 32 patients previously infected with *Giardia* and 19 who had recovered fully (controls) completed symptom questionnaires. Endoscopic duodenal biopsies were obtained from all subjects and immunohistochemically stained for CCK, 5-HT and CgA containing enteroendocrine cells and mast cells. 5-HT content was also assessed. Twenty-one of 32 patients and 19 controls consumed a high-carbohydrate meal, while fasting and postprandial plasma CCK and 5-HIAA were measured.

Results
Post-infectious irritable bowel syndrome patients had increased numbers of CCK cells ($P = 0.02$), but lower numbers of EC cells ($P = 0.009$). Plasma CCK did not differ significantly between the groups, but correlated significantly with postprandial dyspepsia scores ($r = 0.5$, $P = 0.05$). PI-IBS patients had significantly lower plasma 5-HIAA, before and after meal ($P = 0.05$) as well as more dyspepsia ($P < 0.0001$) compared with recovered subjects.

Conclusions
Post-infectious bowel dysfunction following *Giardia* infection is associated with increased duodenal mucosal CCK. Postprandial dyspeptic symptoms correlate better with CCK than measures of 5-HT metabolism.

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INTRODUCTION

Irritable bowel syndrome (IBS)[1, 2] and functional dyspepsia (FD)[3] may follow acute intestinal infection and can persist for months or years.[4] Animal studies had shown that long-term bowel dysfunction can develop after a transient gastrointestinal infection.[5] Human studies following *Campylobacter jejuni* enteritis have shown persistent low-grade rectal mucosal inflammation with increased number of lamina propria T lymphocytes, increased number of enterochromaffin (EC) cells, increased serotonin bioavailability and increased small intestinal permeability in post-infectious IBS (PI-IBS).[2]

However, the clinical features of post-infective bowel dysfunction appear to depend on the type of infection and the site of maximal injury. Thus, rotavirus gastroenteritis which predominantly affects the upper gut is followed by delayed gastric emptying;[6] *Shigella* enteritis affects mainly the left colon causes post-infectious IBS (PI-IBS),[7]whereas *Salmonella* enteritis which causes both terminal ileal and proximal colonic ulceration causes both functional dyspepsia and PI-IBS.[4] Previous studies of bacterial enteritis suggested that an increase in serotonin-containing EC cells was a feature after *Campylobacter* enteritis, but other sorts of enteroendocrine cells such as the PYY-containing cells showed a different profile causing a switch in the normal PYY/5-HT ratio.[2] Other infections such as *H. pylori* elicit a different response with a decrease in somatostatin-containing enteroendocrine cells, but an increase in those containing gastrin.[8] We also found in mice infected with *Trichinella spiralis* that while the numbers of 5-HT-containing EC cells increased in the duodenum, they actually decreased significantly in the colon and hence it appears that the effect of infection on enteroendocrine cells in the gut is complex and depends on both site and type of organism.[9]

A recent prospective cohort study performed in *Giardia lamblia*-induced PI-IBS patients[10] concluded that many previously healthy persons experienced persisting abdominal symptoms after a large waterborne outbreak of giardiasis in Bergen, Norway. Over a 15-month period, 124 referred patients were evaluated and frequent findings were chronic *Giardia* infection and microscopic duodenal inflammation, especially if the evaluation was less than 7 months from the initial illness. Diarrhoea, bloating, nausea, abdominal pain, duodenal mucosal inflammation and elevated levels of faecal calprotectin suggest increased inflammatory response in these patients; however, as time went by, we started seeing more patients in whom the *Giardia* parasite had been eradicated, yet symptoms persisted. A previous pilot study had suggested that increased plasma cholecystokinin (CCK) can occur following acute *Giardia* infection and might mediate the anorexia often associated with this.[11] The aim of our study was therefore to determine the importance of duodenal serotonin (5-HT) and CCK containing cells as well as plasma 5-HT and CCK in patients with prolonged abdominal symptoms after successful treatment of the *Giardia* infection. We hypothesized that altered 5-HT and CCK signalling would be associated with the development of *Giardia*-induced PI-IBS.

MATERIALS AND METHODS

Study subjects

Altogether, as previously described,[10] there were 124 proven cases of giardiasis referred to hospital because of persisting symptoms, mostly bloating and diarrhoea, despite adequate treatment with metronidazole. Forty still had *Giardia* infection leaving 84 cases of post-giardiasis. The 32 consecutive patients included in the current study (see Flow chart, Figure 1) were a subgroup of these 84. All had completed one or more anti-*Giardia* treatment courses and microscopy of later stool samples had been repeatedly negative. After an elaborate clinical workup, they had received a diagnosis of PI-IBS and they met the ROME II criteria for IBS.[12] Twenty one patients also had symptoms compatible with the diagnosis of FD.[13]

The control group consisted of 19 healthy persons, without abdominal or other complaints who had *Giardia* infection at the same time as patients, but recovered rapidly from symptoms after 1–2 courses with metronidazole. These subjects were not referred to our out-patient clinic, but recruited via phone to participate in the study. They had normal endoscopy of the upper GI tract. None of the study participants had previous gastrointestinal surgery (other than appendectomy or cholecystectomy), pregnancy, history of drug or alcohol dependence, other significant illness, or any medication that may alter GI motility (as serotonin reuptake antagonists, TCA and opiates).

Carbohydrate rich test meal

Blood samples were taken for measurements of fasting and postprandial platelet poor plasma (PPP) 5-HT and

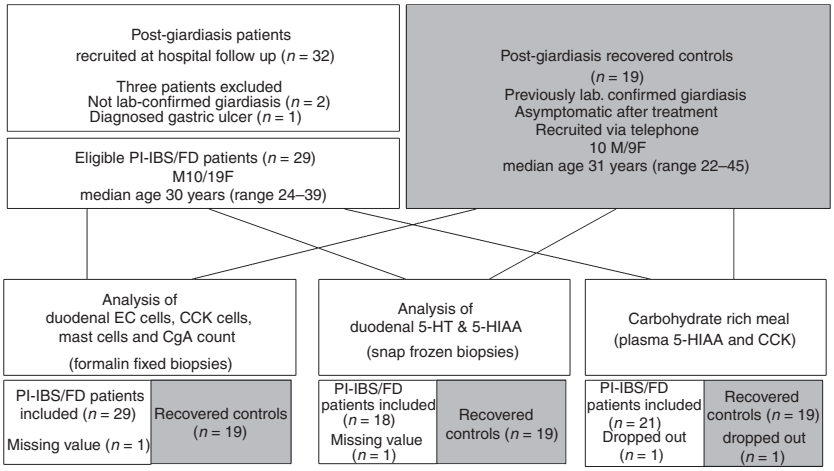

Figure 1. A flowchart of post-giardiasis PI-IBS/FD patients and recovered controls with duodenal histology analysis and meal test.

plasma CCK, whereas platelet 5-HT (platelet-rich plasma)(PRP) was measured at baseline only. The subjects then ingested test meal within 15 min (−15 to 0 min) and samples for plasma 5-HIAA and CCK were taken immediately after meal ingestion (time 0) and every 30 min for 4 h postprandially (time 30, 60, 90, 120, 150, 180, 210, 240). We used a similar carbohydrate-rich meal as Houghton *et al.*,[14] which consists of 100 g boiled pasta in 100 mL tomato soup (Toro, Rieber&Søn, A/S Bergen, Norway), 50 g white bread (two slices), 8 g soft margarine, one wheat bun,10 g jam (500 g sugar/kg) and 300 mL tap-water (calorie content of 557 kcal – carbohydrates, 63%; fat, 27%; protein, 10%).

Assessment of dyspepsia score

Abdominal symptoms (abdominal pain/discomfort, nausea, fullness and bloating) were assessed before (time −30 min) and every 30 min after meal up to 4 h postprandially by using the ROME II dyspepsia questionnaire.[13] Subjects were asked to score the severity of symptoms using the scale 0 = none; 1 = mild; 2 = moderate; 3 = severe. The overall postprandial dyspepsia score was calculated by adding the total scores for each of the four symptoms over the 4-h postprandial period (0–96).

Blood samples for serotonin (5-HT) and cholecystokin analysis

The subjects were asked to refrain from serotonin-rich food for 72 h, and alcohol and nicotine 24 h prior to the study. They arrived at 8 AM after on overnight fast and an 18 guage cannula was inserted in the antecubital vein. Blood samples were taken with the volunteers in a sitting position without using a tourniquet as previously described.[14] A three-way tap was attached to the cannula and the system was flushed with 2.5 mL of sterile normal saline containing heparin 10 units per mL. After every blood sample, the cannula was flushed with heparinised saline to prevent platelet activation. Blood samples were collected into pre-chilled 5 mL plastic syringes containing 0.45 mL of citrate-dipyridamole-adenosine-theophylline (CTAD) platelet stabilizing solution (Diatube H vacutainer tubes, Becton-Dickinson, Franklin Lakes, NJ, USA). During blood withdrawal, care was taken to ensure smooth rapid blood flow while avoiding excessive turbulence. The blood-anticoagulant mixture was placed immediately into the empty vacutainer from which the anticoagulant had been withdrawn, the lid was replaced and the sample was placed on ice. Following blood sampling, the intravenous cannula was flushed with 2.5 mL of normal saline containing 10 units of

heparin per mL. Between sampling, the subjects rested and were kept warm to ensure good peripheral blood flow.

Preparation of platelet-poor and platelet-rich plasma

Platelet-poor plasma was produced in a single step by centrifuging the vacutainer tubes at $3500 \times g$ for 25 min at 4 °C within 5 min of being taken. Platelet-rich plasma was prepared separately by centrifugation at $320 \times g$ for 10 min at 4 °C. Samples were removed promptly from the centrifuge and 1 mL of plasma was removed from the middle third of the supernatant using a plastic pipette and placed into polypropylene cryotubules. A 200 μL sample of platelet rich plasma was sent to the Department of haematology and platelets were counted using an automated cell counter (Sysmex, Kobe, Japan). All samples were frozen immediately at −80 °C and analysed in batches within 3 months of collection.

Extraction of 5-HT from biopsy sample and analysis of 5-HT and 5-HIAA by HPLC

This procedure was performed as previously described.[15] In brief, 5–10 mg of tissue was homogenized in perchloric acid, centrifuged and the supernatant contents analysed using HPLC with electrochemical detection. The limit of detection for 5-HT and 5-HIAA was 4 and 1 nmol/L and the coefficient of variation for the method was 0.8% and 2.1% respectively. Fasting platelet-rich plasma (PRP) samples were analysed for 5-HT while postprandial platelet-poor plasma (PPP) samples were analysed for 5-HIAA using the same technique. We chose to analyse plasma 5-HIAA as it shows a smoother postprandial profile rising steadily and avoids the problems of artifactually high 5-HT due to platelet activation. We therefore analysed plasma 5-HIAA, fasting (−30 min), immediately after meal (0 min) and at the end of the meal (210 and 240 min) as previous data from ourselves and others show that 5-HIAA rises steadily during 4 h postprandially.[15, 16]

Duodenal biopsy specimens

Gastroduodenoscopy was performed in 32 patients with *Giardia*-induced PI-IBS/FD and 19 recovered controls under fasting conditions on a separate day from the meal test. A total of six biopsy specimens were taken from the distal part of duodenum during endoscopy. Three biopsy specimens were placed in 4% buffered formalin for routine histological analysis. Three biopsies were snap frozen in liquid nitrogen and then stored at −80 °C and analysed for 5-HT and 5-HIAA content. A standard technique was used to prepare each biopsy for immunohistochemistry for serotonin producing enterochromaffin (EC) cells, as previously described,[15] CCK producing enteroendocrine cells,[17] mast cells and the nonspecific marker for all enteroendocrine cells, Chromogranin A (CgA).

Immunocytochemistry and image analysis

Nucleated 5-HT-containing cells were counted on coded slides within four non-overlapping high powered fields. Staining was performed using previously described methods[9] using rabbit antihuman serotonin antibody (Serotec Ltd, Oxford, UK) and the AvidinBiotin technique counterstained with haematoxylin. Cells were counted using the Weibel 2 graticule method as previously described and results expressed as cells per mm^2. Mucosal mast cells were stained for their chloroacetate esterase activity using the substrate napthol AS-D chloroacetate and the diazonium salt pararosaniline as previously described,[18] slides were then counterstained in Mayer's haematoxylin and counted using the Weibel graticule.[9] All enteroendocrine cells were stained for the nonspecific marker Chromogranin A (CgA) using rabbit Chromogranin antibody followed by biotinylated swine anti-rabbit antibody followed by AvidinBiotin Complex incubated with diaminobenzidine tetrahydrochloride (DAB) to develop a brown pigment counterstained with haematoxylin (DAKO, Cambridge, UK).

The antiserum against CCK used in this study is directed against gastrin/CCK, C- terminus, cross-reacting with both gastrin and CCK. The cells identified by this serum are mostly CCK cells, as the human duodenum is the main location for CCK cells, with few gastrin cells.[19] CCK immunoreactive cells were detected by Avidin Biotin Complex (ABC) method counterstained with haematoxylin, as described earlier in details,[17] using rabbit antihuman gastrin/CCK antibody (EuroDiagnostica, 804630, Malmö, Sweden).

We used different quantification method for CCK cells.[20] Morphometric analysis for CCK/gastrin, C- terminus immunoreactive cells was performed using Olympus program 'Cell P', with a x 40 objective and

in a frame representing an area of 0.13 mm^2 of the tissue. The number of nucleated CCK cells in the crypts was counted in coded slides from 20 randomly chosen fields from three different sections from each individual. All measurements were performed by the same person (V.D.) and double-checked by an experienced person (M.E.S.).

Questionnaires

Nineteen recovered controls and 21 PI-IBS/FD patients completed a validated Norwegian version of the ROME II questionnaires,[12] HAD (Hospital Anxiety and Depression Scale)[21] and EPQ-N (Eysenck Personality Questionnaire, short 12-item scales).[22] In addition, 14/21 PI-IBS/FD patients and 14/19 recovered controls completed the short form Nepean Dyspepsia Index (SF-NDI).[23] SF-NDI includes five subscales (tension, interference with daily activities, eating/drinking, knowledge/control and work/study). The items were measured by a Likert scale from 1 to 5 and total sum score for each of the five subscales was calculated by adding up scores for each item.

Data and statistical analysis

Demographic data are shown as median (range). Group differences are expressed as the mean + SEM, when distributed normally, otherwise median value ± interquartile range is given. Differences between groups were measured using the unpaired *t*-test (for parametric data) or Mann–Whitney (for non-parametric data). CCK concentrations in plasma were calculated as the area under the postprandial concentration curve (*AUC*) vs. time. Previous studies[11] suggest a mean difference in peak CCK of 10 pM/L (s.d. 10) and hence we calculated that we would require 17 in each group to achieve a power of 80% to detect such a difference. Dyspepsia score was calculated as the *AUC* of the total score. Relationship between dyspepsia scores and CCK or 5-HIAA was calculated using linear regression analysis. A *P*-value ≤ 0.05 was considered significant. Data were analysed using GRAPH PAD PRISM 4 (San Diego, California, USA) and SPSS 14.0 (Chicago, Illinois) for Windows.

Ethics approval

The Regional Committee of Research Ethics approved the study and all subjects gave written informed consent to participate in the study.

RESULTS

Assessment of dyspepsia score

We found that 16/20 (80%) of patients and 5/19 (26%) recovered controls reported mild symptoms immediately before meal ingestion (*P* for difference = 0.01). PI-IBS/FD patients had significantly higher total postprandial symptom score (*AUC*) for pain ($P < 0.005$), nausea ($P < 0.0001$), fullness ($P < 0.0001$) and bloating ($P < 0.0001$) compared with recovered controls (see Table 1). None of the participants reported 'severe' symptoms postprandially.

Psychiatric questionnaires

Post-infectious irritable bowel syndrome/functional dyspepsia patients had a significantly higher score for depression (HAD-D, 5.2 ± 1.0 vs 2.3 ± 0.5, $P = 0.02$) and lower quality of life (SF-NDI, 31.4 ± 1.9 vs 10.7 ± 0.5, $P < 0.0001$) compared to controls. There was a tendency to higher neuroticism scores in the PI-IBS/FD group, but this did not reach significance ($P = 0.06$).

Plasma and platelet 5-HT

There was no significant difference in fasting 5-HT (PRP) levels between patients and recovered controls ($P = 0.3$). The PI-IBS/FD patients had a significantly lower plasma 5-HIAA compared with controls at all time points −30 ($P = 0.05$), 0 min ($P = 0.04$), 210 min ($P = 0.04$) and 240 min ($P = 0.01$) and a reduced *AUC* 0–240 min (Table 2). We found no correlation between symptom scores and 5-HIAA, neither fasting nor postprandially.

Table 1. Dyspepsia score during the 557 kcal carbohydrate rich meal in patients with *Giardia*-induced PI-IBS/FD and recovered controls. Data are expressed as *AUC* (median ± interquartile range)

Symptom score/min	*Giardia* PI-IBS/FD patients ($n = 20$)	Recovered controls ($n = 18$)	*P* value
Abdominal pain	45 (0–218)	0 (0–15)	0.005
Nausea	150 (38–255)	0 (0–8)	0.0001
Bloating	390 (248–525)	30 (0–60)	0.0001
Fullness	248 (120–420)	30 (0–135)	0.0001

Table 2. Fasting and postprandial plasma 5-HT and 5-HIAA concentrations during the 557 kcal carbohydrate-rich meal in *Giardia*-induced PI-IBS/FD patients and recovered controls. Data are shown as median ± inter-quartile range

	Giardia PI-IBS/FD (n = 20)	Recovered controls (n = 18)	P value
5-HT (PRP) nmol/10^9 platelets			
−90 min	3.3 (3.2–3.9)	3.7 (2.7–4.6)	0.3
5-HIAA (PPP) nmol/L			
−30 min	14.9 (13.1–16.8)	17.3 (14.4–20.4)	0.04
0 min	15.1 (12.3–17.9)	18.1 (14.0–21.9)	0.03
210 min	12.0 (10.2–14.2)	14.1 (12.6–17.3)	0.05
240 min	11.8 (10.4–14.9)	15.4 (13.6–18.2)	0. 03
AUC for 5-HIAA (PPP)	3718 (2884–4260)	4386 (3625–4933)	0.03

Plasma cholecystokinin

Figure 2 shows the average plasma CCK levels during the test meal. We found no significant differences at individual time points (ANOVA) between groups. The area under curve (*AUC*) of CCK tended to be higher in the PI-IBS/FD group compared with recovered controls, but this did not reach statistical significance (P = 0.07). In the PI-IBS/FD group, plasma CCK showed a weak, but significant positive correlation to fullness score at 60 min (P = 0.025, r = 0.3), at 120 min (P = 0.04, r = 0.2), at 180 min (P = 0.004, r = 0.4) and at this time also to bloating scores (P = 0.004, r = 0.2).

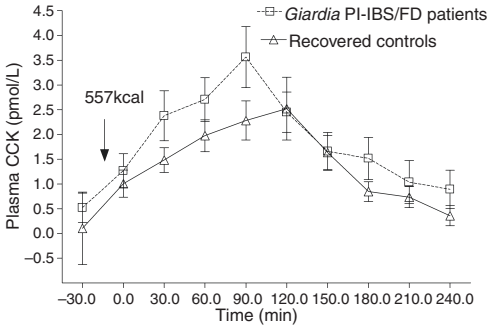

Figure 2. The effect of 557 kcal carbohydrate-rich test meal on plasma CCK levels in *Giardia*-induced PI-IBS/FD (n = 20) and recovered controls (n = 18) (P = 0.07). Data are shown as mean ± S.E.M.

Duodenal histology

We found mild duodenitis in one PI-IBS/FD patient, while others had a normal macroscopic appearance. Five patients had mild chronic inflammation in routine histological analysis of duodenal mucosa. One subject from the recovered controls had mild oesophagitis visible endoscopically, while three other subjects had signs of microscopic duodenitis with mild chronic inflammation.

Duodenal 5-HT content, 5-HIAA, ratio 5-HIAA/5-HT

There was no significant difference of the content of 5-HT (nmol/mg) duodenal tissue between the *Giardia* PI-IBS/FD patients (mean 21.1 ± 4.1) compared to controls (mean 16.8 ± 4.0, P < 0.45) (Table 3). The 5-HIAA tended to be lower in the patients (mean 18.6 ± 3.5 nmol/mg) compared with controls (mean 25.4 ± 3.7), but this difference did not reach a significant level (P = 0.13). However, patients had significantly lower 5-HIAA/5-HT ratio (1.58 ± 0.4) than controls (5.02 ± 1.6) (P = 0.05).

5-HT- and CCK-containing EC cells, chromogranin A and mast cells

The main finding of our study was that 6 months or more after *Giardia* infection, the 5-HT-containing EC cell count was significantly reduced in the PI-IBS/FD patients (mean 27.04 ± 5.8 cells/mm^2) compared with the recovered controls (60.8 ± 11.9), P < 0.009 (Figure 3). There was a weak, but significant, positive

Table 3. Comparison of the duodenal mucosal content of 5-HT, 5-HIAA and 5-HIAA/5-HT ratio from patients with *Giardia*-induced PI-IBS/FD and recovered controls. Data are shown as mean ± S.E.M.

	Giardia PI-IBS/FD patients (n = 17)	Recovered controls (n = 19)	P value
5-HT (pmol/mg)	21.1 ± 4.1	16.8 ± 4.0	0.45
5-HIAA (pmol/mg)	18.6 ± 3.5	25.4 ± 3.7	0.2
5-HIAA/5-HT ratio	1.58 ± 0.4	5.02 ± 1.6	0.05

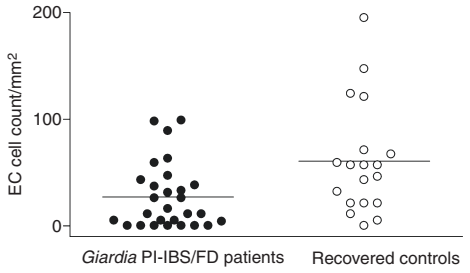

Figure 3. Duodenal 5-HT containing EC cell count/mm^2 (mean ± S.E.M., P = 0.009) in *Giardia*-induced PI-IBS/FD patients (n = 28) and recovered controls (n = 19).

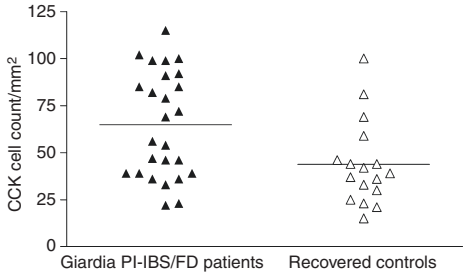

Figure 4. Duodenal CCK containing EC cell count/mm^2 (mean ± S.E.M., P = 0.02) in *Giardia*-induced PI-IBS/FD patients (n = 28) and recovered controls (n = 19).

correlation between EC-cell numbers and mucosal 5-HT content (linear regression r^2 = 0.2, P = 0.05).

However, enteroendocrine cells containing CCK were more numerous in patients with PI-IBS/FD compared with controls (P = 0.02) (Figure 4). Furthermore, the ratio of CCK/5-HT cells was significantly greater in PI-IBS/FD (P < 0.002) (Table 4).

There were no significant differences in the chromogranin A containing cell counts (P = 0.4) or mast cell counts (P = 0.8) between the groups.

DISCUSSION

Most previous studies of post-infectious functional gastrointestinal diseases (PI-FGIDs) have focused on bacterial causes of gastroenteritis, which are characterized by inflammation and mucosal tissue destruction. However, more recently, PI-FGIDs have been described also after *Trichinella* infection[9] and viral gastroenteritis[3, 24] indicating that there are multiple mechanisms

Table 4. Duodenal histology analysis in patients with *Giardia*-induced PI-IBS/FD and recovered controls. Data expressed as mean ± S.E.M.

	Giardia PI-IBS/FD patients (n = 28)	Recovered controls (n = 19)	P value
5-HT producing EC cells count/mm^2	27.04 ± 5.8	60.8 ± 11.9	0.009
CCK producing EC cells count/mm^2	64.9 ± 5.5	43.8 ± 5.4	0.02
CCK/5-HT ratio	17.45 ± 5.85	5.36 ± 4.0	0. 002
Mast cells/mm^2	13.1 ± 0.8	12.6 ± 1.2	0.8
CgA positive cells/mm^2	92.4 ± 14.1	105.6 ± 16.7	0.4

underlying the post-infectious response. *Giardia lamblia* is a common protozoan cause of gut infections, especially in the developing world. Disease severity may differ from asymptomatic to a severe clinical picture with diarrhoea, nausea, abdominal pain and malabsorption. The *Giardia* parasite resides in the lumen and attaches to the epithelium, but does not invade the mucosa. No toxin has been found, and only occasionally does it cause mucosal inflammatory reactions or villous shortening.[25] Factors in both microbe and host are thought to play a role in the variation seen in the clinical and histological picture, and a surprisingly high frequency of mucosal inflammation and prolonged symptoms was observed in a subset of patients after the Bergen outbreak.[10] Many of these went on to develop post-infectious functional gastrointestinal diseases, while the initially inflamed duodenal mucosa normalized.[26] In the present study, where duodenal biopsies were taken 6 months or longer after clearance of the *Giardia* infection, we also found few duodenal inflammatory changes.

EC-cell counts and 5-HT levels

Our study shows that patients with persisting abdominal symptoms after *Giardia* infection have lower number of 5-HT containing enterochromaffin (EC) cells in duodenal mucosa as well as lower plasma 5-HT measured as 5-HIAA during a meal. To our knowledge, this is the first report concerning mucosal and plasma 5-HT changes in patients with *Giardia*-induced PI-IBS/FD. The decrease in EC cells of about 50% was striking and unexpected. CgA positive cells did not change suggesting that overall enteroendocrine cell

numbers were unaltered. Previous studies of PI-IBS after bacterial enteritis have shown greater 5-HT containing EC cell counts and increased 5-HT content in human rectal mucosa.[2, 15, 27, 28] In the present study, we did not assess colonic 5-HT and so cannot exclude the possibility that duodenal and colonic responses might be different.

Studies in mice suggested that while *Trichinella spiralis* infection caused an inflammatory response in the jejunum with an increase in enterochromaffin cells, in the colon, there was a decrease.[9] It is possible that the differing immune responses in different regions of the gut can drive enteroendocrine cell numbers both up and down as they have receptors for cytokines on their surface.[7] Similar contrasting changes can be seen in the antral mucosa with *H. pylori* infection, which causes an increase in gastrin-containing cells, while somatostatin cell numbers decrease.[8]

We also found the duodenal mucosa 5-HT turnover, as assessed by 5-HIAA/5-HT ratio, to be decreased, a feature we previously reported in both PI-IBS and IBS with constipation.[15] As mucosal biopsy 5-HIAA is derived only from metabolism of 5-HT secreted by enterochromaffin cells after uptake by the serotonin transporter (SERT), a reduced 5-HIAA/5-HT ratio implies either impaired release of 5-HT or impaired SERT function. Previous studies do suggest that inflammation depresses SERT levels; an effect again likely to be mediated by local cytokines,[29] but in the current study, impaired release could also play a part.

Cholecystokinin and symptoms

Contrasting our 5HT-findings, duodenal mucosal CCK-containing cells were increased and postprandial plasma CCK tended to increase. Elevated CCK levels have previously been reported both in a pilot study in humans with acute symptomatic giardiasis[11] and in a mouse model with acute giardiasis.[30] Furthermore, previous studies have shown an exaggerated and prolonged postprandial CCK release in IBS patients.[31, 32] Excess of CCK in some IBS patients might explain the benefit of CCK antagonist, dexloxiglumide[33] in IBS, which has also been shown to accelerate gastric emptying in female patients with IBS[34] and also to block the exaggerated postprandial colonic contractions in IBS patients in response to CCK infusions.[35]

Although the increase in plasma CCK in PI-IBS/FD patients was not significant, it should be noted that a local increase could still stimulate vagal afferents in a paracrine fashion. Therefore, we cannot exclude the idea that increased CCK release might be responsible for symptoms. However, definite conclusion on this point will require a randomized placebo-controlled trial of a specific CCK antagonist.

We found that CCK levels in the PI-IBS/FD group were correlated with postprandial fullness, which is an important symptom in functional dyspepsia. Many other studies have suggested that CCK has a major influence on satiety,[36] being released mainly by dietary fat and acting both on vagal afferents[37] as well as the brainstem. The correlation observed between increased release of CCK and a sense of fullness might be mediated by the delay in gastric emptying that we have observed in patients with persistent symptoms following *Giardia* infection[38] and which CCK infusions are known to cause.[39]

Although we based our power calculation on a previous study,[11] we found smaller differences, possibly because our study was performed many months after the initial infection. While we found a numerically higher plasma CCK before and after a carbohydrate rich meal in PI-IBS/FD compared with asymptomatic controls, this failed to reach a statistical significance probably because our study was underpowered.

In summary, we found that patients with long-lasting abdominal symptoms 6 months or more after *Giardia* infection had lower plasma 5-HIAA after a high caloric mixed meal and lower duodenal 5-HT containing EC cells count than recovered controls. Moreover, they had an increased number of duodenal CCK cells and plasma CCK was associated with dyspeptic symptoms. This suggests that CCK could have an important role in generating symptoms in patients with *Giardia*-induced PI-IBS/FD, while 5-HT seems less important in this variety of post-infectious functional gastrointestinal disease, a feature which may explain why 5-HT_3 antagonists show little benefit in this condition.[38]

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The Journal of Infectious Diseases

MAJOR ARTICLE

Prolonged Duodenal Mucosal Lymphocyte Alterations in Patients With and Without Postinfectious Functional Gastrointestinal Disorders After *Giardia* Infection

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Background. Persisting low-grade inflammation is suggested to play a role in postinfectious functional gastrointestinal disorders (PI-FGIDs). The present study examined alterations in duodenal mucosal lymphocytes during and after *Giardia* gastroenteritis in patients who did, or did not, develop PI-FGIDs.

Methods. Duodenal mucosal intraepithelial lymphocytes (IELs) and lamina propria CD3, CD4, CD8, and CD20 lymphocytes were quantified in 28 patients with chronic giardiasis (CG), 66 patients with persistent abdominal symptoms after acute *Giardia* infection (PI-FGID), 19 recovered controls (RCs), and 16 healthy volunteers (HCs). Associations with illness duration, abdominal symptoms, and histology grade were assessed.

Results. Duodenal CD4 IELs were significantly elevated in CG, then decreased, followed by an upward trend after 1 year in both the PI-FGID and RC groups. Duodenal lamina propria crypt CD4 T cells were decreased in CG, and stayed low for about 14 months before normalizing in both the PI-FGID and RC groups. Lamina propria CD20 cells were persistently elevated in all 3 *Giardia*-exposed groups. Biopsies with microscopic inflammation showed increased lamina propria CD20 levels.

Conclusions. Duodenal mucosal lymphocyte alterations were prolonged after *Giardia* infection, but similar in patients who developed PI-FGID and recovered asymptomatic controls.

Keywords. duodenal mucosa; *Giardia*; functional gastrointestinal disorders; PI-IBS; histology; B cell.

Giardia lamblia (synonyms *duodenalis*, *intestinalis*) is an intestinal protozoan parasite that infects the small intestine causing giardiasis, resulting in a variable spectrum of abdominal symptoms. It often causes a gastroenteritis with prolonged diarrheal illness and abdominal cramping, but may also be asymptomatic. It is commonly seen in returning travelers from low-resource settings and a frequent cause of waterborne outbreaks.

Giardiasis has been recognized as a risk for developing postinfectious functional gastrointestinal disorders (PI-FGIDs) [1–4]. Irritable bowel syndrome (IBS) is the most common of these conditions and occurs in 3%–36% of individuals after infectious gastroenteritis [5, 6]. Follow-up studies of laboratory-confirmed *Giardia* infection after an outbreak in Bergen, Norway, found a high prevalence of IBS after 3, 6, and 10 years [4, 7, 8].

The mechanisms behind development of FGID are not known, but are regarded to be multifactorial. Several studies suggest that persisting low-grade inflammation, with increased numbers of mucosal B and T lymphocytes, could be an important contributing factor [6, 9, 10].

Giardia infection is known to elicit both B- and T-cell–dependent immune responses [11–13]. Animal studies have shown that microvillous injury, disaccharidase deficiencies, and increased crypt/villus ratio are mediated by CD8 cells, while CD4 cells contribute to parasite clearance [14]. Mucosal lymphocyte kinetics during and after giardiasis, and their potential association with development of PI-FGID, have not been examined before.

The aim of the present study was to evaluate lymphocyte alterations in the duodenal mucosa in giardiasis and to examine whether such alterations were associated with persisting abdominal symptoms following *Giardia* infection.

MATERIALS AND METHODS

Study Subjects

This study is based on the clinical and research data and specimens obtained during a structured workup and follow-up of

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patients referred to Haukeland University Hospital in Bergen, Norway [15]. Patients were referred due to persisting abdominal symptoms after the *Giardia* outbreak in Bergen in 2004. They were examined and biopsied between January 2005 and April 2006 (Figure 1), at which time they all had been ill for 3 months or more. Biopsies were available from 28 patients with chronic giardiasis (CG) and from 72 patients (66 of these randomly selected for this study) in whom *Giardia* had been successfully eradicated (diagnosed by at least 3 microscopy-negative samples and later verified with negative polymerase chain reaction). A detailed workup including upper endoscopy with duodenal biopsies, serum antiendomysial antibodies, antitissue transglutaminase antibodies, routine blood screening tests, immunoglobulins, immunoglobulin E, and fecal calprotectin did not reveal other organic disease in the *Giardia*-negative patients, who were diagnosed as having PI-FGID. To evaluate possible coinfection, *Helicobacter pylori* was analyzed in frozen stool samples by stool antigen test [16] in 20 randomly selected PI-FGID patients.

Control Groups

Nineteen patients with laboratory-confirmed giardiasis during the outbreak, who had recovered well after treatment, were selected randomly and recruited by invitation letter/telephone and examined 12–19 months after onset of the gastroenteritis [17]. These patients were designated the recovered control (RC) group. Additionally, 16 healthy controls (HCs), with no history of persisting bowel symptoms and not taking immunosuppressive medication, were recruited by advertisements and went through the same investigations as the cases (Figure 1).

All participants provided written informed consent and the study was approved by the Regional Committees for Medical and Health Research Ethics (REC WEST, Norway) number 2016/1632.

Symptoms

Abdominal symptom scores for the last 30 days were recorded on the day biopsies were taken. Nausea, bloating, abdominal pain, diarrhea, and constipation were scored using an ordinal scale from 0 (no symptoms) to 10 (severe symptoms) [18]. Illness duration was defined as the time from the start of acute symptoms of *Giardia* infection until the date of clinical examination with biopsies. At follow-up, PI-FGID patients were asked to complete a validated Norwegian version of the ROME II questionnaire [19].

Duodenal Biopsies and Histology

From all study groups, 3 biopsy specimens were obtained from the second part of the duodenum, embedded in paraffin, and processed for routine hematoxylin and eosin staining and immunohistochemistry. In 11 of the PI-FGID patients examined at 16–19 months of illness duration, biopsies were available from a previous examination 3–7 months after onset of symptoms, when 8 were still *Giardia* positive and 3 were *Giardia* negative.

The severity of duodenal inflammation, as well as the architecture of villi and crypts, was determined by an experienced pathologist in a blinded manner. The routine histological findings were classified as H0 if they were normal and as H1 if there was inflammation with infiltration of leukocytes and increased number of plasma cells in the lamina propria with or without shortening and blunting of intestinal villi (Supplementary Figures 1–3).

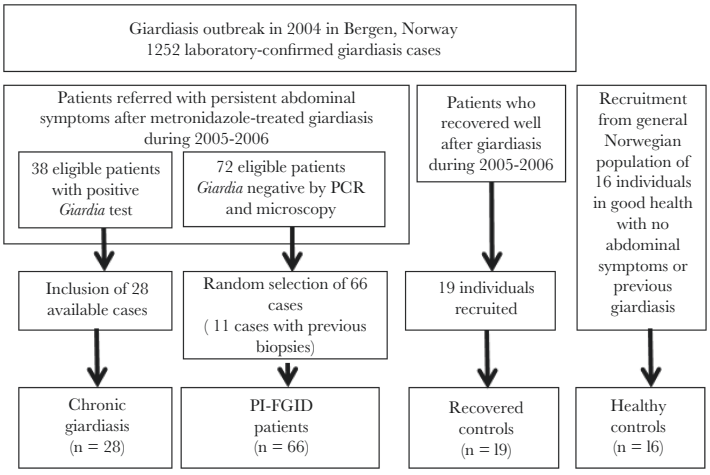

Figure 1. Flowchart of the study population and study design. Abbreviations: PCR, polymerase chain reaction; PI-FGID, postinfectious functional gastrointestinal disorder.

Immunohistochemistry

Formalin-fixed, paraffin-embedded duodenal specimens were cut into 4-µm sections, de-paraffinized in xylene, and rehydrated through graded ethanol series and distilled water.

After heat-induced epitope retrieval in Tris–ethylenediaminetetraacetic acid buffer, pH 9.0 for 15 minutes at 350 W, endogen peroxidase activity was blocked with 0.3% peroxide (Dako) for 5 minutes. Tissue was then incubated with primary antibodies: CD3 (polyclonal rabbit antihuman CD3, 1/400, Dako), CD4 (monoclonal antibody NCL-CD4-IF6, clone IF6, 1/25), CD8 (monoclonal mouse antihuman CD8α, clone C8/144B, 1/100, Dako), and CD20 (monoclonal mouse antihuman CD20, clone L26, 1/1000, Dako).

We used EnVision (Dako 5007) secondary antibody for 30 minutes, with 3,3'-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin (Dako S3301) (Supplementary Figures 4 and 5).

Methods of Cell Counting

Lymphocyte subsets were counted in 3 anatomically defined regions. The numbers of intraepithelial lymphocytes (IELs) located above the basal membrane, per 100 epithelial cells, were counted on 5 well-orientated villi with longitudinal sections and expressed as the number of IELs per 100 epithelial cells [20].

Lamina propria villous (Lpv) lymphocytes located underneath epithelial basal membrane were assessed in 5 villi with results expressed as average cell counts per area (cells/mm^2). Lamina propria crypt (Lpc) lymphocytes were counted per area within 5 consecutive, nonoverlapping ×200 fields of Lpc and the results averaged (cell count/mm^2).

Positive cells in vicinity of lymphoid follicles or clusters were not taken into consideration. Only cells with a visible nucleus were considered as positive and counted.

The cell counts were performed using Olympus program Cell P image analysis software, with a ×200 objective. All measurements were performed in a blinded manner by V. D. and double-checked by a pathologist (O. D. L.). Interobserver agreement (V. D. and O. D. L.) and intraobserver agreement (V. D.) for counting of CD cells was assessed using Bland–Altmann correlations test.

We also analyzed correlation between T- and B-cell data obtained in this study with duodenal enterochromaffin (EC) cell numbers from 19 patients in the PI-FGID group and 19 RCs, which were available from a previous study [21].

Statistical Analysis

Age and illness duration are expressed as median (range). Abdominal symptom score and lymphocyte cell counts are presented as median (interquartile range [IQR]). Differences between groups for age, illness duration, and CD cell counts were assessed using the Kruskal–Wallis test. The Fisher's exact test was used for categorical values. Mann–Whitney unpaired test was used for comparisons of histology and lymphocyte counts. The Wilcoxon paired test was used to compare lymphocyte counts in repeated biopsies. Correlation analyses were performed using the Pearson (parametric data) or Spearman rank test (nonparametric data). All data were analyzed using Graph Pad Prism 4 software. Due to multiple comparisons across 4 groups and 11 locations of lymphocyte subsets, we set an arbitrary P value of <.01 as the level of significance.

RESULTS

As shown in Table 1, there was no significant difference in age or sex between study groups. No correlation was found between participant age or sex and T- and B-cell subsets, except that PI-FGID females had higher levels of CD3 IELs (16 [13–23] vs 11 [8–19]; P = .002) and lower Lpc CD8 (245 [182–349] vs

Table 1. Patient Characteristics by Study Group

Characteristic	Chronic Giardiasis (n = 28)	PI-FGID (n = 66)	Recovered Controls (n = 19)	Healthy Controls (n = 16)	*P* Value
Age, y, median (range)	27 (19–48)	28 (18–51)	29 (22–45)	36 (22–56)	NS[a]
Female sex, No. (%)	17 (61)	40 (61)	9 (48)	11 (69)	
Time since onset of giardiasis, mo, median (range)	6 (3–14)	10 (3–19)	15 (12–19)	NA	< .0001[a]
Duodenal inflammation, No. (%)					
Macroscopic	5 (17.9)	4 (6.1)	0	0	.05[b]
Microscopic	24 (85.7)	19 (28.8)	3 (15.8)	0	< .0001[b]
Symptom score	(n = 22)	(n = 50)	(n = 19)	(n = 16)	
Abdominal pain/discomfort	8 (5–8)	5 (3–8)	0 (0–2)	0	< .0001[a]
Nausea	7 (3–8)	3 (1–6)	0 (0–1)	0	< .0001[a]
Bloating	8 (5–9)	7 (4–8)	2 (0–4)	1 (0–2)	< .0001[a]
Diarrhea	8 (5–10)	6 (3–8)	0 (0–1)	0	< .0001[a]
Constipation	1 (0–3)	1 (0–4)	0 (0–1)	1 (0–1)	.03[a]

Symptom data are shown as median (interquartile range).

Abbreviations: NA, not applicable; NS, not significant; PI-FGID, postinfectious functional gastrointestinal disorder.

[a]Kruskal–Wallis test across 4 groups.

[b]Fisher's exact test.

328 [282–425]; $P = .001$). However, there were no differences in CD3 IEL levels between the 4 study groups.

Microscopic inflammation was seen in a high proportion of patients with CG (85.7%), some of the patients with PI-FGID (28.8%), and a few of the RCs, but not among HCs (Table 1). Twenty PI-FGID patients tested for *H. pylori* were all negative.

Abdominal Symptoms

There were significant differences in abdominal symptoms between the groups (Table 1). Abdominal pain and discomfort, diarrhea, and bloating were the most common symptoms in the patients with CG and PI-FGID. Only diarrhea found to have a significantly higher score among CG cases compared to PI-FGID ($P = .009$). There were few symptoms among recovered and healthy controls. Females scored significantly higher than males for nausea ($P < .0001$) and abdominal pain ($P = .005$) in all 3 *Giardia*-exposed groups.

Of 66 PI-FGID patients, 42 (64%) completed a ROME II form between October 2005 and April 2007, at median illness duration of 19 months (range, 12–34 months). Thirty-seven of 42 (56%) fulfilled criteria for IBS, 3 of 42 (5%) had functional dyspepsia (FD), 5 of 42 (8%) had both FD and IBS, and 2 patients (3%) had functional abdominal bloating. Subtyping of the 37 PI-IBS patients revealed 54% IBS-A, 35% IBS-D, and 11% IBS-C.

Correlation Between T and B Cells and Illness Duration

In the preliminary analysis of lymphocyte subsets, we saw gradual changes over time in the PI-FGID and RC groups. In the PI-FGID group, illness duration was positively correlated with Lpc CD4 cells ($P = .0005$, Spearman $r = 0.4$) and with Lpc CD8 cells ($P = .005$, Spearman $r = 0.3$). Illness duration in RC correlated positively with Lpv CD4 cells ($P = .005$, $r = 0.6$), and Lpc CD4 cells ($P = .0002$, $r = 0.8$). We therefore divided the PI-FGID and RC groups into subgroups of 4-month intervals with regard to onset of symptoms.

In patients with chronic giardiasis, we found a significant correlation between illness duration and a small gradual increase in Lpc CD8 cells ($P = .005$, Spearman $r = 0.5$). The CD3, CD4, or B-cell populations were not altered over time in CG.

Macroscopic and Microscopic Inflammation

Macroscopic duodenitis was not common in the participants (Table 1). Enough patients were available in the PI-FGID group with illness duration 3–10 months to assess duodenal inflammation grade and lymphocyte populations. Histologically normal duodenal biopsies in PI-FGID had lower CD20 cell counts in both villus (0 [0–5] vs 25 [5–43]; $P = .0001$) and crypt (16 [12–26] vs 37 [25–42]; $P = .0002$) than those with microscopic inflammation (Figure 2).

The same trend was seen in the PI-FGID patients with illness duration 11–19 months, in RCs, and in the CG group (Supplementary Table 2). No significant differences in CD4 and CD8 cell counts between histologically normal and inflamed biopsies were found.

Intraepithelial Lymphocytes

When compared to healthy controls, there were no significant differences in CD8 IELs between the 3 *Giardia*-exposed groups (Supplementary Table 1). However, CD4 IELs were significantly increased in chronic giardiasis compared to healthy controls, as shown in Figure 3. Somewhat surprisingly, there was a dip toward normal levels in the PI-FGID group at 7–10 months of illness duration, with a later increase. The same development was seen in RCs with significantly higher levels of CD4 IELs at 15–19 months compared to HCs.

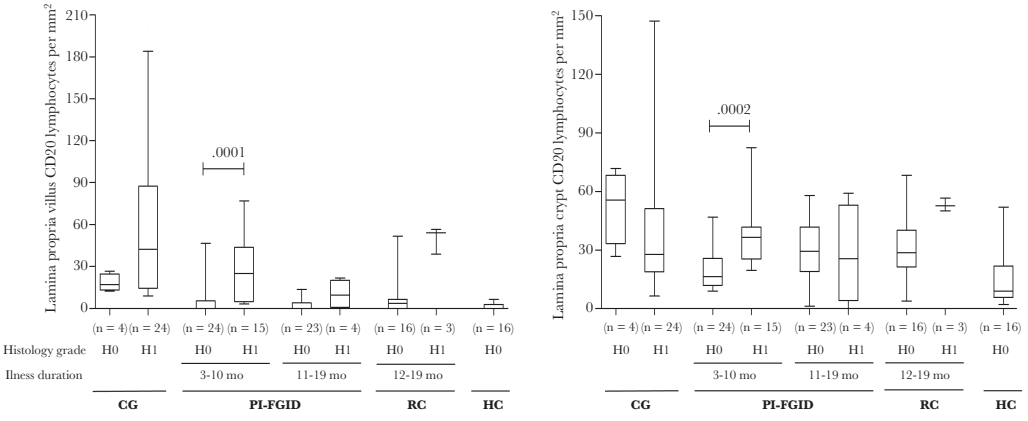

Figure 2. Duodenal lamina propria villus and lamina propria crypt CD20 cells in biopsies with normal histology (H0) and histological inflammation (H1) in patients with chronic giardiasis, those with PI-FGID, recovered controls, and healthy controls. Values above horizontal whiskers are *P* values for comparisons between groups.

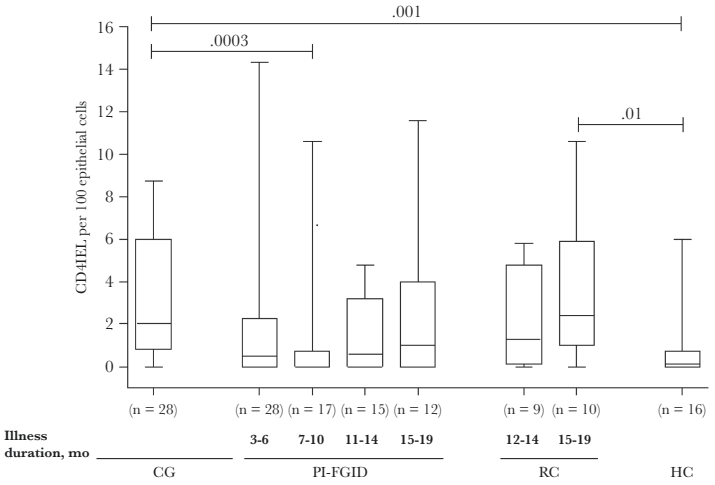

Figure 3. Duodenal CD4 intraepithelial lymphocytes (IELs) in patients with chronic giardiasis (CG), those with postinfectious functional gastrointestinal disorder (PI-FGID), recovered controls (RC), and healthy controls (HC). Values above horizontal whiskers are *P* values for comparisons between groups.

Using 30 IELs per 100 epithelial cells as an upper limit of normal, CD3 IELs were found to be elevated in only 1 patient with chronic giardiasis, 2 with PI-FGID, and 2 HC subjects.

Lamina Propria T Cells

In patients with chronic giardiasis, the number of Lpc CD4 T cells was significantly lower (181 [144–226]) compared to HCs (323 [250–376]) ($P < .0001$) (Figure 4 and Supplementary Table 2). PI-FGID patients with recent *Giardia* infection had low levels of Lpc CD4 cells, similar to CG, but a gradual increase was observed in patients as illness lasted ≥11 months. At 15–19 months it reached the same level as HCs and was significantly increased compared to previous months.

The same pattern was observed in RCs where Lpc CD4 cells at 12–14 months of illness duration were significantly lower compared to HCs ($P = .0004$), but rose to normal levels from 12–14 months to 15–19 months (190 [138–216] vs 321 [254–405]; $P = .0009$).

Lpv CD4 T cells showed were very variable in CG, but decreased after eradication of the parasite, and then gradually increased in both PI-FGID and RC groups (Supplementary Table 1).

We did not observe any significant differences between the 4 study groups regarding CD8 cells in crypts or villus.

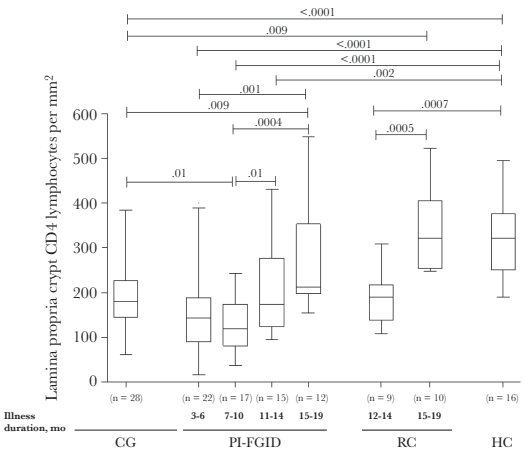

Figure 4. Duodenal lamina propria crypt lymphocytes in patients with chronic giardiasis (CG), those with postinfectious functional gastrointestinal disorder (PI-FGID), recovered controls (RC), and healthy controls (HC). Values above horizontal whiskers are *P* values for comparisons between groups.

Duodenal Lamina Propria B Cells

All 3 *Giardia*-exposed groups had significantly higher number of CD20 B cells in Lpv and Lpc lymphocytes compared with healthy controls (Figure 5).

The CD20 cell counts in the PI-FGID group was lower than in CG, decreased somewhat over time, and became more similar to HC levels. Recovered controls had significantly higher levels of levels of CD20 B cells in both villus and crypt lymphocytes compared to HCs.

Repeated Duodenal Biopsies

In paired samples from the same patient from 2 time points, we found a significant increase in Lpc CD4 cells (from 136

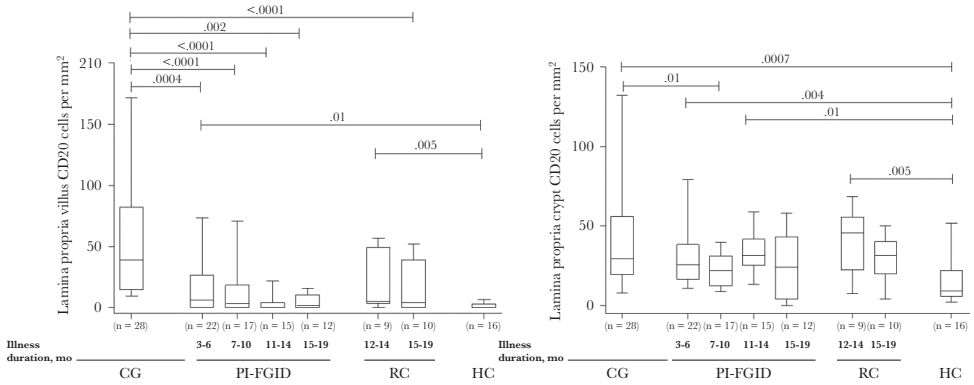

Figure 5. Duodenal lamina propria villus and lamina propria crypt CD20 lymphocytes in patients with chronic giardiasis (CG), those with postinfectious functional gastrointestinal disorder (PI-FGID), recovered controls (RC), and healthy controls (HC). Values above horizontal whiskers are *P* values for comparisons between groups.

[118–193] to 227 [199–371]; $P = .01$) and CD8 cells (from 219 [118–271] to 389 [275–434]; $P = .01$) (Figure 6).

Otherwise, there were no significant differences in duodenal intraepithelial or lamina propria cell counts for CD3, CD4, CD8, or CD20 lymphocytes in these 11 patients.

Correlation Between Duodenal EC Cells and T Cells

A previous study showed that EC cells were reduced in the PI-FGID group (n = 19) compared with the RC group (n = 19). When correlating lymphocyte data with available EC cell data (n = 38), we found EC cells to positively correlate with CD4 IELs ($P = .01$, Spearman $r = 0.4$) and a tendency for correlation with Lpc CD4 cells ($P = .02$, Spearman $r = 0.4$). Looking at the PI-FGID group only (n = 19), there was a positive correlation between EC cell numbers and both Lpv CD4 cells ($P = .01$, Pearson $r = 0.6$) and Lpc CD4 cells ($P = .008$, Pearson $r = 0.6$).

DISCUSSION

In the present study, we identified alterations in duodenal mucosal lymphocyte subsets during symptomatic chronic giardiasis, as well as prolonged differences in these subsets after successful treatment. Importantly, we did not observe important differences in these major lymphocyte populations between patients who did and those who did not develop long-term PI-FGID symptoms. We found increased frequency of CD4 IELs in chronic giardiasis that was sustained over time, especially in recovered asymptomatic patients, but also in patients with PI-FGID. In the lamina propria, CD4 T-cell numbers were high in the villus, but low in the crypt in chronic giardiasis. In both the PI-FGID and RC groups, lamina propria crypt CD4 cells and CD8 cells gradually increased to normal levels >1 year after giardiasis symptoms started. Compared with healthy

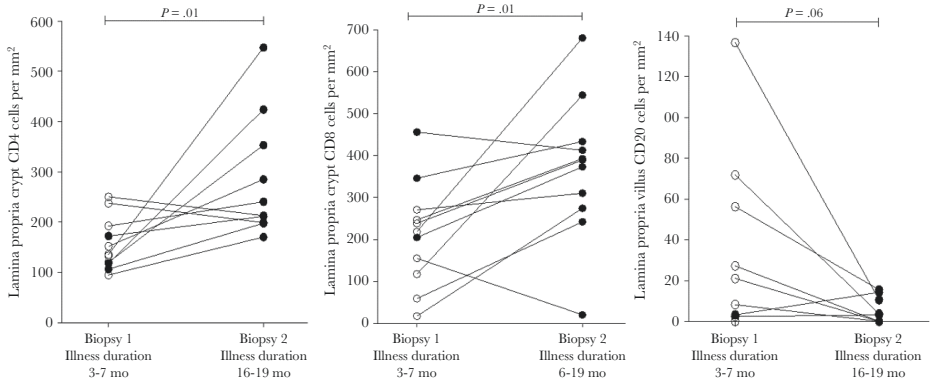

Figure 6. Duodenal lamina propria crypt CD4 cells, lamina propria crypt CD8 cells, and lamina propria villus CD20 cells in biopsy 1 (illness duration between 3 and 7 months) and biopsy 2 (illness duration between 16 and 19 months) in 11 *Giardia*-positive (○) and *Giardia*-negative (●) patients.

controls, lamina propria CD20 cells were elevated during the whole follow-up period in all 3 *Giardia*-exposed groups.

To our knowledge, this is the first study describing duodenal mucosal intraepithelial and Lpv and Lpc lymphocyte kinetics over 1.5 years in patients with and without persistent abdominal symptoms after gastroenteritis with a confirmed intestinal pathogen. A rigorous interpretation of the data was possible due to inclusion of groups with prolonged illness as well as recovered controls exposed to the same pathogen.

Inflammation in Chronic Giardiasis

Previous studies of giardiasis have concluded that there were no specific histological changes in patients with symptomatic giardiasis except slightly increased CD3 IELs [22, 23]. Only rarely there are signs of inflammation and villous flattening [24].

In our study, only a few subjects had increased CD3 IEL counts >30. However, many of the *Giardia* positive cases (by stool examination) presented in this study had duodenal inflammation, but *Giardia* trophozoites could only be identified above the duodenal mucosa in 2 of 28 cases. It is known that some patients may present with a more profound inflammatory reaction to the *Giardia* infection such as this. Recently a similar set of 10 giardiasis cases was reported with inflamed, but parasite-free, duodenal mucosa, but uninflamed ileal mucosa with discernible trophozoites above it [25].

Intestinal T-Cell Alterations

Our results show that duodenal CD4 T cells can be decreased for many months after giardiasis. Most studies of intestinal lymphocytes after gastroenteritis have investigated colonic or rectal mucosa [9]. In patients with persistent abdominal symptoms after *Campylobacter* or *Shigella* infection, an increased count of EC cells and lamina propria T cells have been found in the large intestine when compared with HCs [26–28]. The only study of duodenal lymphocytes in PI-FGIDs patients is a small study of 12 patients with PI-FD of unknown microbial etiology and 12 patients with unspecified FD, and no HCs [29]. A reduced number of intravillar CD4 cells in PI-FD was found. Their conclusion was that PI-FD patients showed an impaired ability of the immune system to terminate the inflammatory response after the acute insult. The long duration of recruitment into our study, and inclusion of a recovered control group exposed to the same infection, allows a better interpretation of the findings. As lower CD4 cell frequencies are present, and gradually increasing, in both the PI-FGID and RC groups, this finding seems to be a prolonged effect of the inflammatory response to the gastroenteritis, rather than associated with the presence of PI-FGID symptoms.

Interestingly, a recent mouse study found increased levels of anti-inflammatory cytokine interleukin 10 (IL-10) to be important in controlling *Giardia*-induced T-cell responses both in the small and large intestine [30]; thus, the prolonged lower T-cell count observed in the present study could be induced via an IL-10–dependent pathway. That mouse study also revealed the possibility that individuals with *Giardia*-induced duodenal inflammation could also have some degree of concomitant colitis. Further research is needed to examine whether this may occur, and if it could trigger a longer-term dysfunction and development of FGID.

Duodenal Lamina Propria B Cells

Antibody-producing B cells have an important role in adaptive immune response and are important in clearing *Giardia* infection [11, 13]. All *Giardia*-exposed participants had elevated CD20 B-cell counts in both Lpv and Lpc compared to healthy controls. CD20 B cells in the lamina propria mainly represent resident local memory B cells, and not plasma cells as these are CD20 negative [31]. These cells are likely to be part of the acquired immune response against *Giardia* observed in epidemiological studies [32].

There was a clear trend of gradual reduction over time of CD20 cells in the villi. However, in crypts, this trend was absent, and the increase was still present 15–19 months after initial infection. Separate counting of the 2 compartments avoided masking these differences.

Mucosal jejunal CD20 B cells have been found moderately elevated in a Spanish study of patients with IBS-D compared to healthy controls [10]. This cohort may have included PI-IBS patients, as IBS-D is a subtype commonly seen after gastroenteritis.

Surprisingly, there was a tendency for the RC group's Lpv B cells not to normalize as well as in the PI-FGID group at 12–19 months. An explanation for this might be that 6 of these 19 RCs had markedly elevated villus B cells compared to the rest of the group, indicating potential intercurrent recent enteric infection.

Duodenal EC Cells

EC cells are dispersed throughout the gut and are the main source of serotonin (5-HT). It has been shown previously that secretory products from CD4 T cells interact with EC cells to enhance the production of 5-HT in the gut via Th2-based mechanisms [33]. Our previous finding of lower number of EC cells that is now found to be associated with a prolonged dip in CD4 cells in the PI-FGID group could indicate a temporarily lower 5-HT production after *Giardia* infection.

Limitations and Strengths

The main strength of this study is the relatively large number of patients in the CG and PI-FGID groups and the long inclusion time, allowing a description of the kinetics of mucosal lymphocyte populations. A similar development in the 11 cases with repeated biopsies supported the finding in individual cases over time. The inclusion of *Giardia*-exposed RCs as well as HCs allowed results to be interpreted with more certainty.

There were some limitations of this study. We were not able to determine exactly when patients became *Giardia* negative after treatment, as many were referred after several courses of

metronidazole due to their prolonged symptoms. We also were not able to collect symptoms scores at the time of biopsy or ROME II follow-up forms from all patients in the CG and PI-FGID groups. Analysis of differences in lymphocyte counts according to histologic inflammation suffered from a low number of cases except in the PI-FGID group with symptom duration of 3–10 months.

Conclusions

Chronic symptomatic *Giardia* infection is associated with elevated CD4 IELs and with elevated B cells and decreased CD4 T cells in the duodenal lamina propria. In patients with PI-FGID symptoms after giardiasis and in RCs the same pattern was seen for more than a year before CD4 T cells were normalizing to levels seen in HCs. The findings implicate a cautionary approach to studies of PI-FGID not including a group of RCs. Further investigations into subsets and activation status of the prolonged alterations of mucosal lymphocyte subsets are warranted and may reveal clues for elucidating the pathogenesis of PI-FGID.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Supplementary material

Dizdar et al, 2018. "Prolonged duodenal mucosal lymphocyte alterations in patients with and without post infectious functional gastrointestinal disorders after *Giardia* infection"

Table S1. Lymphocyte counts per mm^2 (median (IQR)) in duodenal mucosa per study group, with the FGID and RC groups subdivided by illness duration. IEL = Intraepithelial lymphocytes, Lpv = Lamina propria villus, Lpc = Lamina propria crypt, CG= Chronic giardiasis, FGID = Functional gastrointestinal disorder, RC = Recovered controls, HC = Healthy controls, mo = month, IQR = interquartile range.

	CG (n=28)	**FGID** 3-6 mo (n=22)	**FGID** 7-10 mo (n=17)	**FGID** 11-14 mo (n=15)	**FGID** 15-19 mo (n=12)	**RC** 12-14 mo (n=9)	**RC** 15-19 mo (n=10)	**HC** (n=16)	*P value**
IEL CD3	16 (13-21)	17(10-23)	14 (9-21)	16 (10-22)	16 (10-18)	17 (15-23)	21 (17-27)	18 (11-25)	ns
CD3 Lpv	508 (376-718)	382 (316-482)	408 (355-573)	414 (343-481)	444 (359-536)	413 (362-674)	547 (416-665)	438 (345-609)	ns
CD3 Lpc	406 (338-534)	419 (367-535)	450 (382-542)	600 (420-717)	551 (443-745)	582 (372-633)	671 (536-780)	625 (459-736)	0.0003
IEL CD4	2 (1-6)	1 (0-2)	0 (0-1)	1 (0-1)	1 (0-4)	1 (0-5)	2 (1-6)	0 (0-1)	0.002
CD4 Lpv	289 (164-319)	211 (92-248)	156 (97-210)	164 (91-250)	277 (203-355)	155 (133-276)	350 (308-410)	223 (210-272)	<0.0001
CD4 Lpc	181 (144-226)	144 (90-188)	119 (81-174)	174 (123-277)	213 (198-353)	190 (138-216)	321 (254-405)	323 (250-376)	<0.0001
IEL CD8	10 (7-15)	9 (5-15)	9 (7-15)	9 (8-19)	9 (5-18)	4 (3-11)	9 (4-14)	13 (7-20)	ns
CD8 Lpv	236 (157-345)	220 (163-280)	215 (163-277)	227(196-333)	207 (172-261)	320 (239-378)	233 (188-302)	277 (155-313)	ns
CD8 Lpc	240 (132-312)	235 (156-307)	315 (218-341)	298 (250-428)	381 (256-429)	281 (235-360)	210 (189-344)	330 (207-403)	0.02
CD20 Lpv	39 (15-82)	6 (0-26)	4 (0-18)	0 (0-4)	2 (0-11)	5 (3-49)	4 (0-39)	0 (0-3)	<0.0001
CD20 Lpc	29 (19-56)	26 (17-38)	22 (12-31)	32 (25-42)	24 (4-43)	46 (22-56)	32 (20-40)	9 (7-22)	0.005

* Kruskal Wallis test across all groups

Table S2: Duodenal T and B cell counts per mm^2 (median (IQR)) sub-grouped in relation to histology grade (H0 and H1). IEL = Intraepithelial lymphocytes, Lpv = Lamina propria villus, Lpc = Lamina propria crypt, CG= Chronic giardiasis, FGID = Functional gastrointestinal disorder, RC = Recovered controls, HC = Healthy controls.

	CG (n=28)		**PI-FGID** 3-10 months (n=36)		**PI-FGID** 11-19 months (n=57)		**RC** 12-19 months (n=19)		**HC** (n=16)
	H0 (n= 4)	**H1** (n= 24)	**H0** (n=24)	**H1** (n=15)	**H0** (n=23)	**H1** (n=4)	**H0** (n=16)	**H1** (n=3)	**H0** (n=16)
IEL CD3	15 (12-22)	19 (13-21)	13 (9-21)	20 (14-23)	17 (11-19)	8 (2-20)	18 (16-26)	20 (19-26)	18 (11-25)
CD3 Lpv	460 (380-555)	544 (376-756)	358 (312-469)	439 (386-564)	462 (367-538)	362 (82-422)	475 (371-655)	429 (413-690)	438 (345-609)
CD3 Lpc	532 (428-697)	368 (328-508)	486 (376-580)	402 (380-458)	603 (475-719)	322 (77-740)	614(528-736)	329 (211-625)	625 (459-736)
IEL CD4	4 (1-6)	2 (1-6)	0.1(0-1)	1 (0-3)	0 (0-3)	3 (1-10)	2 (0-5)	4 (3-6)	0 (0-1)
CD4 Lpv	316 (169-336)	279 (164-312)	158 (83-221)	209 (97-282)	202 (150-269)	221 (77-375)	308 (179-377)	216 (155-278)	223 (210-272)
CD4 Lpc	180 (155-303)	181 (134-226)	134 (98-183)	119 (58-188)	206 (168-280)	199 (99-389)	254 (192-327)	187 (158-216)	323 (250-376)
IEL CD8	13 (4-23)	10 (7-15)	9 (6-14)	7 (5-15)	9 (8-18)	6 (3-20)	9 (4-13)	3 (3-4)	13 (7-20)
CD8 Lpv	268 (185-317)	228 (154-371)	219 (163-255)	237 (173-300)	227 (179-279)	211 (189-251)	282 (214-372)	225 (153-239)	277 (155-313)
CD8 Lpc	310 (238-375)	212 (123-288)	295 (219-352)	216 (135-315)	327 (261-434)	312 (211-388)	288 (207-358)	198 (186-231)	330 (207-403)
CD20 Lpv	17 (13-25)	42 (15-87)	0 (0-5)	25 (5-44)	0 (0-4)	10 (1-20)	4 (0-7)	54 (39-57)	0 (0-3)
CD20 Lpc	56 (33-68)	28 (19-51)	16 (12-26)	37 (25-42)	29 (19-42)	26 (4-53)	29 (21-40)	53 (50-57)	9 (7-22)

Histological grading examples

Biopsy results were divided into two groups; normal histology (grade H0), mild pathology and moderate to severe pathology (grade H1). These biopsies showed inflammation with oedema and infiltration of leukocytes and increased number of plasma cells in the lamina propria with or without architectural distortion in the form of shortening and blunting of intestinal villi.

Examples of histological grading of hematoxylin-eosin stained biopsies are shown below.

Figure S1. Grade H0 – normal histology
Female, 30 years old: Symptom duration 9 months. No signs of mucosal inflammation. Giardia trophozoites seen above the mucosa.

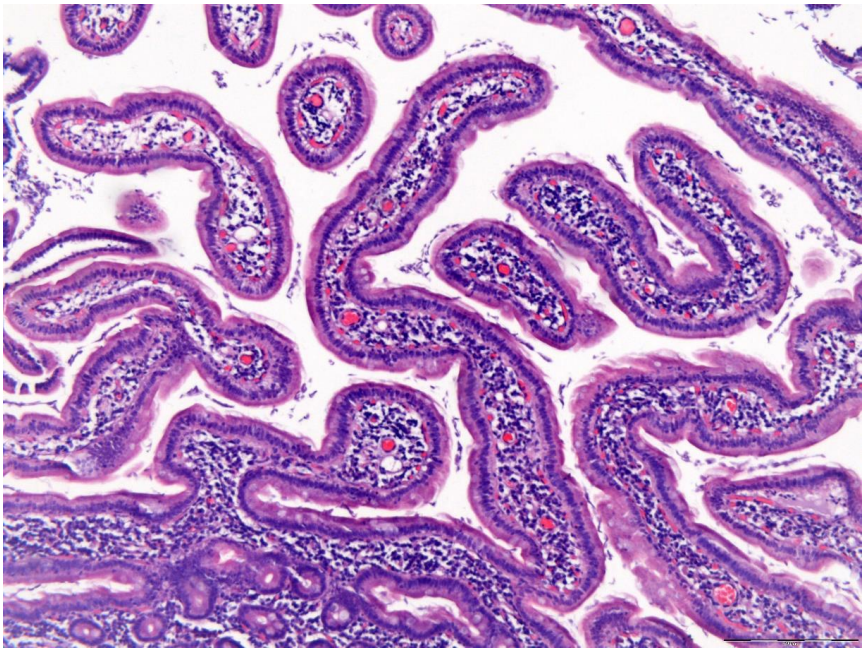


Figure S2. Grade H1 – mild pathology
Female, 47 years old. Symptom duration 8 months. Focal mild active inflammation and increased intraepithelial lymphocytes. Giardia not seen above mucosa, nor in stool samples.

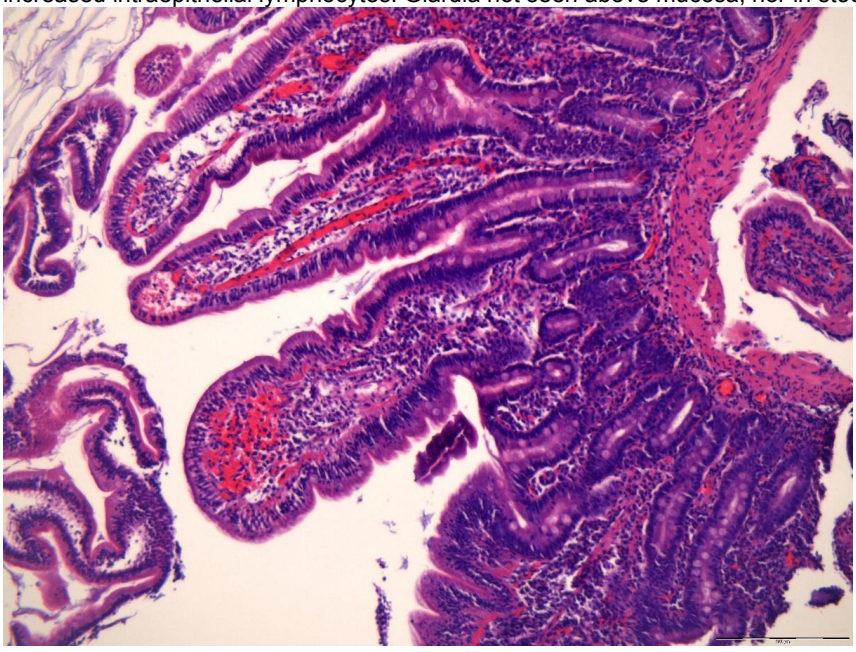


Figure S3. Grade H1 – moderate to severe pathology
Female 31 years old. Symptom duration 4 months. Moderate chronic active inflammation and villous shortening. Giardia not seen. However, stool samples were positive for giardia.

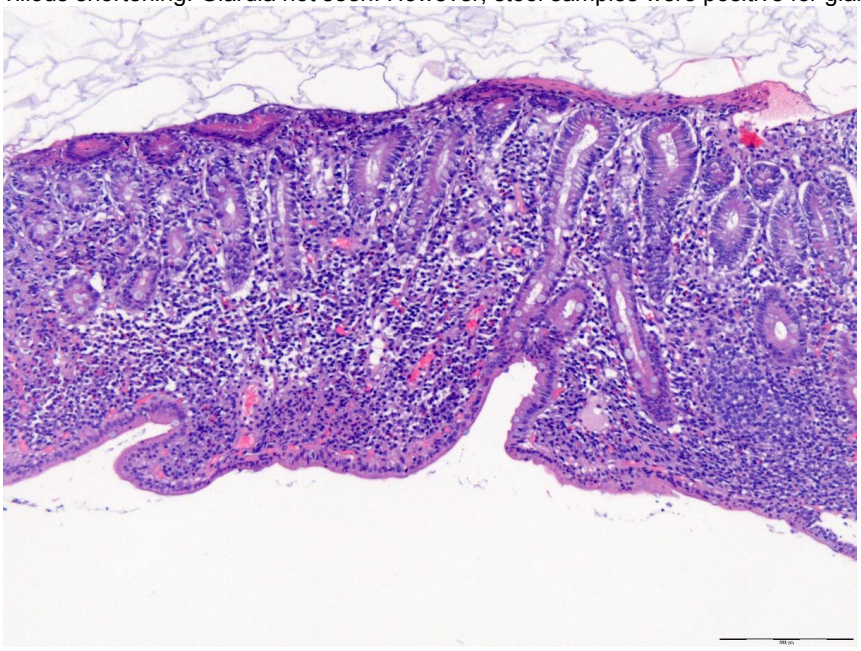
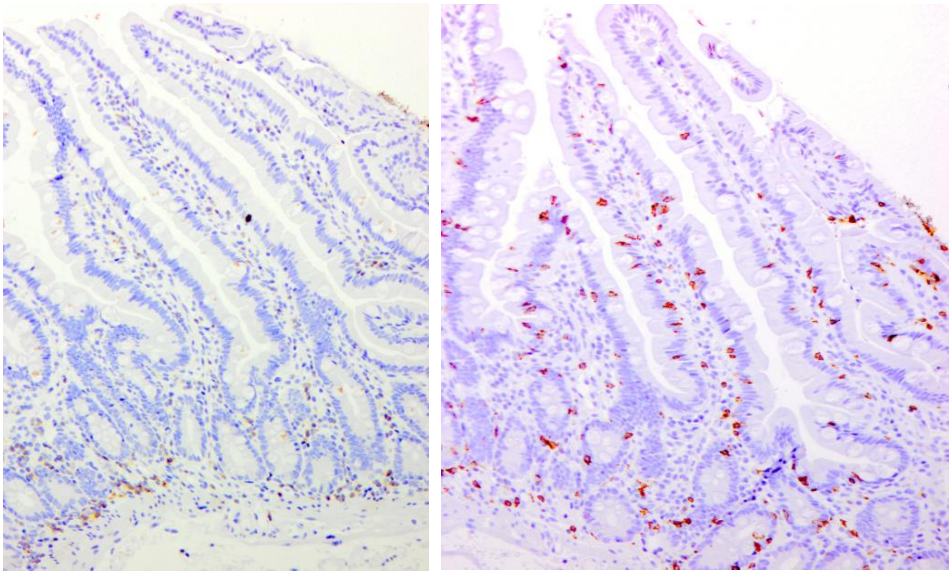
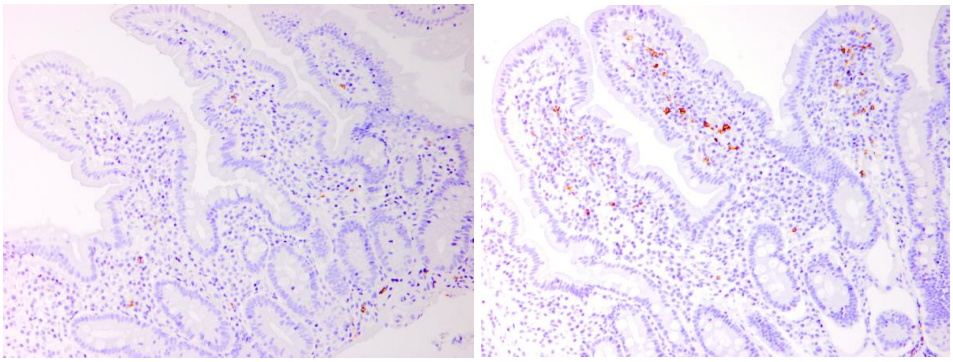


Figure S4. Immunohistochemistry staining of CD4 and CD8 lymphocytes in duodenal mucosa



CD4 CD8

Figure S5. Immunohistochemistry staining of CD20 lymphocytes in duodenal mucosa



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