Post-giardiasis functional gastrointestinal disorders and chronic fatigue syndrome

– clinical symptoms, inflammation and immune responses

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Scientific environment

This study was performed at the Institute of Medicine, University of Bergen and the Department of Medicine, Haukeland University Hospital.

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Bergen, September 2011
Kurt Hanevik

ABBREVIATIONS

CD – Cluster of differentiation

CD4 T-cell – Lymphocyte also called a helper T-cell.

CD8 T-cell – Lymphocyte also called suppressor/cytotoxic T-cell.

CFS – Chronic fatigue syndrome

CF – Chronic fatigue

CMI – Cellular mediated immunity

EC-cell – enterochromaffine cell

FD – functional dyspepsia

FGID – functional gastrointestinal disorder

IBS – irritable bowel syndrome

IBS-D - diarrhea predominant irritable bowel syndrome
IBS-C - constipation predominant irritable bowel syndrome
IBS-A - alternating irritable bowel syndrome

ICF – Idiopathic chronic fatigue

IFNγ – Interferon gamma

MHC class – Major histocompatibility class

NK cell – natural killer cell

PBMC – peripheral blood mononuclear cells

PI-CFS – post-infectious chronic fatigue syndrome

PI-FGID – post-infectious functional gastrointestinal disorder

PI-IBS – post-infectious irritable bowel syndrome

TNFα – Tumor necrosis factor alpha

LIST OF PAPERS

Paper I: Hanevik K, Hausken T, Morken MH, Strand EA, Mørch K, Coll P, Helgeland L, Langeland N. Persisting symptoms and duodenal inflammation related to *Giardia duodenalis* infections. J Infect. 2007 Dec;55(6):524-30.

Paper II: Hanevik K, Dizdar V, Langeland N, Hausken T. Development of functional gastrointestinal disorders after *Giardia lamblia* infection. BMC Gastroenterology 2009, 9:27 doi:10.1186/1471-230X-9-27

Paper III: Hanevik K, Kristoffersen E, Svard S, Bruserud O, Ringqvist E, Sørnes S, Langeland N. Human cellular immune response against *Giardia lamblia* five years after acute giardiasis. J Infect Dis. 2011, in press

Paper IV: Hanevik K, Kristoffersen E, Sørnes S, Mørch K, Rivenes AC, Bødtker J, Næss H, Svard S, Bruserud O, Hausken T, Langeland N. Immunophenotyping and *Giardia* specific immunity in post-giardiasis functional gastrointestinal disease and chronic fatigue syndrome. Submitted.

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1. INTRODUCTION

1.1 *Giardia* outbreak in Bergen

In Norway, *Giardia* infection is most frequently seen in returning travellers, and testing for this parasite is seldomly performed in cases without a travel history. From early October 2004 there was a two-fold increase in gastroenteritis cases at Bergen legevakt (emergency health centre) [1]. At the end of this month an increase was observed in the number of *Giardia* positive faecal samples at the laboratory for infectious diseases at Haukeland University hospital. The 3rd of November the main newspaper in Bergen brought the news of a *Giardia* outbreak. Investigations revealed sewage contamination into Svartediket, an open water reservoir supplying water to around 43000 inhabitants in the city centre [2]. A surplus of 2500 treatments of metronidazole was prescribed during the outbreak period. 1262 outbreak related *Giardia* positive samples were recorded at the laboratory for infectious diseases [3]. Especially young adult women aged 20-29 years were affected by the outbreak, most probably due to water drinking habits in this group. There were few children and elderly [2].

1.2 *Giardia lamblia* and giardiasis

1.2.1 *Giardia* biology

The *Giardia* species are unicellular flagellated eukaryotic microorganisms found to infect many animal species including amphibians, birds and mammals. The species infective to humans is called *Giardia lamblia* (syn. *G. intestinalis*, *G. duodenalis*) while the close relative *G. muris* infects mice and is used in research models of the disease [4].

G. lamblia is a protozoan parasite with two nuclei, eight flagellae and a unique feature; an adhesive disk with which it may adhere to surfaces like the intestinal wall or a cell culture tube. It has two major stages in its living cycle, the vegetative trophozoite (figure 1) and a relatively inert cyst form. The cyst is the infective form, which in the proximal intestine

excysts into the vegetative trophozoite which replicates and lives in the intestinal lumen [5, 6].

Phylogenetic studies have identified seven *G. lamblia* assemblages (A to G) [7] and emerging evidence show variable grades of host specificity. Genotype A and B are known to infect man, with genotype A being the least host specific and some degree of zoonotic transmission may occur [8]. The relatively large genetic differences detected between assemblage A and B, suggest they rather should be considered as two different species [9]. In Norway genotype A is the most prevalent according to a survey of sewage influent performed in 2003 [10].

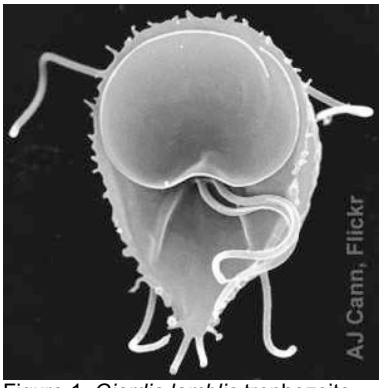


Figure 1. *Giardia lamblia* trophozoite

1.2.2 Clinical presentation and epidemiology

Giardia lamblia is the aetiological agent of giardiasis and was first observed in 1681 by van Leeuwenhoek [11], the inventor of the microscope. *Giardia* infection may cause acute or chronic diarrhea in humans, sometimes with severe disease and malabsorbtion, but may also pass unnoticed by the host as an asymptomatic infection [12]. The symptom picture commonly include diarrhea, abdominal discomfort, flatulence, nausea, weight loss and lassitude [12]. Malabsorbtion of fat and vitamin A [13], as well as vitamin B12 (cobalamin) deficiency has been described [14]. In more prolonged, repeated or severe infections, malnutrition has been observed [15-17] and sequels in the form of retarded growth and development [18, 19] and poor cognitive function [15] have been reported.

Prevalence rates vary from 2-5% in the industrialized world [20], to 20-30% in developing countries, where children are more often infected than adults [21]. The prevalence of *Giardia* infection in Denmark, Finland, Norway and Sweden has been estimated to 3% in the asymptomatic adult population, and 6% in the population with abdominal symptoms [22]. In

Norway around 50 persons per year are infected, most of them abroad [23]. In studies from industrialised countries like New Zealand and USA [24, 25] the incidence shows a bimodal pattern, peaking in the 1-4 years and 30-40 years age groups. The incidence in the youngest age group is doubled compared the other age groups, see figure 2. The parasite is a commonly identified pathogen in waterborne outbreaks [25] and has a global distribution.

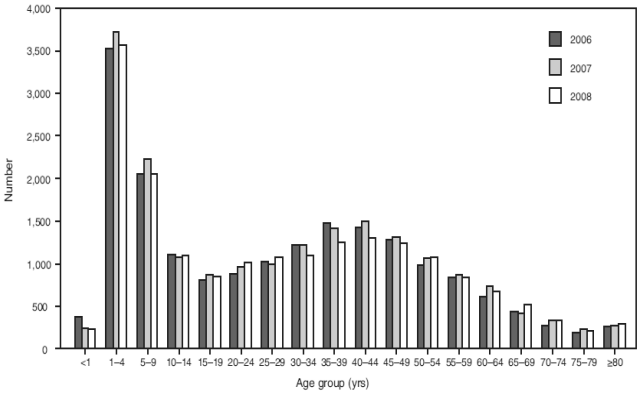


Figure 2. Giardiasis cases (N=58186) in the US between 2006 and 2009 by age group and year. From Yoder et al. 2010

1.2.3 Parasite and host interaction

Until relatively recently the status of *Giardia* as a pathogen was disputed [26]. Former professor Johs Bøe at Haukeland University hospital were in his PhD dissertation in 1941 very skeptical towards the pathogenicity of *Giardia*, but later in 1943 he published 5 pediatric cases that had substantially improved after anti-*Giardia* treatment with atebrine [27]. Although *Giardia* is now recognized as a pathogen that may cause a distinctive disease, the underlying mechanisms by which *Giardia* cause disease is still poorly understood [28].

G.lamblia is a lumen dwelling, non-invasive parasite, and giardiasis symptoms may occur without overt villous atrophy or other signs of mucosal injury [29, 30]. *Giardia* infection may induce diffuse shortening of epithelial microvilli and this together with reduced disaccharidase cause malabsorbtion and maldigestion [31]. In previously infected gerbils the disaccaridase deficiencies occur even when rechallenged with only *Giardia* antigen extracts [32] suggesting an immunologic mechanism that was shown to involve CD8 T-cells in mice [33]. In a rat model small intestinal injury have been shown to be strain dependent [34]. The parasite may

also induce chloride secretion in human cells [31]. The combination of malabsorbtion of sugars, hypersecretion of electrolytes and small intestinal hypermotility [35] are probably the most important mechanisms for diarrhea in *Giardia* infection.

The pathophysiology in some cases involves disruption of the epithelial tight junctions and increased intestinal permeability [36]. In 13 chronic giardiasis cases the loss of epithelial barrier function was shown to be due to down regulation of the tight junction protein claudin 1 and increased enterocyte apoptosis [31]. The resulting increased exposure of any lumen antigens, including *Giardia* antigens, most probably play a role in host immune response and symptomatology [28].

The large variability of clinical illness is likely to be due to differences both in the parasite and host immune responses [37, 38] as well as previous exposure to this pathogen [39]. There are no virulence factors or toxins known. A review of studies investigating the association between *Giardia* genotypes and symptomatology show inconsistent results suggesting that probably several other factors than genotype are also important for symptom development [40].

Reports on duodenal inflammation in humans with giardiasis also show variable results. In a large Austrian study of 462 patients with *Giardia* parasites above the duodenal mucosa, only 3.7% had microscopic duodenitis [29]. Another study found some degree of villous shortening or inflammation in 13 out of 17 patients [41]. A study of 32 duodenal biopsies from children with giardiasis showed villous atrophy in only one child and no signs of intraepithelial lymfocytosis. However, there were 35% showing eosinophilic infiltration and lymph follicle formation in the lamina propria [42].

1.2.4 *Giardia* immunity and immune responses

In most humans, *Giardia* parasites are usually cleared within few weeks [43]. However, in some individuals the infection may persist in immunocompetent individuals [44] who may, or may not, have symptoms from the infection. The infection is common and can be persistent

in immunological disturbances like common variable immunodeficiency (CVID) [45] or IgA-deficiency [46].

Like many other protozoan parasites *G. lamblia* possesses the ability to change its surface proteins in order to evade its host immune system. By a RNA interference machinery it is able to express one variant surface proteins (VSP) at a time from a large repertoire of different VSP genes [47, 48].

In humans, the adult population is less affected than children and specific antibodies against *Giardia* are found in populations in endemic areas [21, 49, 50]. A significantly lower serum IgG and IgA has been reported in Indian children with acute and persistent giardiasis, whereas asymptomatic carriers had levels comparable to healthy controls [51]. Children with persistent giardiasis, despite appropriate chemotherapy, had lower concentrations of *Giardia* membrane protein specific antibodies than acute and asymptomatic cases. Thus, the poor ability to produce specific anti-*Giardia* immunoglobulins could be a risk factor for persistent giardiasis.

The host immune responses to *acute Giardia* infection have mainly been studied in mouse models. In mice αβ-TCR-expressing T-cells are required to control infection [52] and CD4 T-cell depletion results in chronic infection [53]. CD8 T-cells seems not to be important for the control of infection in mice, but contribute to the giardiasis related intestinal mucosal injury [33]. Important roles have also been shown for mast cells and IL-6 [54], as well as for B-cell antibody production [55, 56]. An ex-vivo study of human intestinal lymphocytes stimulated by *Giardia* trophozoites showed importance of IFN-γ, probably secreted by CD4 T-cells [57]. NK-cells do not seem to have a protective role in acute *Giardia* infection, at least not in mice. Beige mice, which are deficient in natural killer cells, cleared *Giardia* infection equally fast as immunocompetent mice [58].

Residents in Creston, Canada who were infected in a waterborne *Giardia* outbreak in 1985 were significantly less likely to be re-infected during a second outbreak five years later [59]. It has also been shown that mice challenged with a second *Giardia* infection shed far less cysts than during the primary infection [60]. When becoming infected with *Giardia* for the

second and third time, fewer Brazilian children had intestinal inflammation measured by faecal lactoferrin compared to first time infections [61]. There are therefore strong indications that acquired immunity towards this non-invasive lumen dwelling parasite is important for protection in many humans. Cellular immunity has previously been described in one case six years after infection [62].

In summary, predisposition for persistent giardiasis is likely to frequently be due not to a single mechanism or host deficiency, but probably to a combination of several minor and often unknown or immeasurable anti-*Giardia* deficiencies interacting with varying degrees of acquired immunity in each individual.

1.2.5 *Giardia* diagnosis and treatment

Giardia lamblia was discovered by the inventor of the microscope [11], and microscopy still remains an important and widely used method to diagnose giardiasis. Microscopy of three stool samples has a sensitivity of 85-90% [63, 64]. There are also immunochromatographic rapid tests with similar sensitivity (81-93.5%) and specificity (<99%) [65, 66]. Alternative methods with increased sensitivity and specificity are direct fluorescence microcopy and enzyme immunoassay (EIA) kits with close to 100% sensitivity and specificity [67].

Polymerase chain reaction (PCR) methods have been mostly used for research purposes, but are slowly entering routine diagnostic facilities in large centers, sometimes coupled with other common stool parasites [68].

Treatment of giardiasis is often successful (in 60-90%) with metronidazole [69], a nitroimidazole derivate. Alternative drugs include tinidazole, quinacrine, albendazole, furazolidone and paromomycin [70].

1.3 Functional gastrointestinal disorders

1.3.1 Criteria and epidemiology

A functional gastrointestinal disorder (FGID) is characterized by recurring or chronic gastrointestinal symptoms without an identifiable disease process [71]. The disorders are hard to classify and early attempts were made in the symptom-based criteria of irritable bowel syndrome (IBS) developed by Manning et al for use in differential diagnosis within the clinical setting [72]. The more comprehensive Rome process resulted in the first Rome I criteria for IBS in 1989, and for the rest of the FGIDs in 1994. The Rome II diagnostic criteria which came in 1999 classified the adult FGIDs into oesophageal, gastroduodenal, bowel, biliary, anorectal and abdominal pain subcategories, see appendix I [73]. A continuous revision process produced a new version of these criteria in 2006 with the Rome III criteria [74, 75] subdividing IBS and functional dyspepsia (FD) in new ways.

The Rome criteria for FGIDs have an important role in standardizing research studies. However, a Norwegian study showed a large discrepancy between the criteria and clinical practice, where general practitioners diagnosed far less cases as IBS than were positive for IBS when completing a Rome II questionnaire. Care should therefore be taken when transferring research findings into clinical practice [76].

IBS and FD are the most well-known and researched FGIDs. IBS is a common disorder characterized by variable abdominal discomfort or pain that occurs in relation to changes in defecation frequency or stool consistency and is often relieved by defecation. FD is characterized by pain or discomfort in the upper abdomen which is not relieved by, or related to changes in stool frequency or form. FD and IBS may both be present in one individual and there are indications that IBS and FD share pathophysiological mechanisms, but at different sites in the gastrointestinal tract [77].

The prevalence of IBS has varied from 0.8 to 28% across population studies performed in many countries [78]. This large variation is mainly explained by use of different criteria and interpretation of these by both investigators and study participants. Adding to this,

longitudinal studies have shown that the symptoms of FGIDs are variable over time and may change from one FGID disorder to another [79]. A prevalence study using the Rome II criteria in 4662 respondents to a mailed questionnaire showed that 8% of Norwegian adults had IBS [80]. Food-related gastrointestinal symptoms are common in the general population, and often coincides with IBS. A previous study of IBS-patients found that 51% considered that their symptoms were linked to individual foods [81], and also improvement following exclusion diets have been reported [82].

1.3.2 Post-infectious FGIDs

When the onset of IBS and/or FD is associated with an acute gastrointestinal infection, it is often termed post-infectious FD or post-infectious IBS (PI-IBS) [83, 84]. This phenomenon was first described by Chaudhary and Truelove in 1962 [85] and has since been shown in numerous studies to occur following viral, bacterial and amoebic gastroenteritis and after trichinellosis [85-87]. A meta-analysis of PI-IBS estimates that the risk of having IBS one year after an acute gastroenteritis is approximately sixfold [88]. Spiller et al summarizes that studies of PI-IBS concludes that between 3.7% - 36% of gastroenteritis cases develop IBS [84].

Between 6 and 17% of IBS patients believe that their symptoms began with an infective illness [89]. A large Belgian study with 400 FD patients found 17% to be associated with acute infections, ie PI-FD [90]. PI-FD seemed to differ from spontaneous FD by showing higher frequency of impaired accommodation, being present in 66% compared with 30% in the remainder. Those with PI-FD also reported more weight loss, early satiety, and nausea. Dunlop et al also found PI-IBS to differ from spontaneous IBS by a higher frequency of diarrhea predominant IBS (D-IBS) and less constipation predominant IBS [91].

Severity of initial illness measures like symptom duration and fever, and also young age, have been found to be associated with later PI-IBS development [88]. A range of other factors such as genetic predisposition for more severe inflammation [92, 93], psychosocial

factors like anxiety somatization and negative illness beliefs [94], smoking [95], aggressiveness of offending pathogen [96], sex [86] was recently reviewed [84]. Whether age [97, 98] and antibiotics [99, 100] are independent risk factors is still not clear. The familial aggregation shown in twin studies [101], and the important role for social learning IBS [102] seen in spontaneous IBS, is still not studied for PI-IBS.

One study has found that fewer patients developing PI-IBS had previous treatment for anxiety and depression than in non-PI-IBS [91]. However, other studies have found co-morbidity with anxiety and depression and stressful life events around the time of gastroenteritis to be independent risk factors also for development of PI-IBS [88, 103-105].

A large study on genetic susceptibility to PI-IBS showed association between three genes; TLR9, which encodes a pattern recognition receptor, IL-6, an inflammatory cytokine and CDH1 which encodes the tight junction protein cadherin-1, and the development of PI-IBS after a large waterborne gastroenteritis outbreak in Walkerton, Canada [106]. The link with a cadherin-1, a protein involved in cell barrier function is particularly interesting as a possible mechanism in giardiasis-induced IBS because disruption of tight junctions has been observed in *in vitro* models of *Giardia* infection [107].

Giardia lamblia infection has not been known to cause PI-IBS before the Bergen outbreak, but it is an important differential diagnosis in patients being evaluated for IBS [108], and it has also been reported to worsen symptoms in patients with previous IBS [109].

1.3.3 IBS and low grade inflammation

A wide range of mechanisms and markers have been investigated to grasp the underlying pathophysiology of FGID symptoms precipitation and continuation. In addition to genetic, environmental and psychosocial risk factors many studies have examined the roles of visceral hypersensitivity, gut motility, intestinal permeability, autonomic nervous system dysfunction and bacterial overgrowth. These have been reviewed recently and will not be detailed further here [110]. The persistence of, or inability to down regulate, the intestinal

inflammatory response is also thought to be an important mechanism behind the development of FGID, and interacts with all the above mentioned factors. Perhaps the strongest evidence for this is the increased risk of developing IBS after a bacterial gastroenteritis shown in several studies [84, 86, 88, 97].

Many studies have looked for the underlying markers and mechanisms for this observation, and have focused on IBS or PI-IBS. Histological examination of mucosal biopsies has given much of the evidence regarding low grade inflammation in IBS. A rare study of full thickness jejunal biopsies from IBS patients indicated that inflammation and neuronal degeneration in the myenteric plexus were involved in the pathogenesis of IBS [111]. In colonic biopsies of 77 IBS patients an increased number of lymphocytes have been reported [112]. A comprehensive study later showed increased levels of CD3, CD4, CD8 and mast cells in biopsies from proximal descending colon of IBS patients [113]. Gastroenteritis patients who developed PI-IBS showed persistently high inflammatory cell counts and increased IL-1β expression in rectal biopsies obtained 3 months after the infection compared to patients who did not [114].

Another study looking at rectal biopsies found EC-cell and T-cell counts to be raised in patients with newly developed PI-IBS 3 months after *Campylobacter* enteritis [115]. The co-occurrence of EC-cells and T-lymphocytes may be explained by CD4 T-lymphocytes stimulating EC-cell hyperplasia and serotonin production via IL-13 [116]. Also, increased intraepithelial CD8 T-cells and EC-cells have been reported in rectal biopsies in post dysenteric, IBS [117]. NK-cell levels in colonic mucosa in IBS were found to be no different than in healthy controls [112].

Altered cytokine profiles have been described in IBS and include studies showing elevated levels of circulating IL-6 and IL-8 [118-120]. However, Kindt et al did not find altered serum IL-6 in 42 FGID patients (including 30 patients with IBS) compared to healthy controls [121]. Mucosal mRNA levels of IL-6 have been similar to healthy controls [122] posing the question about the source for the elevated IL-6 seen in some studies. Liebregts et al found PBMCs from D-IBS patients produced elevated levels of IL-6, IL-1β and TNFα [123]. IBS patients has

also been shown to have a have a proinflammatory IL-10/IL-12 ratio (low IL-10 and raised IL-12) in supernatants from peripheral blood polymorphonuclear cells (PBMC) cultured for 3 days [124].

Also genetic studies are pointing towards a role for an altered cytokine profile in IBS patient. A significant reduction in the high producer IL-10 genotype frequency has been found in IBS patients (21%) compared to controls (32%) [92]. This was not reproduced in another genetic study which found that possession of a low producer IL-10 and high producer TNF*α* genotype was significantly more prevalent in IBS (9%) versus controls (3%) [93]. Proinflammatory polymorphisms in the IL-6 and TNFα genes were shown to increase the risk for IBS in an Iranian study [125].

Two studies investigating peripheral blood percentage and concentrations of T and B lymphocytes and NK-cells in FGID patient groups have not found these to differ from healthy controls [121, 126]. A third study also measured these lymphocyte subsets in IBS patients without psychiatric co-morbidity and found no difference compared to controls, even after a stressful public talking test [127]. However, two Chinese studies investigating D-IBS report differences in CD4/CD8 ratio due to raised CD8 cells [128] or lowered CD4 cells [129]. Regarding NK-cells, one study found normal peripheral blood levels of $CD56^{+}$ NK-cell levels in 12 women with IBS compared to 12 women without IBS, but had increased levels of NK-cells expressing the activation marker CD69 [130]. Another small study has described a post-prandial decrease in NK-cells and monocytes in IBS patients relative to controls, while leukocytes and granulocytes increased [131].

Single studies have found increased levels of B-cells expressing IgG or co-stimulatory molecules CD80 or CD86 and T-cells expressing β7+HLADR+ and CD69+ in IBS-patients compared to controls [126, 132]. Patients with FD have increased levels of peripheral blood small intestine homing T-cells expressing both β7-integrin and chemokine receptor 9 (CCR9) [133]. Differences in regulatory T-cells have not been found in FGID [134].

1.3.4 FGID and co-morbidity

The FGIDs are more often than not associated with other functional illnesses as co morbid conditions like temporomandibular joint disorder, fibromyalgia and the enigmatic disorder chronic fatigue syndrome (CFS) [135]. CFS and FGID shares the characteristic of female preponderance, a diagnosis relying on symptom criteria alone and that in many cases the onset of symptoms is preceded by an acute infection [84, 136].

Fatigue is a common symptom in both FGID and organic gastrointestinal disease [137]. The presence of IBS in CFS patients is reported to be between 35%-92% [138-142] while one study reports that 14% of IBS patients also have CFS [143]. Whether to regard both syndromes, along with multiple other comorbid functional disorders, is a matter of current debate [144, 145]. Although researchers of both FGID and CFS are probing hypotheses around the same underlying pathophysiological mechanisms regarding immune dysfunction, few studies have controlled for the presence of the other condition. A fine exception is the study by Scully et al examining 100 IBS patients with different co-morbidities and finding higher levels of IL-6 and IL-8 compared to healthy controls. Additionally, the cytokines IL-1β and TNFα were found to be increased in several groups with co-morbidity, including the CFS group, but not in the 21 IBS patients lacking co-morbidity [120]. In an equally interesting study in patients with either Epstein Barr virus (EBV) infection or *C.jejuni* infection, EBV virus infection predicted only short term PI-chronic fatigue (CF), while anxiety and depression were stronger predictors for long term PI-CF and CFS. The nature of the infection, gastroenteritis, was the strongest predictor of development of PI-IBS. They concluded that the impact of anxiety and depression was far less for the development of PI-IBS than for PI-CFS [144].

An anatomic correlate for fatigue in IBS-patients has also been found. An increased coecal mucosa cellularity (total number of lymphocytes) correlated with fatigue in a study of 50 IBS patient [146]. Coecal mast cells levels have been found to correlate well with both fatigue and with depression, suggesting psychological factors are associated with the low-grade

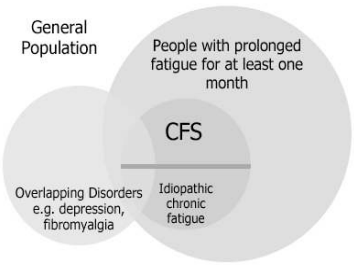
inflammatory infiltrate in IBS. However, in another study of rectal mucosa, depression was not found to be related to mast-cells, T-lymphocytes or EC-cells [147]. There is clearly a complex interplay between psychological, endocrine, immune and neurological factors at play in FGID and its various co-morbid conditions. Ongoing research around neuroendocrine mediators like corticotropin-releasing factor (CRF) and neuropeptide Y (NPY) might provide some answers to this pathophysiological puzzle [148, 149].

1.4 Chronic fatigue syndrome

1.4.1 Criteria and epidemiology

CFS is a diagnosis of exclusion. Its hallmark symptom is recurrent or persistent fatigue for more than 6 months that cannot be explained by specific tests or physical signs. The fatigue onset may have a rather abrupt onset, typically with a flu-like illness. The fatigue is exacerbated after physical exertion, and patients may need several days to recover to their normal fatigued state. Frequently patients also experience concentration and short term memory impairments, intolerance to alcohol, headache, dizziness, and myalgias [150]. The fatigue may lead to social isolation and decreased ability to work. A number of definitions, or sets of criteria, have been developed to further define this enigmatic condition for research purposes and for clinical use. The CFS criteria published in 1994 by Centers for Disease Control and prevention (CDC) in Atlanta USA [151] are the most widely used in research internationally (figure 3). CDC also came up with the term “idiopathic chronic fatigue” (ICF) for unexplained chronic fatigue failing to meet criteria for the chronic fatigue syndrome (see figure 4).

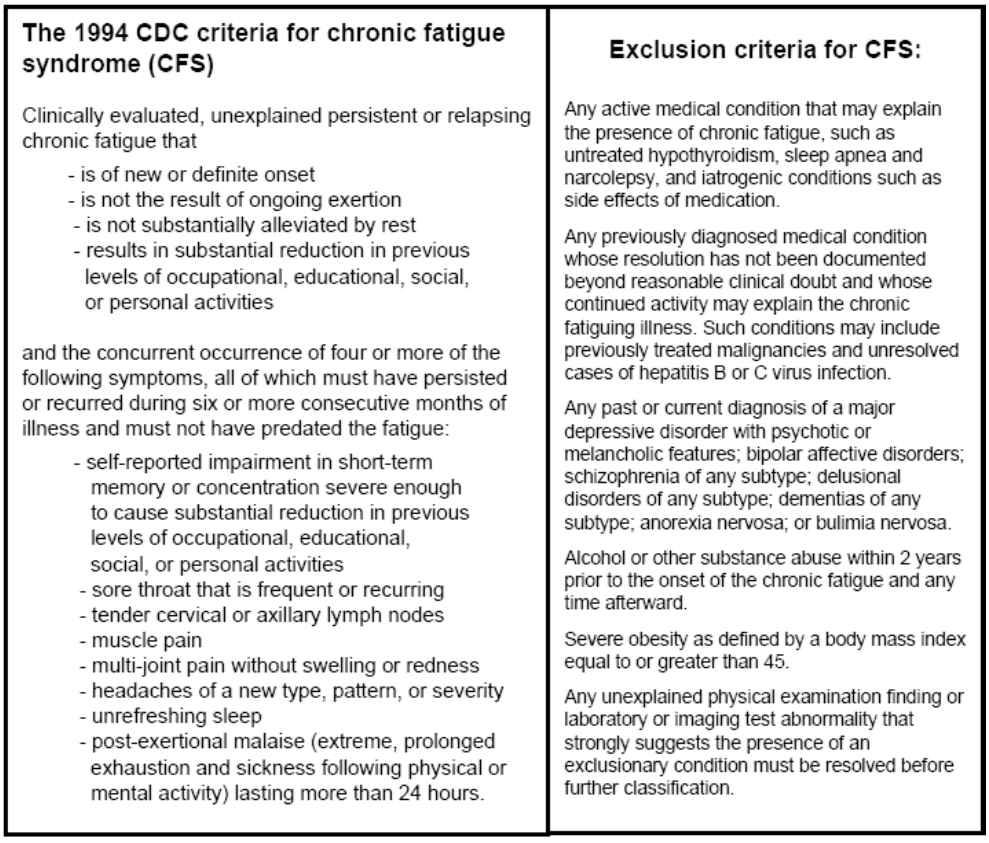
Figure 4. Conceptual Framework for Studying the Chronic Fatigue Syndrome. From *Fukuda et al. 1994*



The prevalence of CFS varies widely from 0.007 to 2.8% [152], but a reasonable estimate from a large population based study in Wichita, Kansas, USA, states a prevalence of 0.37% in women and 0.08% in men [153]. The cause of CFS remains elusive although a many large and small studies have reported abnormalities in brain structure and function, neuroendocrine responses, sleep architecture, immune function, virological findings, and divergent psychological profiles. CFS appears like a combination of predisposing, precipitating and perpetuating factors [152].

The prognosis of untreated CFS is rather poor. A meta analysis concluded with only 5% of patients fully recovering, while around 40% improved during follow-up periods of 1-4 years [154].

Figure 3



1.4.2 Infections and CFS

Many CFS patients experience flu-like symptoms and feel they get colds frequently. A number of infectious agents have been reported to be associated with CFS. These include Epstein Barr virus (EBV) (mononucleosis), dengue virus, Ross-river virus and *Coxiella burnetii* (Q fever) [136]. Parvovirus B19 has also been reported to elicit arthralgia and CFS [155]. Additional infectious agents implied in CFS development are reviewed by Kerr et al and include cytomegalovirus, *Brucellae*, *Toxoplasma gondii*, *C. burnetii*, *C. pneumoniae*, human herpes virus-6(HHV-6), group B coxsackieviruses (CVB), human T cell leukaemia virus II-like virus, spumavirus, hepatitis C virus, human lentiviruses and herpes virus-7 [156]. One report exists about a possible association between a cluster of 9 fatigue cases in Placerville, California and an epidemic of giardiasis [157].

Autum 2010 a paper reported xenotropic murine leukemia virus-related virus (XMRV) to be present at far higher levels in CFS than in healthy controls [158]. This was considered a breakthrough and gave hopes for treatment with anti-retroviral drugs. However, except for one report finding another related gammaretrovirus, murine leukemia virus (MLV) [159], a row of studies have failed to replicate the findings. Instead, contamination with mouse DNA in laboratory reagents has been found to be the likely cause of the association [160].

1.4.3 Immunological markers in CFS

Few studies have been performed regarding the role of immune responses against specific infectious agents. Some early reports claimed that differences in the EBV-specific antibody responses occurred in individuals developing CFS, but have been shown to be unsubstantiated [161]. Analysis of specific humoral immunity in PI-CFS after parvovirus B19 infection also did not identify any distinguishing pattern [155]. No studies on the role of specific CMI towards the suspected eliciting infection have been done in a CFS population.

Studies regarding differences in the general activation and function in peripheral blood lymphocyte subsets have been done since the late 1980ies and have given inconsistent

results. Studies until 2002 are excellently reviewed by Natelson et al [162]. Some studies find altered NK-cell levels and some find lowered CD4:CD8 ratios, but most studies find normal T-, B- and NK cell levels in CFS. HLA-DR expressing CD8 T-cells in CFS have been increased in three studies [163-165], while 6 other studies did not find a difference [166-170]. A Japanese study found significantly reduced NK-cell levels as well as insignificantly elevated CD8 levels in both PI-CFS and non-PI-CFS patients [171]. The same groups also showed the NK-cells levels to return to normal after successful recovery in the PI-CFS group [172].

The most consistent immunological deficit measured in CFS is decreased NK cell cytotoxicity. A more recent study have also found CD8 cell cytotoxicity to be decreased and a decrease in the CD56brightCD16neg NK-cell subset and increased CD4CD25FoxP3 regulatory T-cells in CSF has been reported [173]. Elevated CD26 expression on T-cells and NK-cells (marked with CD2) has been put forward as a promising biomarker in CFS [174].

Some caution needs to be taken regarding these measurements, as NK-cell cytotoxicity is also negatively influenced by chronic stress like unemployment [175]. NK-cells are also influenced by sleep and depression [176] and are known to fluctuate considerably with exercise. However, resting NK-cell levels are not much different in athletes compared to non-athletes [177]. A review article on depression and stressors relationship with immunological assays concluded “in both major depression and naturally occurring stressors the following effects are shared: leukocytosis, increased CD4/CD8 ratios, reduced proliferative response to mitogen, and reduced NK cell cytotoxicity” [178]. Given the relatively frequent co-morbidity with psychiatric illness and life stressors in CFS it is therefore necessary to record and evaluate this co-morbidity in future studies, and plan studies in such ways that stressors before and around blood sampling are avoided or standardized for all participants.

2. AIMS OF THE STUDY

The main aims of this study were:

- To describe symptoms, duodenal biopsies and other clinical findings in patients with persistent abdominal symptoms after *Giardia* infection.

- Characterize and classify the observed post-giardiasis abdominal symptoms into internationally recognized categories of FGID

- To investigate persistence of specific cell mediated immunity against giardiasis, and evaluate its role as a possible risk factor for developing post-giardiasis chronic fatigue syndrome and functional gastrointestinal disease.

- To evaluate peripheral blood lymphocyte subsets reported as markers of immune dysfunction in *Giardia* induced post-infectious functional gastrointestinal disorders and chronic fatigue syndrome

3. MATERIALS AND METHODS

3.1 Study population

The study populations of the studies included in this dissertation are illustrated in figure 5. Participants are drawn from a cohort of patients exposed to *Giardia* infection who were examined at various time points later due to persisting gastrointestinal symptoms (I, II) fatigue symptoms (IV) or because of their past *Giardia* exposure (III). A small *Giardia* exposed, rapidly recovered control group was used for symptom comparison in study I. Except the unexposed controls in study III and IV, all study populations included in these studies had confirmed *Giardia* infection during the outbreak. Knowingly, we thereby excluded a number of referred patients who were exposed to contaminated water, had gastroenteritis

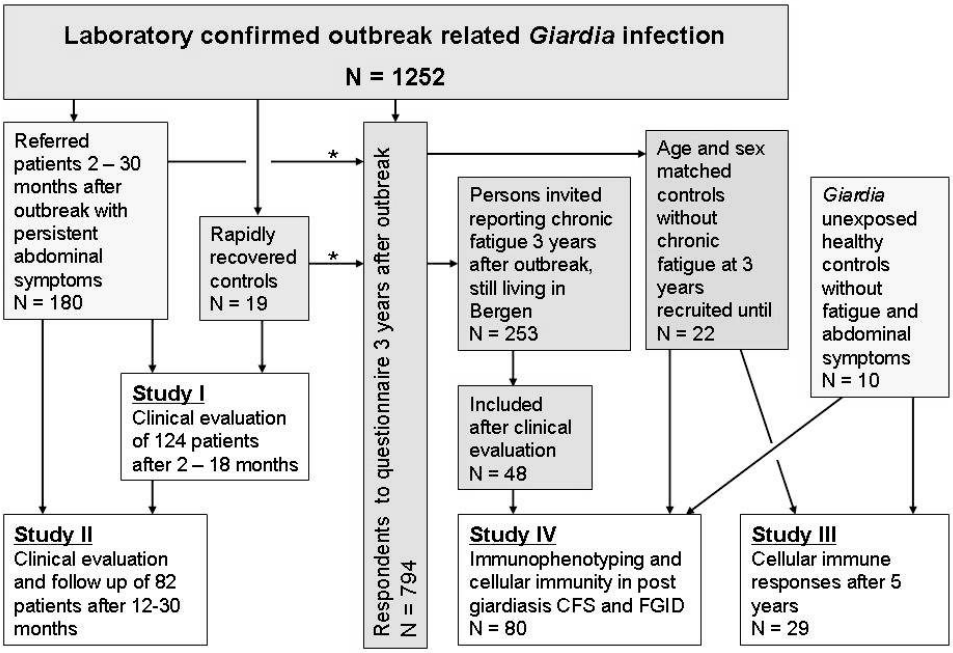


Figure 5. *Giardia* cohort populations included in the four papers of this dissertation.
* All individuals with laboratory confirmed *Giardia* infection received the questionnaire after three years, including the patient and control groups evaluated in the Study I and II.

during the outbreak and therefore were likely to have the same condition, but did not have laboratory confirmed *Giardia*. This was done to be as certain as possible about the role of *Giardia* infection in the studies performed.

The patient groups, and therefore also the matched control groups, were predominantly adult women with mean age range 31.8 - 42.4 years in the four studies. The percentage of females ranged from 61.3 - 79.0%. The female preponderance most probably was due a higher attack rate of the outbreak infection in adult females due to water drinking habits [2].

3.2 Diagnostic criteria and symptom scoring

FGID classification – Patients were given a questionnaire with a selection of questions to diagnose FD and the functional bowel disorders by the Rome II criteria [73]. All FGID diagnoses require the absence of specific organic disease that may explain the symptoms, including upper endoscopy to diagnose FD. We used the Rome II criteria in the first two studies and therefore continued with these criteria in new studies also after the arrival of Rome III criteria.

IBS - At least 12 weeks, which need not be consecutive, in the preceding 12 months of abdominal discomfort or pain that has two of three features:
- relieved with defecation
- onset associated with a change in frequency of stool
- onset associated with a change in form (appearance) of stool

FD - At least 12 weeks, which need not be consecutive, in the preceding 12 months of:
- Persistent or recurrent symptoms (pain or discomfort in the upper abdomen)
- No evidence that dyspepsia is exclusively relieved by defecation or associated with the onset of a change in stool frequency or stool form (i.e., not IBS).

Based on the dominant symptom features IBS can be further subdivided into diarrhea predominant IBS (D-IBS), alternating IBS (A-IBS) and constipation predominant (C-IBS).A detailed description of these IBS subgroups can be found in study II. Patients with prolonged

abdominal symptoms fulfilling neither FD nor IBS criteria were grouped as other FGID including functional abdominal pain, functional diarrhoea, functional bloating and unspecified functional bowel disorder in study II and IV. It is hard for most people to remember and evaluate their abdominal complaints throughout the last 12 months. A more practical timeframe is therefore often used for research purposes, and was also used in our study asking about any abdominal complaint during the last 12 weeks that were present at least one day per week for 3 out of the 12 weeks [80].

Abdominal symptoms - The ordinal scale developed by Kane et al [179] quantifying the subjective severity of nausea, abdominal pain, boating, constipation and anorexia had been used in the study population almost from the start. The scale grades all the mentioned symptoms from 0 - 10 with 0 = no symptoms and 10 = severe symptoms.

CFS - CFS and also ICF were clinically diagnosed according to the Fukuda criteria [151] (figure 2) after a thorough work-up involving consultations with specialists in neurology, psychiatry and internal medicine, routine and clinically indicated blood tests and a magnetic resonance imaging brain scan.

Fatigue symptoms - In study IV we used the validated Fatigue Questionnaire (FQ) [180] which consists of 11 questions addressing different aspects of fatigue. To each question there are four possible answers (“less than normal”, “as normal”, “more than normal”, “much more than normal”) that are scored (0, 1, 2, 3) and added to give a total fatigue score (range 0 to 33). The form has been used previously in a study on fatigue in the general population in Norway [181]. Based on this form one may define “Chronic fatigue” as an added fatigue questionnaire score above four when using a dicotomised score of the 11 questions in the fatigue questionnaire (0 and 1 into 0, 2 and 3 into 1), provided fatigue had been present for at least the last six months.

3.3 Systematic routine examinations

The department of histology performed routine evaluation of all duodenal biopsies. Distal duodenal biopsies were evaluated by an experienced pathologist blinded to other clinical information about the patients. Biopsy results were divided into three groups: normal histology (grade 0), mild pathology (grade 1) and moderate to severe pathology (grade 2) based on the description. Detailed description can be found in paper I.

Blood samples in the work-up and clinical evaluations in Study I and IV were performed at the hospital routine laboratories and included electrolytes, haematology, hormones, immunoglobulins, anti-endomysial and anti-tissue transglutaminase, *Giardia* rapid immunochromatographic test (ImmunoCardSTAT! *Cryptosporidium*/*Giardia* rapid assay; Meridian Bioscience) and microscopy of three stool samples after concentration by formalin-ether concentration technique. In study III & IV we had established a 18S *Giardia* PCR [182] to rule out giardiasis based on one stool sample.

Faecal calprotectin, a marker of intestinal inflammation [183], was analysed by the ELISA-based ϕhiCal Test (NovaTec Immundiagnostica GmbH, Germany) as part of hospital routine work. Faecal samples were also cultured for the presence of pathogenic bacteria in Study I.

3.4 Immunological methods

3.4.1 Mononuclear cell isolation and culture

Blood samples were drawn in the morning in BD Vacutainer Na-citrate CPT tubes. These tubes enabeled isolation of peripheral blood mononuclear cells (PBMC) by direct density gradient separation. PBMC were washed twice in PBS with 1% bovine serum albumin before being stained for flow cytometric analysis. PBMCs were cultured in 96-well U-bottom microtiter plates in X-vivo 15 medium supplemented with L-glutamin, gentamicin and phenol red (figure 6). Cells were cultured in the presence of *Giardia* antigens, control antigens or medium alone for 6 days at 37ºC in a humidified atmosphere of 5% CO_2. All cultures were

prepared in triplicates. Preparation of *Giardia* parasite antigens is described in detail in paper III.

3.4.2 Tritiated thymidine incorporation assay

This assay is a widely used and robust way of measuring T-cell proliferation. The incorporation of ^{3}H thymidine in newly synthesized DNA gives a measure for the cellular proliferative responses [184]. By stimulating cells with the antigen of interest one can then measure the T-cell responses towards that antigen, as a measure of antigen specific cell mediated immunity (CMI). It provides information about the total amount of newly synthesized DNA, but it does not reveal anything about the phenotype of the cells proliferating.

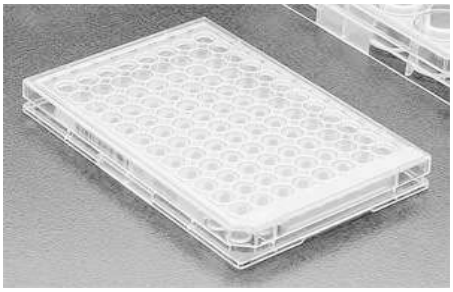


Figure 6. Cells were cultured in triplicates in 96-well plates for both flow cytometry and proliferation assays

We cultured the cells for 5 days and then the PBMC were pulsed with 37kBq/well of ^{3}H-thymidine and harvested 18 hours later onto glass-fibre pads. Radioactivity was determined by liquid scintillation counting in a β-counter. Proliferation was determined as counts per minute. Assay results were expressed as stimulation indices (SI) where responses in stimulated cells were divided by the response for corresponding unstimulated cells.

3.4.3 Flow cytometry assay

A flow cytometer is an instrument identifying cells and other particles passing individually through a narrow tube. Cells are illuminated by a laser beam, enabling cellular characteristics like size and granularity to be recorded, as well as the light emitted from fluorescent dye markers tied to the cellular molecules of interest. Combining many antibody linked dyes

enables detailed phenotyping of cells but need careful planning: Strength of the fluorescent dyes used, amount of target molecules on cells and compensation of interfering dyes are important factors for successful measurements. When properly set up the method can provide detailed information about specific CMI towards viral and bacterial antigens by staining for molecules expressed on the surface of activated or proliferating cells [185-188].

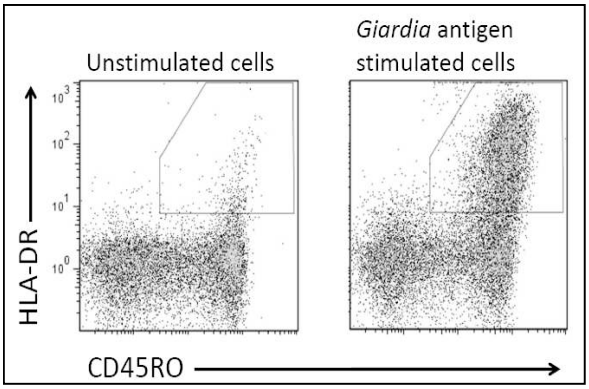


Figure 7. Plot showing CD4 T-cells expressing activation markers CD45RO and HLA-DR and gate for determining the percentage of cells positive for both markers.

We used a Beckman Coulter Cytomics FC 500 MPL flow cytometer to analyse surface markers on CD4 and CD8 T-cells including the late activation markers HLA-DR and CD25 and the recall antigen activation marker CD26, as well as the memory cell marker CD45RO (figure 7). Assessment of surface markers is easy to perform since cells can be stained in a single procedure without permeabilization of cells. There are also some limitations, for example that some markers may be non-specifically upregulated, can also be expressed on resting cells (like CD25), or have high baseline levels.

Lymphocyte subpopulation quantification was performed using the hospital routine laboratory equipment; a BD Multitest 6-color TBNK kit with BD Trucount Tubes for relative and absolute concentration determination. Full blood samples were analysed, after erythrocyte lysis, on a BD Canto II flow cytometer.

3.4.4 Other methods for measuring CMI

In the planning of Study III and IV, other methods for measuring CMI were also evaluated. Identification of proliferating cells is possible by flow cytometric analysis after staining cultured cells with bromodeoxyuridine (BrdU) or Carboxyfluorescein Succinimidyl Ester

(CFSE). Incorporation in DNA of the thymidine-analogue BrdU is an option whereby an antibody against BrdU can later be used to identify proliferating cell subsets [189]. However, this assay is labor intensive and can be unstable. Staining with the fluorescent dye CFSE is another way to follow proliferating cells as this dye is divided between daughter cells, who then stain less strongly, and can thereby reveal the number of cell divisions that have occurred in specific cell populations [190]. The assay is capable of simultaneously evaluating proliferation and phenotype of antigen responsive T-cell clones. It would have been used in Study III and IV if it had been established in our laboratory at the time of these experiments.

Methods avoiding cell stimulation and culture are also available as antigen specific T-cells can be visualized by fluorescently labeled, multimeric peptide-MHC complex that binds to the corresponding specific T-cell receptor if they are present in a patient sample. Tetramers are available both for MHC class I and II enabling analysis of antigen specific CD8 and CD4 cells and to further enumerate, characterize and purify these cells [191]. Disadvantages of this technique includes need for previous knowledge of each subjects HLA haplotype (and availability of the corresponding tetramer), and knowledge of a specific and well-working antigenic peptide. Tetramers do not separate between anergic and activated antigen specific T-cells [192].

A different approach would have been to measure and analyze the cytokine profiles in supernatants above antigen stimulated cells [193]. This method enables a better evaluation of the quality of the response towards the stimulating antigen, but not which cells are contributing to the production of these cytokines. This can, however, be achieved by intracellular cytokine staining followed by flow cytometric analysis. The method requires culture of stimulated cells in the prescence of a protein transport inhibitor, fixation and permeabilization of the cell to allow entry of the fluorescent antibodies targeting key intracellular cytokines like TNFα and IFNγ [194]. The method is labour intensive and not all cytokines are stably measured by this method. Cytokine secreting cells can also be measured with high sensitivity by enzyme-linked immunospot (ELISpot) following antigen stimulation [195].

3.5 Ethics

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

3.6 Statistical analysis

Fisher's exact test was used for testing categorical variables while Mann-Whitney's test was used for comparing continuous variables as they sometimes did not show a satisfactory normal distribution. Kruskal-Wallis test was used to analyse differences in non-parametric variables over more than two groups. In study IV we used linear regression for the correlation between NK-cell levels and symptoms. SPSS versions 14 – 18 were used for all statistical analyses.

4. SUMMARY OF PAPERS

The first two studies included work-up and follow-up of referred patients during the first two years after the outbreak, while the latter two studies examines long-term cellular immune responses and immune dysfunction markers five years after the outbreak in the whole population of *Giardia* outbreak exposed individuals who did or did not report fatigue in a mailed questionnaire three years after the outbreak.

Paper I: Persisting symptoms and duodenal inflammation related to *Giardia duodenalis* infections.

The study describes findings of a prospective standardised investigation procedure in a cohort of 124 referred patients over a 15 months period. All patients had laboratory confirmed giardiasis during the Bergen 2004 *Giardia* outbreak. Procedures included duodenal biopsies and aspirate, routine blood tests and faecal parasite and calprotectin investigations. Symptoms were recorded in 75 of the patients at the time of investigation, and in a control group of *Giardia* patients who rapidly recovered after metronidazole treatment. Chronic *Giardia* infection was found in 40 patients (32.3%). Duodenal biopsies showed signs of inflammation in 57 patients (47.1%), substantially higher in *Giardia* positive (87.2%) compared to *Giardia* negative patients (28.0%). The frequency of duodenal inflammation subsided over time. There were significant associations between persistent *Giardia* positivity, microscopic duodenal inflammation and a positive calprotectin test. Investigations could not determine an organic cause for the persistent symptoms.

Paper II: Development of functional gastrointestinal disorders after *Giardia lamblia* infection.

This study aimed to characterize the persistent abdominal symptoms 12-30 months after the onset of *Giardia* infection, and at least 6 months after *Giardia* eradication, according to the Rome II criteria and symptoms scores, as FGID development after infection with this non-invasive protozoan had not previously been described. It was found that 66 (80.5%) of the 82 patients included had symptoms consistent with irritable bowel syndrome (IBS) and 17 (24.3%) patients had functional dyspepsia (FD). IBS was sub classified into D-IBS (47.0%), A-IBS (45.5%) and C-IBS (7.6%). Food and stress related symptoms in this group were similar to FGID patients in general. Patients were showing clinical symptoms consistent with Rome II FGID categories, and the IBS-subtype pattern was consistent with post-infectious IBS (PI-IBS). With no other specific cause for the symptoms it was concluded that acute *Giardia* infection may elicit functional gastrointestinal diseases.

Paper III: Human cellular immune response against *Giardia lamblia* five years after acute giardiasis.

This was a proof of concept study to show that *Giardia lamblia* infection may induce a long-term cellular mediated immunity and that this may be measured by a ^{3}H-thymidine proliferation assay and flow cytometry analysis of activation markers HLA-DR, CD45RO, CD25 and CD26 in T-cell subsets. Peripheral blood mononuclear cells from 19 individuals with *Giardia* assemblage B infection five years previously and from 10 uninfected controls were cultured for 6 days with various antigen fractions obtained by sonicated *Giardia* assemblage A and B trophozoites. The study shows that proliferation responses were variable, but significantly elevated in the group previously exposed to *Giardia* for nearly all

Giardia antigens tested. Responses to *Giardia* assemblage A and B correlated well, indicating that cellular immune responses are not assemblage specific. Activation marker responses were mainly seen in CD4 T-cells indicating the importance of this T-cell subset in the human immune response against *Giardia*.

Paper IV: Immunophenotyping and *Giardia* specific immunity in post-giardiasis functional gastrointestinal disease and chronic fatigue syndrome.

The aim of this study was to evaluate markers of immune dysfunction and specific anti-*Giardia* CMI in post-infectious functional gastrointestinal disorders (PI-FGID) and chronic fatigue syndrome (PI-CFS) developing after *Giardia* infection. Five years after the outbreak 48 patients reporting chronic fatigue in a questionnaire study three years after the outbreak and 22 *Giardia* exposed non-fatigued individuals and 10 healthy unexposed individuals were included and grouped according to Fukuda criteria for CFS (n=19) and idiopatic chronic fatigue (n=5) and Rome II criteria for FGIDs (n=54).

No difference in *Giardia* specific CMI was found between *Giardia* exposed controls and PI-FGID or PI-CFS patients. However, in peripheral blood, significantly increased levels of CD8 T-cell in the PI-FGID group and significantly lower NK-cell levels in PI-CFS patients were found. NK-cell levels correlated well with both gastrointestinal and fatigue symptoms scores. A trend towards lower T-cell CD26 expression in PI-FGID and increased CD4 T-cell HLA-DR expression was seen.

5. RESULTS AND DISCUSSION

5.1. *Giardia* infection - symptoms and duodenal inflammation

Symptoms in the referred patients in Study I and II were recorded using the same ordinal scale and were most severe in the group with chronic *Giardia* infection and decreased over time in Study I. Symptoms scores, seen in the group as a whole, were largely stabilized in the patient population when measured again at the later time point when study II was performed. At all time points, bloating were the most severe symptom, only surpassed by diarrhea in ongoing giardiasis. Diarrhea figured as the second most severe symptom, closely followed by abdominal pain, in the *Giardia* negative patients at later time points. Similar to a small study of correlation between duodenal histology and diarrhea [41], we found abdominal pain and diarrhea to be associated with duodenal mucosal inflammation in study I. However, both these symptoms persisted even if inflammatory changes in routine biopsy findings normalized in the 11 repeated biopsies mentioned in Study II.

Studies of assemblage and symptoms development have shown inconsistent results, reviewed in [40]. In three of these studies [196-198], it is the assemblage found to be less prevalent in each study population that is also found to be causing most symptoms. This was also the case in Bergen where assemblage A was found to be the most prevalent in sewage the year before the outbreak ref [199], and the outbreak *Giardia* strain was genotyped to be assemblage B [200]. An inverse relationship between community prevalence and the development of symptomatic infection for any given *Giardia* genotype is therefore an intriguing possibility.

Differences in virulence in the outbreak strain cannot be excluded as a cause of variation in the clinical picture and duodenal inflammation, although there is little evidence for this. An indication for such differences is a recent study which found decreased post infection levels of the disaccaridases sucrase, maltase, and lactase in a mice model infected with the assemblage B strain, but not with the assemblage A strain [201]. Disaccaridase activities in

humans are well correlated with the level of duodenal inflammation [202]. The high degree of duodenal inflammation in adults presented in Study I was similar to another small study [41], but considerably higher than what has been reported in two large studies where genotype was not known [29, 203]. It is also plausible that the low percentage of duodenal inflammation in these two large studies was due because *Giardia* was a well-tolerated commensal in a part of the patients included, and that other pathology was leading to endoscopic examination. Another possible explanation is that the Bergen outbreak was caused by a more virulent assemblage B strain than seen in other studies.

Hardly any intestinal inflammation was found in a study of children with giardiasis [42], suggesting a milder inflammatory reaction in lower age groups. In developing countries, the childhood *Giardia* prevalence is much higher than in adults [21] and the risk of going through several *Giardia* infections in childhood and develop a degree of immunity is high. Our study population resided in a non-endemic area and consisted mainly of adults. Possibly, a first infection with *Giardia* in adulthood more often results in symptomatic infection and stronger inflammatory reaction than a first *Giardia* infection in childhood. Such age dependent differences in disease presentation are well known for example in polio and hepatitis A viral infections [204, 205]. Another possible cause could be a higher infective dose in a waterborne outbreak, and symptomatic and inflammatory responses are reflecting differences in water intake habits.

5.2 Abdominal symptoms and fatigue after giardiasis - consistent with PI-FGID and PI-CFS?

No other pathogen was found in the many fecal samples taken before and during the outbreak. Although it cannot be totally excluded, there is therefore limited potential for a possible accompanying other factor to have a role in the persisting symptoms observed in our study population. After thorough clinical work-up and follow up of around 180 patients referred to the Department of Medicine at Haukeland University Hospital during the two first

years after the *Giardia* outbreak in 2004, we were not able to find a discernible organic diagnosis to explain their abdominal symptoms and fatigue (study I and II). Additionally we documented a pattern of IBS-subtypes with a high frequency of diarrhoeal symptoms and little constipation. This agrees well with previous descriptions of PI-IBS as a distinctive subgroup of IBS patients [91].

It is known from clinical experience that *Giardia* may cause prolonged symptoms for several weeks after successful treatment due to secondary lactose intolerance [12]. In Study II we found that many patients reported different kinds of food to worsen symptoms. Testing of duodenal lactase activity in 10 patients with PI-FGID at our hospital did not confirm lactase deficiency (unpublished data). Lactose-intolerance has also not been found to be a factor in the aetiology of PI-IBS after bacterial gastroenteritis in a previous study [206].

We therefore concluded that abdominal complaints in this post-giardiasis cohort were consistent with development of post-infectious functional gastrointestinal disorders and, in patients evaluated for fatigue at the department of neurology, also with CFS.

The referred patients who reported persistent abdominal symptoms and fatigue were a subgroup of all individuals who had acute giardiasis during the outbreak. In a questionnaire study to all individuals with laboratory confirmed outbreak related giardiasis 2 years after the outbreak, the same pattern of abdominal symptoms was seen in 38% of the 1017 respondents. Fatigue was reported in 41% of respondents [3]. Severe and protracted infection, measured as the number of treatment courses and delayed education, were associated with the development of abdominal symptoms and fatigue in the same population [207].

A follow up questionnaire study in the same population three years after the outbreak used validated Rome III criteria [74] and a validated fatigue scale [180] and also included a control group. This study found that 46.1% of previously *Giardia* infected patients suffered from IBS compared to 14.0% in unexposed controls, and that 62.6% of these also experienced chronic fatigue [208].

Five years after the outbreak all individuals in this exposed population, who reported chronic fatigue and who still lived in the vicinity of Bergen, as well as controls reporting no fatigue, were invited to participate in a new study. These individuals formed the study population of Study IV where we again found abdominal symptoms consistent with FGID and were able to also diagnose concurrent CFS, ICF and other medical causes that could plausibly explain the fatigue. In both the questionnaire studies and in the Study IV population, FGID and chronic fatigue were associated with each other. Only one of the 19 patients diagnosed with CFS in study IV did not have FGID.

The development of PI-IBS after gastroenteritis is widely reported and acknowledged, and co-morbidity with depression and anxiety has been well investigated. The co-morbidity with CFS observed in our patient population has not been well-studied, with the two exceptions mentioned in the introduction [120, 144].

Moss-Morris et al found that the nature of the infection was a strong predictor of IBS, while premorbid levels of anxiety and depression better predicted CFS. The very prolonged FGID symptoms seen after *Giardia*-gastroenteritis in our patient population is consistent with this study. It would have been interesting to document the role of premorbid distress in the development of PI-FGID and PI-CFS in our population, but this would have been methodologically difficult to retrospectively. Although not a focus of the studies included in this dissertation we have experienced that many PI-CF/CFS patients recovered 2-4 years after the outbreak. It will be possible, in future studies, to look for differences in cytokine profiles of PI-FGID and PI-CFS as was done by Scully et al [120] by using stored plasma samples from the Study IV population.

5.3 Cellular immunity as mechanism for PI-FGID and PI-CFS

Some epidemiologic data support long term immunity against the non-invasive parasite *Giardia* [59, 61] and there are studies showing gradually increasing antibody titers with age in developing countries. Seroprevalence in a large Mexican study showed that 10% of one year

old infants, 40% of children < 10 years and 70% of adults > 25 years had IgG antibodies against *G. lamblia* in an ELISA assay with *Giardia* trophozoite soluble extract as antigen [50]. In addition to humoral immunity, a role for CMI could be assumed to be present, given the important role for CD4 T-cells to clear infection in mice [52, 53]. We were inspired by a human giardiasis case report showing a strong CMI measured 6 years later when stimulated with antigens from the infecting strain of *Giardia* in a ^{3}H-thymidine assay [62]. We hypothesized that a stronger CMI might be a mechanism for development of PI-CFS and PI-FGID which we investigated Study IV.

First it was necessary to validate the assays used and to ascertain the presence of a *Giardia* specific CMI 5 years after giardiasis. This was done by recruiting individuals who had laboratory confirmed giardiasis during the outbreak and a control group of people without prior known *Giardia* infection, and at low risk for having had it. Comparing the exposed no-fatigue control group versus the presumed unexposed controls we found that assemblage A and B independent, significantly stronger responses in the group exposed to giardiasis five years previously (Study III). In the flow cytometric assay mainly CD4 cells were seen to become activated. This is consistent with the results obtained in mice and in the case report by Gottstein et al [52, 53, 62]. The triggering of responses both to assemblage A and B in this population who had gone through an assemblage B infection is indicating some shared antigens by both assemblages which could be possible candidates for vaccine development.

In study IV, which was performed simultaneously with study III, we added data from 38 patients who reported chronic fatigue 3 years after the outbreak. They were categorized according to clinical evaluation using the Fukuda criteria regarding CFS and ICF [151] and according to responses to Rome II questionnaires regarding FGID. We evaluated the strength and quality of the CMI in these groups. While we still found a significant difference in CMI between all *Giardia* exposed participants and the *Giardia* unexposed, we did not find differences between the PI-CFS/ICF and PI-FGID patient groups and exposed controls.

Evaluation of the specific CMI towards the infectious agent eliciting PI-FGID or PI-CFS has not been done before. Our result does not exclude an altered CMI against the culprit

pathogen, as even if equal to exposed healthy controls measured 5 years later it may still have caused a psyhconeuroendocrine dysfunction resulting in prolonged symptoms. In IBS a lower proliferative response in T-cells has been observed in IBS patients when PBMC were stimulated by anti-CD3 and anti-CD28 antibodies for 3 days [126]. Similar assays have largely shown similar or decreased proliferative responses in CFS, reviewed by Natelson et al [162]. We saw a weak decrease in the proliferative responses after stimulation with anti-CD3 and anti-CD28 antibodies in the exposed patient groups, but this difference may have been larger if measured after 3 days in culture. It is therefore possible that a stronger specific CMI in the PI-FGID of PI-CFS groups is masked by unknown common factors also contributing to the symptomatology of CFS and IBS.

5.4 Inflammation markers in PI-FGID and PI-CFS

The large waterborne outbreak in Bergen led to a numerous people suffering from PI-CFS and PI-FGID. These conditions were described based on initial workup and follow up, and seeing some patients still reporting chronic fatigue led us to try to further clinically describe their illness and look for markers of underlying pathophysiology. Low grade inflammation has been put forward as having an important role in both CFS [156, 209] and FGID [84, 110], but may not be specific to these conditions [210]. A number of markers of immune activation in peripheral blood have been reported to differ between healthy subjects and patients with FGID or CFS. We therefore evaluated some of these in our study population and found similar levels of almost all markers in our small study population.

The reported elevated levels of CD26 in NK and T-cells [174] were not found in our PI-CFS group, rather a tendency for decreased levels in PI-FGID was found. HLA-DR expression on CD8 cells, which has been reported to be increased in three out of nine CFS-studies analyzing this marker [162], was not found. Instead a tendency towards increased HLA-DR expression on CD4 cells in the combined PI-CFS/ICF&FGID compared to the control group, a significant reduction in the small group recovered from fatigue compared to the PI-

CFS/ICF&FGID group. Regarding published FGID markers like β7 expressing CD4 or CD8 T-cells [211], we found normal levels of this lymphocyte subset.

In contrast to the decrease in NK-cells and increase in CD4 cells expressing HLA-DR in PI-CFS, these markers were seen to be at normal levels in the groups who had recovered from chronic fatigue during the two years between the questionnaire and the clinical evaluation. NK-cell levels correlated significantly with fatigue scores. These data suggest that decreased NK-cells and elevated HLA-DR expressing CD4 T-cells are associated with ongoing fatigue in PI-CFS after *Giardia* infection. However, NK-cell levels also correlated well with abdominal symptoms scores which again correlated well with fatigue scores. There is therefore also a possibility that NK-cell levels are linked to the severity of the overall condition of the patients.

While CD4 and CD8 T-cell numbers and percentages may fluctuate considerably within an individual over time, the CD4:CD8 ratio is found to be relatively stable [212]. CD4 and CD8 T-cell subsets and ratio in FGID have been looked at in a few studies which did not find differences in T-cell subsets [121, 126]. However, two Chinese studies found lowered CD4/CD8 ratio in IBS patients; one due to high CD8 cell levels [128] and one due to low CD4 cell levels [129].

The peripheral blood T-cell pattern with elevated CD8 levels resulting in a reduced CD4:CD8 ratio is seen in patients suffering from a number of viral diseases like mononucleosis, dengue, RSV and cytomegalovirus infection and herpes simplex recrudescence as well as in chronic toxoplasmosis infection [212, 213]. In our patient population this pattern was related to PI-FGID and not to PI-CFS, even though the hypothesis of low grade viral infection/reactivation is a hypothesis of CFS morbidity [214]. In our population of PI-CFS we did find a lower level of NK-cells, which is another cell subset important for the elimination of virally infected/altered host cells, and it is possible that impaired NK cell function allows the persistence of chronic viral infection in CFS.

5.5 Conclusions and future perspectives

PI-FGID has not been reported in the scientific literature before, but is recognized by clinicians. This may be because *Giardia* infection only occasionally cause the strong inflammatory response observed and documented in Study I. The development of PI-FGID and PI-CFS could be documented after the outbreak only because there were a large number of people simultaneously being infected and a substantial proportion were developing the conditions described in these studies.

In summary, there were no other pathogen found during the outbreak, and a strong and prolonged intestinal inflammatory reaction has been documented in many of the patients with persistent or chronic inflammation (Study I). No discernable cause for symptoms were found (Study II) and symptoms were consistent with *Giardia* induced post-infectious FGID and PI-CFS that for some patients were still present five years after the outbreak. The outbreak infection caused a long term cellular immunity that was not a predictor of development of the post-infectious sequels observed. In patients 5 years after the outbreak we found signs of immune dysfunction with low NK-cell levels in PI-CFS and elevated levels of CD8 in PI-FGID. It is not clear whether these findings are cause or effect of these disorders.

At this time, 7 years after the outbreak it is heartening to see many of the referred patients who developed severe FGID and debilitating CSF have recovered or improved considerably. We sincerely hope the research that has taken place alongside the clinical work-up and follow-up of the patients will be benefitting them and future patients with these disorders. Future research in collected materials and follow up studies will hopefully provide more knowledge on mechanisms and potential treatment options.

REFERENCES

(1) Steen K, Damsgaard E. [The Giardia epidemic in 2004 and out-of-hours service in Bergen]. Tidsskr Nor Laegeforen **2007 Jan 18**; 127(2):187-9.

(2) Nygard K, Schimmer B, Sobstad O, et al. A large community outbreak of waterborne giardiasis- delayed detection in a non-endemic urban area. BMC Public Health **2006 May 25**; 6(1):141.

(3) Morch K, Hanevik K, Rortveit G, Wensaas KA, Langeland N. High rate of fatigue and abdominal symptoms 2 years after an outbreak of giardiasis. Trans R Soc Trop Med Hyg **2009 Jan 29**.

(4) Roberts-Thomson IC, Stevens DP, Mahmoud AA, Warren KS. Giardiasis in the mouse: an animal model. Gastroenterology **1976 Jul**; 71(1):57-61.

(5) Adam RD. The Biology of Giardia Spp. Microbiological Reviews **1991 Dec**; 55(4):706-32.

(6) Ankarklev J, Jerlstrom-Hultqvist J, Ringqvist E, Troell K, Svard SG. Behind the smile: cell biology and disease mechanisms of Giardia species. Nat Rev Microbiol **2010 Jun**; 8(6):413-22.

(7) Monis PT, Caccio SM, Thompson RC. Variation in Giardia: towards a taxonomic revision of the genus. Trends Parasitol **2009 Feb**; 25(2):93-100.

(8) Thompson RC, Hopkins RM, Homan WL. Nomenclature and genetic groupings of Giardia infecting mammals. Parasitol Today **2000 May**; 16(5):210-3.

(9) Franzen O, Jerlstrom-Hultqvist J, Castro E, et al. Draft genome sequencing of giardia intestinalis assemblage B isolate GS: is human giardiasis caused by two different species? PLoS Pathog **2009 Aug**; 5(8):e1000560.

(10) Robertson LJ, Hermansen L, Gjerde BK. Occurrence of Cryptosporidium oocysts and Giardia cysts in sewage in Norway. Appl Environ Microbiol **2006 Aug**; 72(8):5297-303.

(11) Dobell C. The Discovery of the Intestinal Protozoa of Man. Proc R Soc Med **1920**; 13(Sect Hist Med):1-15.

(12) Farthing MJ. Giardiasis. Gastroenterol Clin North Am **1996 Sep**; 25(3):493-515.

(13) Al-Mekhlafi HM, Surin J, Sallam AA, Abdullah AW, Mahdy MA. Giardiasis and poor vitamin A status among aboriginal school children in rural Malaysia. Am J Trop Med Hyg **2010 Sep**; 83(3):523-7.

(14) Olivares JL, Fernandez R, Fleta J, Ruiz MY, Clavel A. Vitamin B12 and folic acid in children with intestinal parasitic infection. J Am Coll Nutr **2002 Apr**; 21(2):109-13.

(15) Berkman DS, Lescano AG, Gilman RH, Lopez SL, Black MM. Effects of stunting, diarrhoeal disease, and parasitic infection during infancy on cognition in late childhood: a follow-up study. Lancet **2002 Feb 16**; 359(9306):564-71.

(16) Botero-Garces JH, Garcia-Montoya GM, Grisales-Patino D, Aguirre-Acevedo DC, Alvarez-Uribe MC. Giardia intestinalis and nutritional status in children participating in the complementary nutrition program, Antioquia, Colombia, May to October 2006. Rev Inst Med Trop Sao Paulo **2009 May**; 51(3):155-62.

(17) Newman RD, Moore SR, Lima AA, Nataro JP, Guerrant RL, Sears CL. A longitudinal study of Giardia lamblia infection in north-east Brazilian children. Trop Med Int Health **2001 Aug**; 6(8):624-34.

(18) Sullivan PB, Marsh MN, Phillips MB, et al. Prevalence and treatment of giardiasis in chronic diarrhoea and malnutrition. Arch Dis Child **1991 Mar**; 66(3):304-6.

(19) Farthing MJG, Mata L, Urrutia JJ, Kronmal RA. Natural-History of Giardia Infection of Infants and Children in Rural Guatemala and Its Impact on Physical Growth. American Journal of Clinical Nutrition **1986 Mar**; 43(3):395-405.

(20) Ortega YR, Adam RD. Giardia: Overview and update. Clinical Infectious Diseases **1997 Sep**; 25(3):545-9.

(21) Oyerinde JP, Ogunbi O, Alonge AA. Age and sex distribution of infections with Entamoeba histolytica and Giardia intestinalis in the Lagos population. Int J Epidemiol **1977 Sep**; 6(3):231-4.

(22) Horman A, Korpela H, Sutinen J, Wedel H, Hanninen ML. Meta-analysis in assessment of the prevalence and annual incidence of Giardia spp. and Cryptosporidium spp. infections in humans in the Nordic countries. Int J Parasitol **2004 Nov**; 34(12):1337-46.

(23) Robertson LJ, Forberg T, Hermansen L, Gjerde BK, Langeland N. Demographics of Giardia infections in Bergen, Norway, subsequent to a waterborne outbreak. Scand J Infect Dis **2007 Jul 31**;1-4.

(24) Hoque ME, Hope VT, Scragg R, Kjellstrom T. Children at risk of giardiasis in Auckland: a case-control analysis. Epidemiol Infect **2003 Aug**; 131(1):655-62.

(25) Yoder JS, Harral C, Beach MJ. Giardiasis surveillance - United States, 2006-2008. MMWR Surveill Summ **2010 Jun 11**; 59(6):15-25.

(26) Fantham HB, Porter A. The pathogenicity of Giardia (lamblia) intestinalis to men and to experimental animals. Br Med J **1916 Jul 29**; 2(2900):139-41.

(27) Bøe J, Rinvik R. Infection with Lamblia Intestinalis in Children: its Clinical Significance and Treatment. Acta Paediatrica **1943**; 31(1):125-46.

(28) Buret AG. Mechanisms of epithelial dysfunction in giardiasis. Gut **2007 Mar**; 56(3):316-7.

(29) Oberhuber G, Kastner N, Stolte M. Giardiasis: a histologic analysis of 567 cases. Scand J Gastroenterol **1997 Jan**; 32(1):48-51.

(30) Faubert G. Immune response to Giardia duodenalis. Clin Microbiol Rev **2000 Jan**; 13(1):35-54, table.

(31) Troeger H, Epple HJ, Schneider T, et al. Effect of chronic Giardia lamblia infection on epithelial transport and barrier function in human duodenum. Gut **2007 Mar**; 56(3):328-35.

(32) Belosevic M, Faubert GM, MacLean JD. Disaccharidase activity in the small intestine of gerbils (Meriones unguiculatus) during primary and challenge infections with Giardia lamblia. Gut **1989 Sep**; 30(9):1213-9.

(33) Scott KG, Yu LC, Buret AG. Role of CD8+ and CD4+ T lymphocytes in jejunal mucosal injury during murine giardiasis. Infect Immun **2004 Jun**; 72(6):3536-42.

(34) Cevallos A, Carnaby S, James M, Farthing JG. Small intestinal injury in a neonatal rat model of giardiasis is strain dependent. Gastroenterology **1995 Sep**; 109(3):766-73.

(35) Andersen YS, Gillin FD, Eckmann L. Adaptive immunity-dependent intestinal hypermotility contributes to host defense against Giardia spp. Infect Immun **2006 Apr**; 74(4):2473-6.

(36) Scott KG, Meddings JB, Kirk DR, Lees-Miller SP, Buret AG. Intestinal infection with Giardia spp. reduces epithelial barrier function in a myosin light chain kinase-dependent fashion. Gastroenterology **2002 Oct**; 123(4):1179-90.

(37) Aggarwal A, Nash TE. Comparison of two antigenically distinct Giardia lamblia isolates in gerbils. Am J Trop Med Hyg **1987 Mar**; 36(2):325-32.

(38) Gottstein B, Harriman GR, Conrad JT, Nash TE. Antigenic variation in Giardia lamblia: cellular and humoral immune response in a mouse model. Parasite Immunol **1990 Nov**; 12(6):659-73.

(39) Islam A, Stoll BJ, Ljungstrom I, Biswas J, Nazrul H, Huldt G. Giardia lamblia infections in a cohort of Bangladeshi mothers and infants followed for one year. J Pediatr **1983 Dec**; 103(6):996-1000.

(40) Robertson LJ, Hanevik K, Escobedo AA, Morch K, Langeland N. Giardiasis - why do the symptoms sometimes never stop? Trends Parasitol **2010 Jan 5**.

(41) Duncombe VM, Bolin TD, Davis AE, Cummins AG, Crouch RL. Histopathology in giardiasis: a correlation with diarrhoea. Aust N Z J Med **1978 Aug**; 8(4):392-6.

(42) Koot BG, ten Kate FJ, Juffrie M, Rosalina I, Taminiau JJ, Benninga MA. Does Giardia lamblia cause villous atrophy in children?: A retrospective cohort study of the histological abnormalities in giardiasis. J Pediatr Gastroenterol Nutr **2009 Sep**; 49(3):304-8.

(43) Rendtorff RC. The experimental transmission of human intestinal protozoan parasites. II. Giardia lamblia cysts given in capsules. Am J Hyg **1954 Mar**; 59(2):209-20.

(44) Chester AC, MacMurray FG, Restifo MD, Mann O. Giardiasis as a chronic disease. Dig Dis Sci **1985 Mar**; 30(3):215-8.

(45) Oksenhendler E, Gerard L, Fieschi C, et al. Infections in 252 patients with common variable immunodeficiency. Clin Infect Dis **2008 May 15**; 46(10):1547-54.

(46) Cunningham-Rundles C. Physiology of IgA and IgA deficiency. J Clin Immunol **2001 Sep**; 21(5):303-9.

(47) Rivero FD, Saura A, Prucca CG, Carranza PG, Torri A, Lujan HD. Disruption of antigenic variation is crucial for effective parasite vaccine. Nat Med **2010 May**; 16(5):551-7, 1p.

(48) Nash T. Surface antigen variability and variation in Giardia lamblia. Parasitol Today **1992 Jul**; 8(7):229-34.

(49) Istre GR, Dunlop TS, Gaspard GB, Hopkins RS. Waterborne giardiasis at a mountain resort: evidence for acquired immunity. Am J Public Health **1984 Jun**; 74(6):602-4.

(50) Cedillo-Rivera R, Leal YA, Yepez-Mulia L, et al. Seroepidemiology of giardiasis in Mexico. Am J Trop Med Hyg **2009 Jan**; 80(1):6-10.

(51) Kumkum, Khanna R, Nain CK, Mehta S, Vinayak VK. Depressed humoral immune responses to surface antigens of Giardia lamblia in persistent giardiasis. Pediatr Infect Dis J **1988 Jul**; 7(7):492-8.

(52) Singer SM, Nash TE. T-cell-dependent control of acute Giardia lamblia infections in mice. Infect Immun **2000 Jan**; 68(1):170-5.

(53) Heyworth MF, Carlson JR, Ermak TH. Clearance of Giardia muris infection requires helper/inducer T lymphocytes. J Exp Med **1987 Jun 1**; 165(6):1743-8.

(54) Li E, Zhou P, Petrin Z, Singer SM. Mast cell-dependent control of Giardia lamblia infections in mice. Infect Immun **2004 Nov**; 72(11):6642-9.

(55) Langford TD, Housley MP, Boes M, et al. Central importance of immunoglobulin a in host defense against Giardia spp. Infection and Immunity **2002 Jan**; 70(1):11-8.

(56) Palm JE, Weiland ME, Griffiths WJ, Ljungstrom I, Svard SG. Identification of immunoreactive proteins during acute human giardiasis. J Infect Dis **2003 Jun 15**; 187(12):1849-59.

(57) Ebert EC. Giardia induces proliferation and interferon gamma production by intestinal lymphocytes. Gut **1999 Mar**; 44(3):342-6.

(58) Heyworth MF, Kung JE, Eriksson EC. Clearance of Giardia muris infection in mice deficient in natural killer cells. Infect Immun **1986 Dec**; 54(3):903-4.

(59) Isaac-Renton JL, Lewis LF, Ong CS, Nulsen MF. A second community outbreak of waterborne giardiasis in Canada and serological investigation of patients. Trans R Soc Trop Med Hyg **1994 Jul**; 88(4):395-9.

(60) Abdul-Wahid A, Faubert G. Characterization of the local immune response to cyst antigens during the acute and elimination phases of primary murine giardiasis. Int J Parasitol **2008 May**; 38(6):691-703.

(61) Kohli A, Bushen OY, Pinkerton RC, et al. Giardia duodenalis assemblage, clinical presentation and markers of intestinal inflammation in Brazilian children. Trans R Soc Trop Med Hyg **2008 Jul**; 102(7):718-25.

(62) Gottstein B, Stocks NI, Shearer GM, Nash TE. Human cellular immune response to Giardia lamblia. Infection **1991 Nov**; 19(6):421-6.

(63) Goka AK, Rolston DD, Mathan VI, Farthing MJ. The relative merits of faecal and duodenal juice microscopy in the diagnosis of giardiasis. Trans R Soc Trop Med Hyg **1990 Jan**; 84(1):66-7.

(64) Hiatt RA, Markell EK, Ng E. How many stool examinations are necessary to detect pathogenic intestinal protozoa? Am J Trop Med Hyg **1995 Jul**; 53(1):36-9.

(65) Garcia LS, Shimizu RY, Novak S, Carroll M, Chan F. Commercial assay for detection of Giardia lamblia and Cryptosporidium parvum antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. J Clin Microbiol **2003 Jan**; 41(1):209-12.

(66) Johnston SP, Ballard MM, Beach MJ, Causer L, Wilkins PP. Evaluation of three commercial assays for detection of Giardia and Cryptosporidium organisms in fecal specimens. J Clin Microbiol **2003 Feb**; 41(2):623-6.

(67) Garcia LS, Shimizu RY. Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of Giardia lamblia and Cryptosporidium parvum in human fecal specimens. J Clin Microbiol **1997 Jun**; 35(6):1526-9.

(68) Verweij JJ, Blange RA, Templeton K, et al. Simultaneous detection of Entamoeba histolytica, Giardia lamblia, and Cryptosporidium parvum in fecal samples by using multiplex real-time PCR. J Clin Microbiol **2004 Mar**; 42(3):1220-3.

(69) Gardner TB, Hill DR. Treatment of giardiasis. Clinical Microbiology Reviews **2001 Jan**; 14(1):114-+.

(70) Escobedo AA, Cimerman S. Giardiasis: a pharmacotherapy review. Expert Opin Pharmacother **2007 Aug**; 8(12):1885-902.

(71) Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel disorders and functional abdominal pain. Gut **1999 Sep**; 45 Suppl 2:II43-II47.

(72) Manning AP, Thompson WG, Heaton KW, Morris AF. Towards positive diagnosis of the irritable bowel. Br Med J **1978 Sep 2**; 2(6138):653-4.

(73) Drossman DA. The functional gastrointestinal disorders and the Rome II process. Gut **1999 Sep**; 45 Suppl 2:II1-II5.

(74) Drossman DA. Rome III: the new criteria. Chin J Dig Dis **2006**; 7(4):181-5.

(75) Drossman DA. The functional gastrointestinal disorders and the Rome III process. Gastroenterology **2006 Apr**; 130(5):1377-90.

(76) Vandvik PO, Aabakken L, Farup PG. Diagnosing irritable bowel syndrome: poor agreement between general practitioners and the Rome II criteria. Scand J Gastroenterol **2004 May**; 39(5):448-53.

(77) Cremonini F, Talley NJ. Review article: the overlap between functional dyspepsia and irritable bowel syndrome -- a tale of one or two disorders? Aliment Pharmacol Ther **2004 Nov**; 20 Suppl 7:40-9.

(78) Corazziari E. Definition and epidemiology of functional gastrointestinal disorders. Best Pract Res Clin Gastroenterol **2004 Aug**; 18(4):613-31.

(79) Agreus L, Svardsudd K, Nyren O, Tibblin G. Irritable bowel syndrome and dyspepsia in the general population: overlap and lack of stability over time. Gastroenterology **1995 Sep**; 109(3):671-80.

(80) Vandvik PO, Lydersen S, Farup PG. Prevalence, comorbidity and impact of irritable bowel syndrome in Norway. Scand J Gastroenterol **2006 Jun**; 41(6):650-6.

(81) Simren M, Mansson A, Langkilde AM, et al. Food-related gastrointestinal symptoms in the irritable bowel syndrome. Digestion **2001**; 63(2):108-15.

(82) Nanda R, James R, Smith H, Dudley CR, Jewell DP. Food intolerance and the irritable bowel syndrome. Gut **1989 Aug**; 30(8):1099-104.

(83) Mearin F. Postinfectious Functional Gastrointestinal Disorders. J Clin Gastroenterol **2011 Aug**; 45 Proceedings from the Pan-European Conference on Irritable Bowel Syndrome December 10, 2010 Vienna, Austria:S102-S105.

(84) Spiller R, Garsed K. Postinfectious irritable bowel syndrome. Gastroenterology **2009 May**; 136(6):1979-88.

(85) Chaudhary NA, Truelove SC. The irritable colon syndrome. A study of the clinical features, predisposing causes, and prognosis in 130 cases. Q J Med **1962 Jul**; 31:307-22.

(86) Marshall JK, Thabane M, Garg AX, Clark WF, Salvadori M, Collins SM. Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery. Gastroenterology **2006 Aug**; 131(2):445-50.

(87) Soyturk M, Akpinar H, Gurler O, et al. Irritable bowel syndrome in persons who acquired trichinellosis. Am J Gastroenterol **2007 May**; 102(5):1064-9.

(88) Thabane M, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome. Aliment Pharmacol Ther **2007 Aug 15**; 26(4):535-44.

(89) Longstreth GF, Hawkey CJ, Mayer EA, et al. Characteristics of patients with irritable bowel syndrome recruited from three sources: implications for clinical trials. Aliment Pharmacol Ther **2001 Jul**; 15(7):959-64.

(90) Tack J, Demedts I, Dehondt G, et al. Clinical and pathophysiological characteristics of acute-onset functional dyspepsia. Gastroenterology **2002 Jun**; 122(7):1738-47.

(91) Dunlop SP, Jenkins D, Spiller RC. Distinctive clinical, psychological, and histological features of postinfective irritable bowel syndrome. Am J Gastroenterol **2003 Jul**; 98(7):1578-83.

(92) Gonsalkorale WM, Perrey C, Pravica V, Whorwell PJ, Hutchinson IV. Interleukin 10 genotypes in irritable bowel syndrome: evidence for an inflammatory component? Gut **2003 Jan**; 52(1):91-3.

(93) van der Veek PP, van den BM, de Kroon YE, Verspaget HW, Masclee AA. Role of tumor necrosis factor-alpha and interleukin-10 gene polymorphisms in irritable bowel syndrome. Am J Gastroenterol **2005 Nov**; 100(11):2510-6.

(94) Spence MJ, Moss-Morris R. The cognitive behavioural model of irritable bowel syndrome: a prospective investigation of patients with gastroenteritis. Gut **2007 Aug**; 56(8):1066-71.

(95) Parry SD, Barton JR, Welfare MR. Factors associated with the development of post-infectious functional gastrointestinal diseases: does smoking play a role? Eur J Gastroenterol Hepatol **2005 Oct**; 17(10):1071-5.

(96) Thornley JP, Jenkins D, Neal K, Wright T, Brough J, Spiller RC. Relationship of Campylobacter toxigenicity in vitro to the development of postinfectious irritable bowel syndrome. J Infect Dis **2001 Sep 1**; 184(5):606-9.

(97) Mearin F, Perez-Oliveras M, Perello A, et al. Dyspepsia and irritable bowel syndrome after a Salmonella gastroenteritis outbreak: One-year follow-up cohort study. Gastroenterology **2005 Jul**; 129(1):98-104.

(98) Neal KR, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: Postal survey of patients. British Medical Journal **1997 Mar 15**; 314(7083):779-82.

(99) Maxwell PR, Rink E, Kumar D, Mendall MA. Antibiotics increase functional abdominal symptoms. Am J Gastroenterol **2002 Jan**; 97(1):104-8.

(100) Barbara G, Stanghellini V, Berti-Ceroni C, et al. Role of antibiotic therapy on long-term germ excretion in faeces and digestive symptoms after Salmonella infection. Aliment Pharmacol Ther **2000 Sep**; 14(9):1127-31.

(101) Morris-Yates A, Talley NJ, Boyce PM, Nandurkar S, Andrews G. Evidence of a genetic contribution to functional bowel disorder. Am J Gastroenterol **1998 Aug**; 93(8):1311-7.

(102) Levy RL, Jones KR, Whitehead WE, Feld SI, Talley NJ, Corey LA. Irritable bowel syndrome in twins: heredity and social learning both contribute to etiology. Gastroenterology **2001 Oct**; 121(4):799-804.

(103) Dunlop SP, Jenkins D, Neal KR, Spiller RC. Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. Gastroenterology **2003 Dec**; 125(6):1651-9.

(104) Gwee KA, Graham JC, McKendrick MW, et al. Psychometric scores and persistence of irritable bowel after infectious diarrhoea. Lancet **1996 Jan 20**; 347(8995):150-3.

(105) Gwee KA, Leong YL, Graham C, et al. The role of psychological and biological factors in postinfective gut dysfunction. Gut **1999 Mar**; 44(3):400-6.

(106) Villani AC, Lemire M, Thabane M, et al. Genetic risk factors for post-infectious irritable bowel syndrome following a waterborne outbreak of gastroenteritis. Gastroenterology **2010 Apr**; 138(4):1502-13.

(107) Buret AG, Mitchell K, Muench DG, Scott KG. Giardia lamblia disrupts tight junctional ZO-1 and increases permeability in non-transformed human small intestinal epithelial monolayers: effects of epidermal growth factor. Parasitology **2002 Jul**; 125(Pt 1):11-9.

(108) Grazioli B, Matera G, Laratta C, et al. Giardia lamblia infection in patients with irritable bowel syndrome and dyspepsia: a prospective study. World J Gastroenterol **2006 Mar 28**; 12(12):1941-4.

(109) D'Anchino M, Orlando D, De Feudis L. Giardia lamblia infections become clinically evident by eliciting symptoms of irritable bowel syndrome. J Infect **2002 Oct**; 45(3):169-72.

(110) Ohman L, Simren M. Pathogenesis of IBS: role of inflammation, immunity and neuroimmune interactions. Nat Rev Gastroenterol Hepatol **2010 Mar**; 7(3):163-73.

(111) Tornblom H, Lindberg G, Nyberg B, Veress B. Full-thickness biopsy of the jejunum reveals inflammation and enteric neuropathy in irritable bowel syndrome. Gastroenterology **2002 Dec**; 123(6):1972-9.

(112) Chadwick VS, Chen W, Shu D, et al. Activation of the mucosal immune system in irritable bowel syndrome. Gastroenterology **2002 Jun**; 122(7):1778-83.

(113) Cremon C, Gargano L, Morselli-Labate AM, et al. Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms. Am J Gastroenterol **2009 Feb**; 104(2):392-400.

(114) Gwee KA, Collins SM, Read NW, et al. Increased rectal mucosal expression of interleukin 1 beta in recently acquired post-infectious irritable bowel syndrome. Gut **2003 Apr**; 52(4):523-6.

(115) Dunlop SP, Jenkins D, Spiller RC. Distinctive clinical, psychological, and histological features of postinfective irritable bowel syndrome. Am J Gastroenterol **2003 Jul**; 98(7):1578-83.

(116) Wang H, Steeds J, Motomura Y, et al. CD4+ T cell-mediated immunological control of enterochromaffin cell hyperplasia and 5-hydroxytryptamine production in enteric infection. Gut **2007 Jul**; 56(7):949-57.

(117) Spiller RC, Jenkins D, Thornley JP, et al. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. Gut **2000 Dec**; 47(6):804-11.

(118) Dinan TG, Quigley EM, Ahmed SM, et al. Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? Gastroenterology **2006 Feb**; 130(2):304-11.

(119) Dinan TG, Clarke G, Quigley EM, et al. Enhanced cholinergic-mediated increase in the pro-inflammatory cytokine IL-6 in irritable bowel syndrome: role of muscarinic receptors. Am J Gastroenterol **2008 Oct**; 103(10):2570-6.

(120) Scully P, McKernan DP, Keohane J, et al. Plasma cytokine profiles in females with irritable bowel syndrome and extra-intestinal co-morbidity. Am J Gastroenterol **2010 Oct**; 105(10):2235-43.

(121) Kindt S, Van OL, Broekaert D, et al. Immune dysfunction in patients with functional gastrointestinal disorders. Neurogastroenterol Motil **2009 Apr**; 21(4):389-98.

(122) MacSharry J, O'Mahony L, Fanning A, et al. Mucosal cytokine imbalance in irritable bowel syndrome. Scand J Gastroenterol **2008**; 43(12):1467-76.

(123) Liebregts T, Adam B, Bredack C, et al. Immune activation in patients with irritable bowel syndrome. Gastroenterology **2007 Mar**; 132(3):913-20.

(124) O'Mahony L, McCarthy J, Kelly P, et al. Lactobacillus and Bifidobacterium in irritable bowel syndrome: Symptom responses and relationship to cytokine profiles. Gastroenterology **2005 Mar**; 128(3):541-51.

(125) Barkhordari E, Rezaei N, Ansaripour B, et al. Proinflammatory cytokine gene polymorphisms in irritable bowel syndrome. J Clin Immunol **2010 Jan**; 30(1):74-9.

(126) Ohman L, Isaksson S, Lindmark AC, et al. T-cell activation in patients with irritable bowel syndrome. Am J Gastroenterol **2009 May**; 104(5):1205-12.

(127) Elsenbruch S, Lucas A, Holtmann G, et al. Public speaking stress-induced neuroendocrine responses and circulating immune cell redistribution in irritable bowel syndrome. Am J Gastroenterol **2006 Oct**; 101(10):2300-7.

(128) Linghu EQ, Yang Y.S. Analysis of peripheral blood lymphocytes subgroup in irritable bowel syndrome. Chin J Dig **2002**; 22:423-5.

(129) Jin HB, Gu ZY, Zhao HW, Wang QY, Wu W, Li YM. [T lymphocyte subgroups and serum levels of trace elements in patients with diarrhea type of irritable bowel syndrome]. Zhejiang Da Xue Xue Bao Yi Xue Ban **2008 Nov**; 37(6):634-7.

(130) Motzer SA, Jarrett M, Heitkemper MM, Tsuji J. Natural killer cell function and psychological distress in women with and without irritable bowel syndrome. Biol Res Nurs **2002 Jul**; 4(1):31-42.

(131) Elsenbruch S, Holtmann G, Oezcan D, et al. Are there alterations of neuroendocrine and cellular immune responses to nutrients in women with irritable bowel syndrome? Am J Gastroenterol **2004 Apr**; 99(4):703-10.

(132) Ohman L, Lindmark AC, Isaksson S, et al. B-cell activation in patients with irritable bowel syndrome (IBS). Neurogastroenterol Motil **2009 Jun**; 21(6):644-50, e27.

(133) Liebregts T, Adam B, Bredack C, et al. Small bowel homing T cells are associated with symptoms and delayed gastric emptying in functional dyspepsia. Am J Gastroenterol **2011 Jun**; 106(6):1089-98.

(134) Holmen N, Isaksson S, Simren M, Sjovall H, Ohman L. CD4+CD25+ regulatory T cells in irritable bowel syndrome patients. Neurogastroenterol Motil **2007 Feb**; 19(2):119-25.

(135) Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology **2002 Apr**; 122(4):1140-56.

(136) Hickie I, Davenport T, Wakefield D, et al. Post-infective and chronic fatigue syndromes precipitated by viral and non-viral pathogens: prospective cohort study. BMJ **2006 Sep 16**; 333(7568):575.

(137) Simren M, Svedlund J, Posserud I, Bjornsson ES, Abrahamsson H. Predictors of subjective fatigue in chronic gastrointestinal disease. Aliment Pharmacol Ther **2008 Sep 1**; 28(5):638-47.

(138) Aaron LA, Burke MM, Buchwald D. Overlapping conditions among patients with chronic fatigue syndrome, fibromyalgia, and temporomandibular disorder. Arch Intern Med **2000 Jan 24**; 160(2):221-7.

(139) Aaron LA, Herrell R, Ashton S, et al. Comorbid clinical conditions in chronic fatigue: a co-twin control study. J Gen Intern Med **2001 Jan**; 16(1):24-31.

(140) Endicott NA. Chronic fatigue syndrome in psychiatric patients: lifetime and premorbid personal history of physical health. Psychosom Med **1998 Nov**; 60(6):744-51.

(141) Korszun A, Papadopoulos E, Demitrack M, Engleberg C, Crofford L. The relationship between temporomandibular disorders and stress-associated syndromes. Oral Surg Oral Med Oral Pathol Oral Radiol Endod **1998 Oct**; 86(4):416-20.

(142) Morriss RK, Ahmed M, Wearden AJ, et al. The role of depression in pain, psychophysiological syndromes and medically unexplained symptoms associated with chronic fatigue syndrome. J Affect Disord **1999 Oct**; 55(2-3):143-8.

(143) Jones KR, Palsson OS, Levy RL, et al. Comorbid disorders and symptoms in irritable bowel syndrome (IBS) compared to other gastroenterology patients. Gastroenterology **2001**; 120(Suppl 1):A66.

(144) Moss-Morris R, Spence M. To "lump" or to "split" the functional somatic syndromes: can infectious and emotional risk factors differentiate between the onset of chronic fatigue syndrome and irritable bowel syndrome? Psychosom Med **2006 May**; 68(3):463-9.

(145) White PD. Chronic fatigue syndrome: Is it one discrete syndrome or many? Implications for the "one vs. many" functional somatic syndromes debate. J Psychosom Res **2010 May**; 68(5):455-9.

(146) Piche T, Saint-Paul MC, Dainese R, et al. Mast cells and cellularity of the colonic mucosa correlated with fatigue and depression in irritable bowel syndrome. Gut **2008 Apr**; 57(4):468-73.

(147) Lee KJ, Kim YB, Kim JH, Kwon HC, Kim DK, Cho SW. The alteration of enterochromaffin cell, mast cell, and lamina propria T lymphocyte numbers in irritable bowel syndrome and its relationship with psychological factors. J Gastroenterol Hepatol **2008 Nov**; 23(11):1689-94.

(148) Vicario M, Alonso C, Guilarte M, et al. Chronic psychosocial stress induces reversible mitochondrial damage and corticotropin-releasing factor receptor type-1 upregulation in the rat intestine and IBS-like gut dysfunction. Psychoneuroendocrinology **2011 Jun 3**.

(149) Simren M, Stotzer PO, Sjovall H, Abrahamsson H, Bjornsson ES. Abnormal levels of neuropeptide Y and peptide YY in the colon in irritable bowel syndrome. Eur J Gastroenterol Hepatol **2003 Jan**; 15(1):55-62.

(150) Komaroff AL, Buchwald D. Symptoms and signs of chronic fatigue syndrome. Rev Infect Dis **1991 Jan**; 13 Suppl 1:S8-11.

(151) Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. Ann Intern Med **1994 Dec 15**; 121(12):953-9.

(152) Afari N, Buchwald D. Chronic fatigue syndrome: a review. Am J Psychiatry **2003 Feb**; 160(2):221-36.

(153) Reyes M, Nisenbaum R, Hoaglin DC, et al. Prevalence and incidence of chronic fatigue syndrome in Wichita, Kansas. Arch Intern Med **2003 Jul 14**; 163(13):1530-6.

(154) Cairns R, Hotopf M. A systematic review describing the prognosis of chronic fatigue syndrome. Occup Med (Lond) **2005 Jan**; 55(1):20-31.

(155) Kerr JR, Bracewell J, Laing I, et al. Chronic fatigue syndrome and arthralgia following parvovirus B19 infection. J Rheumatol **2002 Mar**; 29(3):595-602.

(156) Devanur LD, Kerr JR. Chronic fatigue syndrome. J Clin Virol **2006 Nov**; 37(3):139-50.

(157) Levine PH, Jacobson S, Pocinki AG, et al. Clinical, Epidemiologic, and Virological Studies in 4 Clusters of the Chronic Fatigue Syndrome. Archives of Internal Medicine **1992 Aug**; 152(8):1611-6.

(158) Lombardi VC, Hagen KS, Hunter KW, et al. Xenotropic murine leukemia virus-related virus-associated chronic fatigue syndrome reveals a distinct inflammatory signature. In Vivo **2011 May**; 25(3):307-14.

(159) Lo SC, Pripuzova N, Li B, et al. Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors. Proc Natl Acad Sci U S A **2010 Sep 7**; 107(36):15874-9.

(160) Knox K, Carrigan D, Simmons G, et al. No evidence of murine-like gammaretroviruses in CFS patients previously identified as XMRV-infected. Science **2011 Jul 1**; 333(6038):94-7.

(161) Buchwald D, Sullivan JL, Komaroff AL. Frequency of 'chronic active Epstein-Barr virus infection' in a general medical practice. JAMA **1987 May 1**; 257(17):2303-7.

(162) Natelson BH, Haghighi MH, Ponzio NM. Evidence for the presence of immune dysfunction in chronic fatigue syndrome. Clin Diagn Lab Immunol **2002 Jul**; 9(4):747-52.

(163) Klimas NG, Salvato FR, Morgan R, Fletcher MA. Immunologic abnormalities in chronic fatigue syndrome. J Clin Microbiol **1990 Jun**; 28(6):1403-10.

(164) Landay AL, Jessop C, Lennette ET, Levy JA. Chronic fatigue syndrome: clinical condition associated with immune activation. Lancet **1991 Sep 21**; 338(8769):707-12.

(165) Lloyd AR, Wakefield D, Boughton CR, Dwyer JM. Immunological abnormalities in the chronic fatigue syndrome. Med J Aust **1989 Aug 7**; 151(3):122-4.

(166) Hassan IS, Bannister BA, Akbar A, Weir W, Bofill M. A study of the immunology of the chronic fatigue syndrome: correlation of immunologic parameters to health dysfunction. Clin Immunol Immunopathol **1998 Apr**; 87(1):60-7.

(167) Lloyd A, Hickie I, Hickie C, Dwyer J, Wakefield D. Cell-mediated immunity in patients with chronic fatigue syndrome, healthy control subjects and patients with major depression. Clin Exp Immunol **1992 Jan**; 87(1):76-9.

(168) Peakman M, Deale A, Field R, Mahalingam M, Wessely S. Clinical improvement in chronic fatigue syndrome is not associated with lymphocyte subsets of function or activation. Clin Immunol Immunopathol **1997 Jan**; 82(1):83-91.

(169) Swanink CM, Vercoulen JH, Galama JM, et al. Lymphocyte subsets, apoptosis, and cytokines in patients with chronic fatigue syndrome. J Infect Dis **1996 Feb**; 173(2):460-3.

(170) Zhang Q, Zhou XD, Denny T, et al. Changes in immune parameters seen in Gulf War veterans but not in civilians with chronic fatigue syndrome. Clin Diagn Lab Immunol **1999 Jan**; 6(1):6-13.

(171) Masuda A, Munemoto T, Yamanaka T, Takei M, Tei C. Psychosocial characteristics and immunological functions in patients with postinfectious chronic fatigue syndrome and noninfectious chronic fatigue syndrome. J Behav Med **2002 Oct**; 25(5):477-85.

(172) Masuda A, Nakayama T, Yamanaka T, Koga Y, Tei C. The prognosis after multidisciplinary treatment for patients with postinfectious chronic fatigue syndrome and noninfectious chronic fatigue syndrome. J Behav Med **2002 Oct**; 25(5):487-97.

(173) Brenu EW, van Driel ML, Staines DR, et al. Immunological abnormalities as potential biomarkers in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. J Transl Med **2011**; 9:81.

(174) Fletcher MA, Zeng XR, Maher K, et al. Biomarkers in chronic fatigue syndrome: evaluation of natural killer cell function and dipeptidyl peptidase IV/CD26. PLoS One **2010**; 5(5):e10817.

(175) Cohen F, Kemeny ME, Zegans LS, Johnson P, Kearney KA, Stites DP. Immune function declines with unemployment and recovers after stressor termination. Psychosom Med **2007 Apr**; 69(3):225-34.

(176) Irwin M, Lacher U, Caldwell C. Depression and reduced natural killer cytotoxicity: a longitudinal study of depressed patients and control subjects. Psychol Med **1992 Nov**; 22(4):1045-50.

(177) Gleeson M, Bishop NC. The T cell and NK cell immune response to exercise. Ann Transplant **2005**; 10(4):43-8.

(178) Zorrilla EP, Luborsky L, McKay JR, et al. The relationship of depression and stressors to immunological assays: a meta-analytic review. Brain Behav Immun **2001 Sep**; 15(3):199-226.

(179) Kane SV, Sandborn WJ, Rufo PA, et al. Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. Am J Gastroenterol **2003 Jun**; 98(6):1309-14.

(180) Chalder T, Berelowitz G, Pawlikowska T, et al. Development of a fatigue scale. J Psychosom Res **1993**; 37(2):147-53.

(181) Loge JH, Ekeberg O, Kaasa S. Fatigue in the general Norwegian population: normative data and associations. J Psychosom Res **1998 Jul**; 45(1 Spec No):53-65.

(182) Verweij JJ, Schinkel J, Laeijendecker D, van Rooyen MA, van LL, Polderman AM. Real-time PCR for the detection of Giardia lamblia. Mol Cell Probes **2003 Oct**; 17(5):223-5.

(183) Costa F, Mumolo MG, Bellini M, et al. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. Dig Liver Dis **2003 Sep**; 35(9):642-7.

(184) Jordan GW, Merigan TC. Cell-mediated immunity to varicella-zoster virus: in vitro lymphocyte responses. J Infect Dis **1974 Nov**; 130(5):495-501.

(185) Weil AA, Arifuzzaman M, Bhuiyan TR, et al. Memory T-cell responses to Vibrio cholerae O1 infection. Infect Immun **2009 Nov**; 77(11):5090-6.

(186) Gaines H, Biberfeld G. Measurement of lymphoproliferation at the single-cell level by flow cytometry. Methods Mol Biol **2000**; 134:243-55.

(187) Svahn A, Linde A, Thorstensson R, Karlen K, Andersson L, Gaines H. Development and evaluation of a flow-cytometric assay of specific cell-mediated immune response in activated whole blood for the detection of cell-mediated immunity against varicella-zoster virus. J Immunol Methods **2003 Jun 1**; 277(1-2):17-25.

(188) Ohnuma K, Takahashi N, Yamochi T, Hosono O, Dang NH, Morimoto C. Role of CD26/dipeptidyl peptidase IV in human T cell activation and function. Front Biosci **2008**; 13:2299-310.

(189) Mehta BA, Maino VC. Simultaneous detection of DNA synthesis and cytokine production in staphylococcal enterotoxin B activated CD4+ T lymphocytes by flow cytometry. J Immunol Methods **1997 Oct 13**; 208(1):49-59.

(190) Fulcher D, Wong S. Carboxyfluorescein succinimidyl ester-based proliferative assays for assessment of T cell function in the diagnostic laboratory. Immunol Cell Biol **1999 Dec**; 77(6):559-64.

(191) Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science **1996 Oct 4**; 274(5284):94-6.

(192) Lee PP, Yee C, Savage PA, et al. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med **1999 Jun**; 5(6):677-85.

(193) Street NE, Mosmann TR. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. FASEB J **1991 Feb**; 5(2):171-7.

(194) Horton H, Thomas EP, Stucky JA, et al. Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. J Immunol Methods **2007 May 31**; 323(1):39-54.

(195) Herr W, Linn B, Leister N, Wandel E, Meyer zum Buschenfelde KH, Wolfel T. The use of computer-assisted video image analysis for the quantification of CD8+ T lymphocytes producing tumor necrosis factor alpha spots in response to peptide antigens. J Immunol Methods **1997 Apr 25**; 203(2):141-52.

(196) Read C, Walters J, Robertson ID, Thompson RC. Correlation between genotype of Giardia duodenalis and diarrhoea. Int J Parasitol **2002 Feb**; 32(2):229-31.

(197) Gelanew T, Lalle M, Hailu A, Pozio E, Caccio SM. Molecular characterization of human isolates of Giardia duodenalis from Ethiopia. Acta Trop **2007 May**; 102(2):92-9.

(198) Haque R, Roy S, Kabir M, Stroup SE, Mondal D, Houpt ER. Giardia assemblage A infection and diarrhea in Bangladesh. J Infect Dis **2005 Dec 15**; 192(12):2171-3.

(199) Robertson LJ, Forberg T, Gjerde BK. Giardia cysts in sewage influent in Bergen, Norway 15-23 months after an extensive waterborne outbreak of giardiasis. J Appl Microbiol **2008 Apr**; 104(4):1147-52.

(200) Robertson LJ, Hermansen L, Gjerde BK, Strand E, Alvsvag JO, Langeland N. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Appl Environ Microbiol **2006 Mar**; 72(3):2212-7.

(201) Solaymani-Mohammadi S, Singer SM. Host Immunity and Pathogen Strain Contribute to Intestinal Disaccharidase Impairment following Gut Infection. J Immunol **2011 Aug 26**.

(202) Gupta SK, Chong SK, Fitzgerald JF. Disaccharidase activities in children: normal values and comparison based on symptoms and histologic changes. J Pediatr Gastroenterol Nutr **1999 Mar**; 28(3):246-51.

(203) Oberhuber G, Stolte M. Giardiasis: analysis of histological changes in biopsy specimens of 80 patients. J Clin Pathol **1990 Aug**; 43(8):641-3.

(204) Bunimovich-Mendrazitsky S, Stone L. Modeling polio as a disease of development. J Theor Biol **2005 Dec 7**; 237(3):302-15.

(205) Shapiro CN, Margolis HS. Worldwide epidemiology of hepatitis A virus infection. J Hepatol **1993**; 18 Suppl 2:S11-S14.

(206) Parry SD, Barton JR, Welfare MR. Is lactose intolerance implicated in the development of post-infectious irritable bowel syndrome or functional diarrhoea in previously asymptomatic people? Eur J Gastroenterol Hepatol **2002 Nov**; 14(11):1225-30.

(207) Morch K, Hanevik K, Rortveit G, et al. Severity of Giardia infection associated with post-infectious fatigue and abdominal symptoms two years after. BMC Infect Dis **2009 Dec 15**; 9(1):206.

(208) Wensaas KA, Langeland N, Hanevik K, Morch K, Eide GE, Rortveit G. Irritable bowel syndrome and chronic fatigue 3 years after acute giardiasis: historic cohort study. Gut **2011 Sep 12**.

(209) Aspler AL, Bolshin C, Vernon SD, Broderick G. Evidence of inflammatory immune signaling in chronic fatigue syndrome: A pilot study of gene expression in peripheral blood. Behav Brain Funct **2008**; 4:44.

(210) Raison CL, Lin JM, Reeves WC. Association of peripheral inflammatory markers with chronic fatigue in a population-based sample. Brain Behav Immun **2009 Mar**; 23(3):327-37.

(211) Ohman L, Isaksson S, Lundgren A, Simren M, Sjovall H. A controlled study of colonic immune activity and beta7+ blood T lymphocytes in patients with irritable bowel syndrome. Clin Gastroenterol Hepatol **2005 Oct**; 3(10):980-6.

(212) Laurence J. T-cell subsets in health, infectious disease, and idiopathic CD4+ T lymphocytopenia. Ann Intern Med **1993 Jul 1**; 119(1):55-62.

(213) Liu CC, Huang KJ, Lin YS, Yeh TM, Liu HS, Lei HY. Transient CD4/CD8 ratio inversion and aberrant immune activation during dengue virus infection. J Med Virol **2002 Oct**; 68(2):241-52.

(214) Ablashi DV. Viral studies of chronic fatigue syndrome. Clin Infect Dis **1994 Jan**; 18 Suppl 1:S130-S133.

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Persisting symptoms and duodenal inflammation related to *Giardia duodenalis* infection

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Summary *Objectives:* After a large waterborne outbreak of *Giardia* infection in Bergen, some patients experienced persisting abdominal symptoms despite metronidazole treatment. This study aimed at investigating possible causes for their symptoms.
Methods: Over a 15 months period, 124 referred patients were evaluated in a prospective cohort analysis with a standardised investigation including duodenal biopsies and aspirate, blood tests and faecal parasite and calprotectin tests. Recovered subjects were recruited for symptom analysis.
Results: Persisting *Giardia duodenalis* infection was found in 40 patients (32.3%). Duodenal biopsies showed signs of inflammation in 57 patients (47.1%). Microscopic duodenal inflammation was present in 34 (87.2%) of the *Giardia* positive and 23 (28.0%) of the *Giardia* negative patients. There were significant associations between persistent *Giardia* positivity, microscopic duodenal inflammation and a positive calprotectin test. Duodenal aspirate and duodenal biopsies performed poorly in diagnosis of persistent giardiasis.
Conclusions: In patients with persisting symptoms after metronidazole treated *Giardia* infection we commonly found chronic *Giardia* infection and microscopic duodenal inflammation, especially in illness duration less than 7 months. Both these findings subsided over time. Increasingly, investigations could not determine a definite cause for the persistent symptoms. The very long-term post-giardiasis diarrhoea, bloating, nausea and abdominal pain documented here need further study.

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Introduction

Giardia duodenalis is a major cause of waterborne outbreaks of gastroenteritis.[1] The clinical picture varies from asymptomatic infection in more than half of infected cases to severe illness and hospitalisation.[2–4] Common symptoms include diarrhoea, flatulence, excessive fatigue, nausea, foul smelling stools, abdominal cramps and weight loss.

Giardia infection is normally a self-limiting disease with symptoms spontaneously resolving and stool samples becoming negative after around 3 weeks.[5] Symptoms may last for several weeks even after clearance of the parasite, due to acquired lactose intolerance which may occur in 20–40% of cases.[6] One study has shown that 16% of infected individuals became chronically infected.[5] These patients experience variable symptoms of loose stools, weight loss, abdominal pain, malabsorbtion and malaise. Symptoms of chronic giardiasis are often indistinguishable from irritable bowel syndrome.[7]

In autumn 2004, after a period of heavy rainfall, an outbreak of giardiasis due to contaminated drinking water occurred in the inner city of Bergen, a coastal city in Western Norway. Around 2500 cases were treated with metronidazole.[8] The outbreak subsided with few new cases after November 2004. A total of 1300 laboratory confirmed cases were reported. The outbreak strain was genotyped to be assemblage B.[9,10]

In some patients symptoms persisted even after several treatment courses with metronidazole. They were referred from general practitioners for specialist evaluation. The magnitude of the outbreak offered a unique opportunity to increase our knowledge about *Giardia* infection and its manifestations in a developed country setting. The objective of the present study was to investigate possible causes for the persisting symptoms in this group of patients.

Patients and methods

Study patients

From January 2005 to March 2006 altogether 139 patients were referred to Haukeland University Hospital and examined according to a standardised procedure at the outpatient department. These patients had:

1. Clinically suspected giardiasis with onset of symptoms (including two or more of the following five symptoms; loose stools, nausea, weight loss, fatigue, foul smelling faeces) from June 2004 until February 2005.
2. Confirmed laboratory diagnosis of giardiasis at least once during the initial illness.
3. Persisting abdominal symptoms after one or more treatment courses of metronidazole.

Twelve of the referred patients did not fulfil these criteria and were excluded from the present evaluation. One patient had never received metronidazole and 11 did not have laboratory confirmed giardiasis. Investigations concluded with inflammatory bowel disease in two patients and one patient had considerable comorbidity and could not complete the investigations. The remaining 124 patients were included in this analysis. They had received from one up to four (mean 2.0) courses of metronidazole before referral. Metronidazole is the only licensed drug for giardiasis in Norway.

Study design

This was a prospective cohort study with standardised investigations of patients with persisting symptoms following *Giardia* infection. Only findings and symptoms at the initial visit are included in this report. All examinations and investigations were performed as part of the clinical evaluation. For analysis of symptom scores, a control group of 19 individuals were recruited. All of them had had laboratory confirmed giardiasis during the outbreak, and they rapidly recovered after metronidazole treatment and they had three microscopy negative faecal samples at the time of symptom recording 12–18 months after their *Giardia* infection. The Regional ethics committee approved the data collection and analysis of the study.

Symptoms recording

The severity of abdominal symptoms was determined by an irritable bowel syndrome symptom questionnaire[11] including nausea, bloating, abdominal pain, diarrhoea, constipation and anorexia. Patients and controls graded these symptoms on an ordinal scale from 0 to 10 with 0 = no symptoms and 10 = severe symptoms.

Blood samples

Levels of haemoglobin, mean corpuscular volume, ESR, s-calcium, s-B12, s-ferritin, s-iron, s-zinc, thyroidea stimulating hormone, parathyroid hormone, immunoglobulins A, G and M, serum anti-endomysial and anti-tissue transglutaminase antibodies were measured.

Upper endoscopy

Upper endoscopy was performed in 121 patients. Biopsies were taken from the distal part of duodenum and were evaluated by an experienced pathologist blinded to other clinical information about the patients. Biopsy results were divided into three groups: normal histology (grade 0), mild pathology (grade 1) and moderate to severe pathology (grade 2). Grade 1 biopsies showed inflammation with oedema and infiltration of leukocytes and increased number of plasma cells in the lamina propria. Grade 2 biopsies showed in addition to the inflammatory changes of grade 1, architectural distortion with shortening and blunting of intestinal villi.

Duodenal content was collected after instillation of 5 ml 0.9% NaCl, and examined by microscopy for *Giardia* trophozoites after centrifugation. This was discontinued after being performed in 65 patients, as all samples until then had been negative.

Faecal samples

Three faecal samples from three different defecations, normally collected over a period of 2–3 days, were

analysed by microscopy and one sample for culture of bacterial pathogens. Conventional formalin-ether concentration technique was used before microscopy. A faecal antigen test (ImmunoCardSTAT! *Cryptosporidium*/*Giardia* rapid assay; Meridian Bioscience) was performed in the first 109 patients, but was then discontinued due to poor performance compared with microscopy (unpublished observations). Calprotectin, a marker of intestinal inflammation,[12] was analysed by the ELISA-based ϕhiCal Test (NovaTec Immundiagnostica GmbH, Germany) from one faecal sample per patient.

Statistical methods

Over time, substantial changes in the analysed parameters were observed. Data were therefore grouped after illness duration into three-month periods for analysis. Illness duration was defined as the time from onset of symptoms until investigations in this study were performed. Cases referred during the last periods were combined in some analyses, as there were few patients referred in these periods.

Fisher's exact test was used for testing independence between histology, calprotectin and *Giardia* result. Wilcoxon-Mann–Whitney's test was used for comparing blood and calprotectin tests, and for analysis of variance for the level of symptoms. Linear regression analysis was used to investigate the potential influence of *Giardia* results on the symptoms adjusted for biopsy result. Values are given as mean with standard error unless otherwise stated. Level of significance was set at $p < 0.05$. SPSS 14 was used for all statistical analyses.

Results

There were 48 men (38.7%) and 76 women (61.3%), mean age 31, ranging from 16 to 79 years (Table 1), similar to the age distribution of the patient population of the epidemic.[8] No previous abdominal complaints were stated by 85% of the patients, the rest reporting various kinds of mild to moderate abdominal symptoms, evenly distributed within age groups. In the control group there were 10 men (52.6%) and 9 women (47.4%), mean age 32, ranging from 23 to 46 years old. The population was generally healthy young adults with little co-morbidity.

Faecal samples

Giardia cysts were found by microscopy of faecal samples in 37 patients and faecal antigen test identified 3 additional patients as *Giardia* positive. Thus, chronic *Giardia* infection was found in 40 of 124 patients (32.3%). In the remaining patients both microscopy and antigen tests were negative. 37.5% of the male patients were *Giardia* positive compared to 28.9% of the female patients, the difference not being statistically significant. There were no significant differences between age groups. Almost all patients found to be *Giardia* positive (32 out of 40) presented with an illness duration of less than 7 months. The number of *Giardia* positive patients, and patients with pathological duodenal biopsies decreased with increasing illness duration (Fig. 1). Microscopy of duodenal aspirate was negative in all 65 samples where this analysis was performed, including 25 patients with *Giardia* positive faecal samples. The patient samples tested for *Cryptosporidium* were all negative, either with the antigen-test alone, or by control Ziehl-Nilsen staining of samples in cases of weakly positive antigen test.

Faecal calprotectin levels were elevated in 26 (23.6%) of the patients. Seventeen out of 39 *Giardia* positive cases (43.6%) had pathological levels compared to 9 out of 71 *Giardia* negative cases (12.7%). Positive calprotectin tests were associated with pathological biopsy ($p = 0.003$) and chronic *Giardia* infection ($p < 0.001$) (Fisher's exact test)

Table 1 Study population characteristics

	Giardia status		All[a]
	Giardia positive[a]	*Giardia* negative[a]	
Females, *n*	22 (55.0%)	54 (64.3%)	76 (61.3%)
Age 16–25 years, *n*	15 (37.5%)	35 (41.7%)	50 (40.3%)
Age 25–35 years, *n*	16 (40.0%)	25 (29.8%)	41 (33.1%)
Age 35–79 years, *n*	9 (22.5%)	24 (28.6%)	33 (26.6%)
Previous minor abdominal symptoms, *n*	7 (20.0%)	9 (12.5%)	16 (15.0%)
Disease duration (days), mean ± SE*	197 ± 16	261 ± 14	240 ± 11
Total symptoms score, mean ± SE	27.9 ± 2.2	23.3 ± 1.3	24.6 ± 1.1
Calprotectin positive (>50 mg/kg), *n**	17 (43.6%)	9 (12.7%)	26 (23.6%)
F-calprotectin mg/kg, median (75%ile)[b,*]	28 (119.0)	<20 (<20)	<20 (44.8)
Haemoglobin g/dL, mean ± SE	14.5 ± 0.2	14.1 ± 0.1	14.2 ± 0.1
s-ferritin μg/L, mean ± SE	60.8 ± 6.0	76.2 ± 7.6	71.3 ± 5.6
s-B12 pmol/L, mean ± SE*	281.8 ± 24	334.3 ± 15	316.8 ± 13
s-zinc μmol/L, mean ± SE	11.7 ± 0.3	12.5 ± 0.2	12.2 ± 0.1
ESR mm/h, mean ± SE	10.0 ± 1.6	10.0 ± 1.4	10.0 ± 1.1

*Significant differences between *Giardia* positive and *Giardia* negative with *p*-value <0.05.
[a] All percentages given as percentage of *Giardia* positive or *Giardia* negative or all.
[b] Results outside of the quantitative range were set to 10 when <20 mg/kg and to 1275 when >1275 mg/kg.

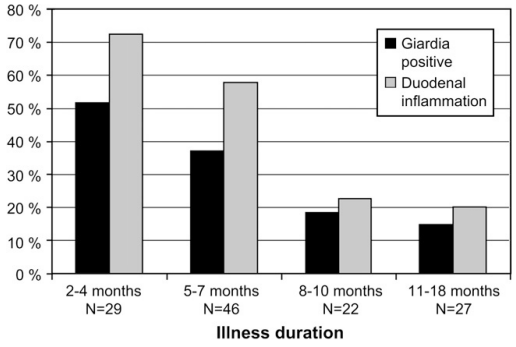

Figure 1 Percentage of patients with *Giardia* positive stool samples and microscopic duodenal inflammation by illness duration.

and were evenly distributed among sex and age groups. Faecal samples from all patients were negative for growth of bacterial pathogens.

Upper endoscopy and histology

Only occasionally were there macroscopic signs of duodenitis (Table 2). It was found more often in *Giardia* positive patients than in *Giardia* negative patients, but the difference was not significant. Microscopic duodenal inflammation was found in 72.4% of patients with illness duration of 2–4 months. In the whole cohort, 57 patients (47.1%) had either grade 1 or grade 2 pathological duodenal biopsies. A higher percentage of men (52.2%) compared to women (44.0%) had pathological duodenal biopsies, although not statistically significant. Throughout the study period, duodenal inflammation was more frequently found than *Giardia* positivity. With increasing illness duration an increasing fraction of patients had normal duodenal biopsies (Fig. 1). We found a significant association between *Giardia* positivity and pathological histology (grade 1 and 2 combined), the strongest association occurring early after the acute infection (Table 3). *Giardia* trophozoites were visible in duodenal biopsies in only 4 (10%) of the 40 cases with *Giardia* positive faecal samples.

Blood tests

One patient had IgA deficiency with non-measurable IgA levels and had visible *Giardia* trophozoites and grade 2 duodenal inflammation in the duodenal biopsy. In nine other patients the levels of IgA were below the normal range (1.00–4.10 g/L) with values in the range 0.57–0.86. Only one of these was *Giardia* positive and three had grade 2 pathological biopsies.

B12 levels were significantly lower ($p = 0.008$) in the *Giardia* positive group (mean: 281.8 ± 24 pmol/L) than in the *Giardia* negative group (mean: 334.3 ± 15 pmol/L). The normal range is 175–700 pmol/L. There was no correlation between B12 levels and duodenal inflammation. Other blood tests were generally within normal ranges, and showed neither significant differences over time nor any correlation with *Giardia* presence or with histological findings (Table 1).

Eight patients in this study had elevated anti-tissue transglutaminase antibodies (relative OD 22.3–441.7, median 29.9). Five of these patients were also positive for anti-endomysial antibodies and had pathological duodenal biopsies. Four of these were also *Giardia* positive at the time of investigation.

Symptoms

The symptom questionnaire was answered by 75 (60.5%) of patients at the time of investigation. Bloating was reported to be the most severe symptom (mean 6.2 ± 0.36), followed by diarrhoea (mean 5.6 ± 0.36), abdominal pain (mean 5.2 ± 0.31) and nausea (mean 4.1 ± 0.36). Constipation (mean 1.8 ± 0.29) and anorexia (mean 1.7 ± 0.25) were less often reported.

Total symptoms scores changed over time with the highest scores in the periods 2–4 months (mean 27.9 ± 3.2) and 5–7 months (28.0 ± 1.4). Patients with illness duration 8–10 months reported a lower total symptom score of 22.2 ± 2.9. For illness duration of 11–18 months, a total score of 17.3 ± 1.7 was reported. This was still significantly higher ($p = 0.002$) than the total symptom score in the control group which was 7.1 ± 2.2. Individual symptom scores among patients were significantly higher than symptom scores in the control group for all symptoms except for constipation (Table 4).

We analysed symptom scores from patients with illness duration 2–7 months separately, to avoid the influence of the overall decrease in symptom scores. The patients with microscopic duodenal inflammation (grade 1 and 2 combined), reported more abdominal pain ($p = 0.046$) and more diarrhoea ($p = 0.05$) than patients with normal histology. With regard to *Giardia* result or calprotectin result we found no significant differences in symptom scores.

Table 2 Histology related to *Giardia* status

	Giardia positive[a]				*Giardia* negative[b]			
	Macroscopic duodenitis	Histology			Macroscopic duodenitis	Histology		
		Normal	Grade 1	Grade 2		Normal	Grade 1	Grade 2
No of patients	6	5	13	21	5	59	9	14
Percent[c]	15.4%	12.8%	33.3%	53.8%	6.1%	72.0%	11.0%	17.1%

[a] $N = 39$ as upper endoscopy not performed in one of these patients.
[b] $N = 82$ as upper endoscopy was not performed in two of these patients.
[c] Percent of *Giardia* positive or *Giardia* negative patients where upper endoscopy was performed.

Table 3 Histological findings among *Giardia* positive and *Giardia* negative cases by illness duration

Illness duration	Duodenal biopsy pathological		Duodenal biopsy normal		*p*-value* Fisher's exact test
	Giardia positive	*Giardia* negative	*Giardia* positive	*Giardia* negative	
2–4 months	15	6	0	8	0.001
5–7 months	14	12	2	17	0.004
8–18 months	5	5	3	34	0.007

*p-value for association between *Giardia* positivity and pathological duodenal biopsy.

In patients with illness duration 8–18 months, abdominal pain was reported to be significantly more severe in *Giardia* positive patients. No significant differences were found with regard to nausea, bloating, constipation and anorexia.

Discussion

Reports on pathology in duodenal biopsies in giardiasis patients vary greatly. Different virulence of *Giardia* strains, host factors or differences in infection duration and severity may explain these variable results. Our patient population was a selection of cases with persisting symptoms, which were severe enough for the patient to be referred for specialist evaluation. Duodenal inflammation was found in 87.2% of *Giardia* positive and 28% of *Giardia* negative patients. The relatively high number of patients with abnormal duodenal histology in our study is consistent with some earlier reports of the prevalence of mucosal pathology with 13 out of 17 patients having some degree of villous shortening or inflammation.[13] However, it is much higher than results from a large Austrian study of 462 patients with *Giardia* parasites above the duodenal mucosa, where only 3.7% had microscopic duodenitis.[14] Elevated levels of calprotectin correlated well with findings of histological duodenal inflammation and chronic *Giardia* infection, supporting our findings of an increased inflammatory reaction due to giardiasis in these patients.

The outbreak strain was genotyped to be assemblage B. We are not aware of any studies of relations between genotype and duodenal inflammation, but it is an interesting possibility that assemblage B may cause a higher degree of duodenal inflammation in adults than what has been reported in previous studies where genotype was not known.

The patients in our study were adults with symptoms of bloating, diarrhoea, abdominal pain and nausea, and they had ongoing or recent assemblage B infection. There are a few studies relating *Giardia* genotype to symptoms. A study of 18 Dutch patients between 8 and 60 years of age reported patients with assemblage B infection to have a more acute and severe symptomatology with persistent diarrhoea, than patients with assemblage A who had milder, intermittent diarrhoea.[15] A study in children under 5 years of age in Western Australia found assemblage A to be correlated with diarrhoea, while the more common assemblage B was often asymptomatic.[16] Also a study from Bangladesh showed that assemblage B infection was often asymptomatic, but it was associated with a higher parasite load. Infection with the less common assemblage A

Table 4 Symptom scores of patients by *Giardia* result and symptom scores from recovered controls

Illness duration	Symptom[a]	Patients		Recovered controls
		Giardia positive	*Giardia* negative	
2–7 months		$N = 17$	$N = 28$	
	Nausea	5.1 ± 0.7	5.0 ± 0.6	na
	Bloating	6.8 ± 0.8	6.5 ± 2.9	na
	Abd. pain	6.1 ± 0.6	5.5 ± 0.6	na
	Constipation	1.8 ± 0.6	2.3 ± 0.5	na
	Diarrhoea	7.6 ± 0.5	5.9 ± 0.6	na
	Anorexia	1.5 ± 04	2.4 ± 0.5	na
8–18 months		$N = 4$	$N = 27$	$N = 19$
	Nausea	6.3 ± 2.2	2.4 ± 0.5	0.8 ± 0.4[c]
	Bloating	5.8 ± 2.1	5.5 ± 0.6	2.3 ± 0.6[c]
	Abd. pain	7.0 ± 1.1[b]	4.0 ± 0.5	1.4 ± 0.4[c]
	Constipation	0.3 ± 0.3	1.5 ± 0.5	1.5 ± 0.3
	Diarrhoea	4.3 ± 2.1	4.1 ± 0.5	1.2 ± 0.5[c]
	Anorexia	1.5 ± 0.9	1.1 ± 0.4	0.1 ± 0.1[c]

[a] Symptom scores given as mean $\pm$ SE of the mean.
[b] Abdominal pain was reported significantly more often ($p = 0.026$) among *Giardia* positive vs *Giardia* negative patients with illness duration 8–18 months, although caution should be taken as there were only 4 patients in the *Giardia* positive group.
[c] Values for patient symptom scores in both periods were significantly higher than control symptom scores except for constipation.

genotype was more often associated with diarrhoea. However, in a recent study from Ethiopia, assemblage B was found to give symptomatic infection more often than the commoner assemblage A.[17] Interestingly, two of these assemblage B subtypes were also found during the Bergen outbreak. Also in Norway assemblage A is the genotype most commonly found.[18]

In three of the studies mentioned, it is the assemblage found to be less prevalent in each community that is also found to be causing most symptoms. An inverse relationship between community prevalence and the development of symptomatic infection for any given *Giardia* genotype is an intriguing possibility.

Our study population consisted of relatively young adults, with an age distribution similar to that reported from the epidemic. One possible explanation for the frequency of persisting symptoms and duodenal inflammation could be that many of our patients most likely have never been exposed to *Giardia duodenalis*, or its assemblage B subtype before. The *Giardia* infection may therefore have elicited a different immune response in these persons compared to those who have been exposed to this antigen in childhood. Another possible cause could be a higher infective dose in this age group, due to differences in water intake habits.

The finding of inflammation in the duodenal mucosa of some *Giardia* negative patients might be caused by the intestinal mucosa recovering slowly after the infection has been cleared. Severity and duration of mucosal inflammation may be a risk factor for development of long term symptoms. This would be in line with recent research linking prolonged intestinal inflammation to the development of post-infectious functional bowel disorders.[19]

B12 deficiency has been described in giardiasis.[20,21] This agrees with our findings of lower B12 levels among the *Giardia* positive patients. IgA deficiency is a risk factor for persistent *Giardia* infection in mice[22] and this is consistent with one patient in our study who had non-measurable IgA and persistent infection. We did not find any plausible host related explanation for the lack of response to metronidazole treatment in the other cases of chronic *Giardia* infection. None of the patients had known HIV-infection or used immunosuppressive drugs. Less severe IgA deficiency seems from our results not to be associated with persistent infection, and further studies are needed to determine if it may be associated with persistent symptoms. The elevated levels of tissue transglutaminase antibodies in eight patients are interesting and further follow up may reveal if *Giardia* infection can play a role in the onset of coeliac disease.

Duodenal aspirate microscopy showed no positive results. Another study has also found duodenal aspirate to be of doubtful value in diagnosis of giardiasis compared to faecal samples with a sensitivity of 20.5% for wet mount of duodenal fluid.[23] The method of NaCl-instillation and then suction of fluid rather than direct suction may be an explanation for the poor outcome of this test in our study. The findings of negative duodenal aspirates and very few *Giardia* positive cases showing visible trophozoites in duodenal biopsies suggest a low parasite burden in the duodenum in our patient population, which may be due to long standing or subsiding infection and previous treatment attempts. This is supported by a study investigating patients with long lasting abdominal symptoms where duodenal biopsies were not a very sensitive marker of giardiasis.[24] Out of nine stool microscopy positive patients only two patients had visible *Giardia* trophozoites upon histological examination.

Increased severity of diarrhoea correlating with increasing duodenal mucosal pathology in giardiasis has been shown previously.[13] This is consistent with our results, and we also find a correlation to abdominal pain. An Italian study[25] showed that acute giardiasis may worsen symptoms in patients with a previous diagnosis of irritable bowel syndrome. However, in our study population only 15.0% reported mild to moderate previous bowel complaints. This is only slightly more than the background prevalence of irritable bowel syndrome of around 10% in our patients' age group in Norway.[26]

Microscopy of three stool samples for *Giardia* cysts has a sensitivity of 85–90%.[27,28] The faecal-antigen test did not improve the diagnostic yield (data not shown). In the present study 85% of patients with illness duration of 11 months or more were found to be *Giardia* negative. Most of them had been tested repeatedly with *Giardia* negative faecal samples in the time before referral. We are therefore convinced that these patients are truly no longer harbouring *Giardia* parasites. Symptoms have been reported to resolve in 60–70% of patients with chronic giardiasis when the parasites have been successfully eradicated.[29,30] Persistent abdominal symptoms in travellers, where *Giardia* may be an etiological agent, has been described,[31] but to our knowledge no long term follow-up or description of post-treatment symptomatic *Giardia* negative cases has been published.

We studied 124 patients presenting over a period of $1\frac{1}{4}$ year with persistent abdominal symptoms after a *Giardia* outbreak. In the first months of this study many patients were found to have persisting *Giardia* infection and even more patients had duodenal microscopic inflammation. These findings subsided over time and patients increasingly presented without detectable *Giardia* parasites.

With the investigations performed in this study, we have not been able to positively identify the cause of the very prolonged abdominal symptoms in the *Giardia* negative patients. Prolonged lactose intolerance, an undiagnosed pathogen, bacterial overgrowth, a change in mucosal architecture, or development of a post-infectious functional bowel disorder are possible explanations that should be considered. Studies to further explore this phenomenon are needed.

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References

1. Craun GF, Calderon RL, Craun MF. Outbreaks associated with recreational water in the United States. *Int J Environ Health Res* 2005;**15**(4):243–62.
2. Nash TE, Herrington DA, Losonsky GA, Levine MM. Experimental human infections with *Giardia lamblia*. *J Infect Dis* 1987; **156**(6):974–84.
3. Lopez CE, Dykes AC, Juranek DD, Sinclair SP, Conn JM, Christie RW, et al. Waterborne giardiasis: a community wide outbreak of disease and a high rate of asymptomatic infection. *Am J Epidemiol* 1980;**112**(4):495–507.
4. Faubert G. Immune response to *Giardia duodenalis*. *Clin Microbiol Rev* 2000;**13**(1):35–54 [table].
5. Rendtorff RC. The experimental transmission of human intestinal protozoan parasites. II. *Giardia lamblia* cysts given in capsules. *Am J Hyg* 1954;**59**(2):209–20.
6. Farthing MJ. Giardiasis. *Gastroenterol Clin North Am* 1996; **25**(3):493–515.
7. Stark D, van Hal S, Marriott D, Ellis J, Harkness J. Irritable bowel syndrome: a review on the role of intestinal protozoa and the importance of their detection and diagnosis. *Int J Parasitol* 2007;**37**(1):11–20.
8. Nygard K, Schimmer B, Sobstad O, Walde AK, Tveit I, Langeland N, et al. A large community outbreak of waterborne giardiasis-delayed detection in a non-endemic urban area. *BMC Public Health* 2006;**6**(1):141.
9. Robertson LJ, Hermansen L, Gjerde BK, Strand E, Alvsvag JO, Langeland N. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. *Appl Environ Microbiol* 2006;**72**(3): 2212–7.
10. Thompson RC, Monis PT. Variation in Giardia: implications for taxonomy and epidemiology. *Adv Parasitol* 2004;**58**:69–137.
11. Kane SV, Sandborn WJ, Rufo PA, Zholudev A, Boone J, Lyerly D, et al. Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. *Am J Gastroenterol* 2003; **98**(6):1309–14.
12. Costa F, Mumolo MG, Bellini M, Romano MR, Ceccarelli L, Arpe P, et al. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. *Dig Liver Dis* 2003;**35**(9): 642–7.
13. Duncombe VM, Bolin TD, Davis AE, Cummins AG, Crouch RL. Histopathology in giardiasis: a correlation with diarrhoea. *Aust N Z J Med* 1978;**8**(4):392–6.
14. Oberhuber G, Kastner N, Stolte M. Giardiasis: a histologic analysis of 567 cases. *Scand J Gastroenterol* 1997;**32**(1):48–51.
15. Homan WL, Mank TG. Human giardiasis: genotype linked differences in clinical symptomatology. *Int J Parasitol* 2001;**31**(8): 822–6.
16. Read C, Walters J, Robertson ID, Thompson RC. Correlation between genotype of *Giardia duodenalis* and diarrhoea. *Int J Parasitol* 2002;**32**(2):229–31.
17. Gelanew T, Lalle M, Hailu A, Pozio E, Caccio SM. Molecular characterization of human isolates of *Giardia duodenalis* from Ethiopia. *Acta Trop* 2007;**102**(2):92–9.
18. Robertson LJ, Hermansen L, Gjerde BK. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. *Appl Environ Microbiol* 2006;**72**(8):5297–303.
19. Tornblom H, Abrahamsson H, Barbara G, Hellstrom PM, Lindberg G, Nyhlin H, et al. Inflammation as a cause of functional bowel disorders. *Scand J Gastroenterol* 2005;**40**(10): 1140–8.
20. Cowen AE, Campbell CB. Giardiasis—a cause of vitamin B 12 malabsorption. *Am J Dig Dis* 1973;**18**(5):384–90.
21. Olivares JL, Fernandez R, Fleta J, Ruiz MY, Clavel A. Vitamin B12 and folic acid in children with intestinal parasitic infection. *J Am Coll Nutr* 2002;**21**(2):109–13.
22. Langford TD, Housley MP, Boes M, Chen JZ, Kagnoff MF, Gillin FD, et al. Central importance of immunoglobulin a in host defense against Giardia spp. *Infect Immun* 2002;**70**(1): 11–8.
23. Wahnschaffe U, Ignatius R, Loddenkemper C, Liesenfeld O, Muehlen M, Jelinek T, et al. Diagnostic value of endoscopy for the diagnosis of giardiasis and other intestinal diseases in patients with persistent diarrhea from tropical or subtropical areas. *Scand J Gastroenterol* 2007;**42**(3):391–6.
24. Grazioli B, Matera G, Laratta C, Schipani G, Guarnieri G, Spiniello E, et al. *Giardia lamblia* infection in patients with irritable bowel syndrome and dyspepsia: a prospective study. *World J Gastroenterol* 2006;**12**(12):1941–4.
25. D'Anchino M, Orlando D, De Feudis L. *Giardia lamblia* infections become clinically evident by eliciting symptoms of irritable bowel syndrome. *J Infect* 2002;**45**(3):169–72.
26. Vandvik PO, Lydersen S, Farup PG. Prevalence, comorbidity and impact of irritable bowel syndrome in Norway. *Scand J Gastroenterol* 2006;**41**(6):650–6.
27. Goka AK, Rolston DD, Mathan VI, Farthing MJ. The relative merits of faecal and duodenal juice microscopy in the diagnosis of giardiasis. *Trans R Soc Trop Med Hyg* 1990;**84**(1):66–7.
28. Hiatt RA, Markell EK, Ng E. How many stool examinations are necessary to detect pathogenic intestinal protozoa? *Am J Trop Med Hyg* 1995;**53**(1):36–9.
29. Oberhuber G, Stolte M. Symptoms in patients with giardiasis undergoing upper gastrointestinal endoscopy. *Endoscopy* 1997;**29**(8):716–20.
30. Chester AC, MacMurray FG, Restifo MD, Mann O. Giardiasis as a chronic disease. *Dig Dis Sci* 1985;**30**(3):215–8.
31. Stermer E, Lubezky A, Potasman I, Paster E, Lavy A. Is traveler's diarrhea a significant risk factor for the development of irritable bowel syndrome? A prospective study. *Clin Infect Dis* 2006;**43**(7):898–901.

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Development of functional gastrointestinal disorders after *Giardia lamblia* infection

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Abstract

Background: Functional gastrointestinal disorders (FGID) may occur following acute gastroenteritis. This long-term complication has previously not been described after infection with the non-invasive protozoan *Giardia lamblia*. This study aims to characterize persistent abdominal symptoms elicited by *Giardia* infection according to Rome II criteria and symptoms scores.

Methods: Structured interview and questionnaires 12–30 months after the onset of *Giardia* infection, and at least 6 months after *Giardia* eradication, among 82 patients with persisting abdominal symptoms elicited by the *Giardia* infection. All had been evaluated to exclude other causes.

Results: We found that 66 (80.5%) of the 82 patients had symptoms consistent with irritable bowel syndrome (IBS) and 17 (24.3%) patients had functional dyspepsia (FD) according to Rome II criteria. IBS was sub classified into D-IBS (47.0%), A-IBS (45.5%) and C-IBS (7.6%). Bloating, diarrhoea and abdominal pain were reported to be most severe. Symptoms exacerbation related to specific foods were reported by 45 (57.7%) patients and to physical or mental stress by 34 (44.7%) patients.

Conclusion: In the presence of an IBS-subtype pattern consistent with post-infectious IBS (PI-IBS), and in the absence of any other plausible causes, we conclude that acute *Giardia* infection may elicit functional gastrointestinal diseases with food and stress related symptoms similar to FGID patients in general.

Background

Long term abdominal symptoms may develop after acute gastroenteritis and was first described in 1962 [1]. These symptoms are clinically similar to functional gastrointestinal diseases (FGID) and may be classified using the Rome II criteria for such illnesses. Symptoms often fulfil the criteria for irritable bowel syndrome (IBS) and the term post-infectious irritable bowel syndrome (PI-IBS) is often used for this condition [2]. It has been shown to occur following viral, bacterial and amoebic gastroenteritis and after trichinellosis [1,3,4]. A meta-analysis found the odds of developing irritable bowel syndrome (IBS) to be increased sixfold after acute gastroenteritis [5]. Previously, *Giardia* infection has been found to trigger abdominal symptoms in patients with established IBS[6], and *Giardia* should be ruled out as a possible cause in patients

with IBS-like symptoms[7]. However, post-infectious functional gastrointestinal diseases elicited by infection with the non-invasive protozoan *Giardia lamblia* have not been described before. Similarly, the relation of patients' abdominal symptoms and food types and the influence of physical or mental stress have been well researched among IBS-patients in general[8,9], but little data exists regarding such relations in post-infectious FGIDs.

Giardia lamblia is a parasite of the small intestine occurring endemically, or as the cause of waterborne outbreaks. The parasite is commonly found in children in developing countries and in travellers to endemic regions. It causes infections varying from asymptomatic to protracted and severe illness with diarrhoea, weight loss and malabsorbtion[10]. After an outbreak in autumn 2004 of assemblage B giardiasis in Bergen, Norway, many patients experienced long-lasting abdominal symptoms despite one or several courses of metronidazole. They were referred to the local university hospital for evaluation. Extensive work-up revealed a surprisingly high rate of duodenal inflammation both in patients with and without evidence of chronic infection [11]. All *Giardia* positive patients were treated successfully[12]. However, symptoms remained in many patients despite eradication of the parasite, and they were followed up regularly.

The main aim of this study was to evaluate the abdominal symptoms according to the Rome II criteria for FGID among patients with persisting abdominal symptoms 12–30 months after the onset of *Giardia* infection, and more than 6 months after *Giardia* eradication. Secondarily, we included some questions about the symptoms relation to food types and stress.

Methods

This is a prospective study describing the results of questionnaires and structured interviews during follow-up visits of patients with persisting symptoms following *Giardia* infection. The data were collected during a 16 month period from January 2006 until April 2007.

All 82 patients included in this study had laboratory confirmed giardiasis during an outbreak in 2004. They had been successfully treated and were confirmed *Giardia* negative by microscopy of three or more faecal samples at least 6 months prior to inclusion in this study. An extensive work-up including upper endoscopy with duodenal biopsies, routine blood screening tests, immunoglobulins, IgE, serum anti-endomysial, anti-tissue transglutaminase antibodies and faecal calprotectin had not produced any findings to explain their symptoms [11]. In 11 patients included in this study, repeated routine duodenal biopsies were taken approximately one year after the initial work-up, and these were reviewed with regard to inflammatory changes.

After the initial extensive work-up, patients came for follow up visits where, in addition to routine clinical examination, a structured interview was performed with regard to previous abdominal symptoms, and symptom exacerbation related to food types or physical or mental stress. In the interview most patients denied previous abdominal illness, while 14 (17.1%) had experienced some previous abdominal symptoms. All these 14 patients reported considerable more abdominal problems after *Giardia* infection than they had had before. Only 5 of them reported previous contact with a physician about their abdominal problems. For clarity we chose to give results for patients without previous abdominal complaints separately in the analysis.

Patients also filled in a questionnaire regarding their current abdominal symptoms allowing evaluation of IBS according to the Rome II criteria[13]. As it became evident that functional dyspepsia (FD) was occurring in our patient population, Rome II FD criteria questions were included in the questionnaires. IBS patients were sub classified into diarrhoea-predominant IBS (D-IBS: two or more diarrhoea symptoms and a maximum of one symptom of constipation), constipation-predominant IBS (C-IBS: two or more constipation symptoms and a maximum of one symptom of diarrhoea) and alternating IBS (A-IBS: all subjects with IBS not qualifying for D-IBS or C-IBS). Symptom severity, for the last month, of nausea, bloating, abdominal pain, diarrhoea, constipation and anorexia was quantified by patients grading these symptoms on an ordinal scale from 0 – 10 with 0 = no symptoms and 10 = severe symptoms.

Statistical analysis was performed using SPSS 16.0. The Regional ethics committee and the Norwegian Social Science Data Services approved the data collection and analysis of the data.

Results

There were 52 females (63.4%) and the mean age was 31.8 years (range 18–61). Analysis of the collected data showed a high frequency of patient's symptoms fulfilling the Rome II IBS criteria with D-IBS and A-IBS of similar prevalence (Table 1). IBS-C was uncommon. Regarding FD, 17 cases were identified and 10 of these also had concurrent IBS. The 6 cases fulfilling neither FD nor IBS criteria could be put into one or more of the Rome II categories; functional abdominal pain, functional diarrhoea and functional bloating.

No significant sex differences were found regarding IBS subtypes, symptom scores, influence of stress or subjective food intolerance, only trends towards more FD among females ($p = 0.09$) and more females reporting previous abdominal symptoms ($p = 0.07$). Patients with FD had similar symptom scores as non-FD patients except for the

Table 1: Frequency of FGID and relation of abdominal symptoms to food types and stress

	Patients **without** previous abdominal symptoms N = 68		All patients N = 82	
	N	%	*N*	%
IBS all subtypes	54	79.4	66	80.5
D – IBS[1]	25	46.3	31	47.0
A – IBS[1]	27	50.0	30	45.5
C – IBS[1]	3	5.6	5	7.6
FD[2]	14	21.9	17	24.3
IBS & FD[2]	8	12.5	10	14.3
Other FGID (not IBS/FD)	6	8.8	6	7.3
Food related symptoms[3]	37	56.1	45	57.7
Stress related symptoms[4]	26	40.6	34	44.7

[1] Percentages within subgroup with IBS
[2] 70 patients with available FD data
[3] 78 patients with available food related data
[4] 76 patients with available stress related data

constipation score which was 2.85 in FD patients and 0.65 in non-FD patients with a p-value < 0.001.

Bloating was the symptom reported to be most severe (Table 2). In fact only two patients reported nil bloating. Symptoms of diarrhoea and constipation varied consistently with IBS subtype. No gender differences were found, only a trend towards nausea being more severe among females (p = 0.08).

The question of food related worsening of symptoms was answered by 78 patients. The majority of patients (57.7%) reported their post-giardiasis abdominal problems to worsen after intake of certain food items. They were asked to name the food items they had begun to avoid. Milk and milk products were mentioned spontaneously by 27% of the patients. Other common food items mentioned were alcohol containing beverages (18.4%), wheat flour products (14.5%) and coffee (6%).

In 76 patients who responded to the question about the influence of physical or mental stress on their abdominal symptoms, 44.7% felt that their abdominal illness was worsened by stress. No correlations were found between IBS-subtypes and symptoms exacerbation related to stress and food types.

Signs of inflammation with infiltration of inflammatory cells, with or without shortening and blunting of intestinal villi, were found in 8 out of 11 of these patients during the first workup in spring 2005. One year later, repeated routine duodenal biopsies were normal in ten and improved in one of these patients, although all these patients still had troubling abdominal symptoms (data not shown).

Discussion

Clinical characteristics of a relatively large number of patients seeking help for long lasting abdominal symptoms after *Giardia* infection are described in this study. An extensive follow-up of these patients over three years has not revealed any specific illness, which can explain the symptoms seen in our study population. The prolonged symptoms in parasitologically successfully treated patients came as a surprise, as such complications have not been described after *Giardia* infection before.

Table 2: Symptom scores in IBS subtypes and in the group with other FGIDs.

Symptom	**D-IBS** N = 31	**A-IBS** N = 30	**C-IBS** N = 5	**Other FGID** N = 16	**All** N = 82
Nausea	2.8 ± 0.4	3.1 ± 0.5	3.4 ± 1.5	3.0 ± 0.8	3.0 ± 0.3
Bloating	6.2 ± 0.4	7.0 ± 0.4	8.0 ± 0.8	5.4 ± 0.8	6.4 ± 0.2
Abd.pain	3.9 ± 0.5	4.6 ± 0.4	5.2 ± 1.0	3.8 ± 0.6	4.2 ± 0.3
Constipation	1.7 ± 0.5	3.1 ± 0.5	3.8 ± 1.2	0.9 ± 0.3	2.2 ± 0.3
Diarrhea	5.4 ± 0.4	4.5 ± 0.5	3.4 ± 1.0	4.3 ± 0.7	4.8 ± 0.3
Anorexia	2.0 ± 0.5	1.5 ± 0.4	2.4 ± 0.8	1.1 ± 0.5	1.7 ± 0.3
Total score	22.1 ± 1.5	23.9 ± 1.6	26.2 ± 5.0	19.1 ± 2.1	22.3 ± 1.0

Values given are mean scores ± standard error.

There have been several *Giardia* outbreaks described, among others in Solna, Sweden[14], Creston, Canada [15] and Aspen highlands, USA[16]. However, no follow-up studies have looked at persistent symptoms after eradication of the parasite. An epidemiological study in Michigan, USA[17] did not find any link between *Giardia* infection and IBS, by correlating new *Giardia* cases with prescriptions of three drugs (dicyclomine, tegaserod and alosetron) used in IBS. In our study population any such correlation would probably also not have been found as patients were previously largely healthy, active young people unaccustomed to taking drugs daily. Only a few had abdominal complaints of a severity that any of these medications would be considered.

In our study population we find a pattern of IBS-subtypes with a high frequency of diarrhoeal symptoms and little constipation. This agrees well with previous descriptions of PI-IBS as a distinctive subgroup of IBS patients [18]. In the general population in Norway a recent study with 4622 respondents showed that 10% in the relevant age group fulfilled the IBS criteria and the pattern of subtypes contrasts our findings with subtype A-IBS most commonly reported (53%), followed by similar prevalence of the other two subtypes D-IBS (23%) and C-IBS (24%) [19].

It is known that *Giardia* may cause prolonged symptoms for several weeks after successful treatment due to secondary lactose intolerance [20]. In the present study we found that many patients reported many different kinds of food to worsen symptoms and that this persisted for years after infection. A previous study has not found lactose-intolerance to be factor in the aetiology of PI-IBS after bacterial gastroenteritis[21]. Preliminary data from duodenal lactase activity testing in newly referred patients with post-giardiasis IBS at our hospital support this finding (unpublished).

It is known that food-related gastrointestinal symptoms are common in the general population and often coincides with IBS. A previous study found that 51% of IBS-patient considered that their symptoms were linked to individual foods [8], and improvement following exclusion diets have also been reported [22]. However, objective measurements methods, like skin prick test or intestinal permeability have not been consistent with the reported food-intolerance [23]. Outside of this study many of the patients were referred to allergologic evaluation with no specific findings. It thus seems that the food-related symptoms seen in our patient population may be intrinsically linked with the development of FGID.

The finding that around half the patients felt that physical or mental stress influence their abdominal symptoms is consistent with previous findings in patients with IBS of all causes seeking medical care in Norway [9].

It remains speculative what may be the reason for development of FGID after *Giardia* infection. Previous studies have pointed to psychological factors, young age, and severity and duration of the acute infection as factors increasing the risk of FGID after bacterial gastroenteritis [2,5]. We showed in a former study that a high frequency of microscopic duodenal inflammation was found in our study population when illness duration was 2–4 months, indicating that the severity of host response may be a risk factor for FGID in our population [11]. The initially high frequency of duodenal microscopic inflammation normalised within a year, but subtle low-grade inflammation, not recognised by routine microscopy might be ongoing.

The particular genotype of *Giardia* responsible for the epidemic may also be a relevant risk factor in itself, as different strains have been shown to differ in their ability to induce small intestinal injury in rats [24]. The higher proportion of females in our study population is probably not indicating an increased risk, but rather a reflection of more females contracting giardiasis during the epidemic, probably as a consequence of higher water intake [25]. Host factors like age, no previous *Giardia* exposure or infection, pre-existing intestinal microbiota, immunologic and genetic predisposition may also play a role. These will be interesting issues for further research.

All patients were treated with one or more courses of metronidazole for their giardiasis. Although one study points to a link between antibiotic treatment in general and FGID[26], prolonged gastrointestinal symptoms is not reported as a side effect of metronidazole. Although we can not fully exclude this possibility, we think the *Giardia* infection is a far more plausible cause of the later FGID than its treatment.

A strength of this study is the relatively large number of laboratory confirmed giardiasis cases who have been thoroughly investigated and followed for a long period of time. However, it is therefore also important to note that our data are drawn from a population likely to be the more severely affected, and may not represent patients with milder post-giardiasis abdominal symptoms. A control group presented with the same follow-up and questionnaire would have been desirable.

Patients were asked about previous abdominal complaints retrospectively, one to two years after their *Giardia* infection. Recall bias might influence the answers given. Some caution should also be taken regarding the exclusion of chronic giardiasis. Microscopy of three stool samples for *Giardia* cysts has a sensitivity of 85 – 90%[27].

Our patient population had repeated series of *Giardia* negative faecal samples before referral and during the hospital workup, thus we consider the risk of low-grade chronic infections to be very small.

Conclusion

Due to the subtype pattern consistent with PI-IBS, no other discernable cause, and symptoms being elicited by a symptomatic, laboratory confirmed *Giardia* infection, we here document for the first time that the non-invasive protozoan pathogen *Giardia lamblia* may induce irritable bowel syndrome and functional dyspepsia. Utilising the unique setting of around 1300 laboratory confirmed *Giardia* cases, we are planning follow up studies of the frequency and characteristics of FGID after *Giardia* infection including population based approaches.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Data collection was done by KH and VD. TH and NL supervised the study. All participated in the writing and finalisation of the paper. All authors have approved the final draft submitted.

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References

1. Chaudhary NA, Truelove SC: **The irritable colon syndrome. A study of the clinical features, predisposing causes, and prognosis in 130 cases.** *Q J Med* 1962, **31:**307-322.
2. Spiller RC: **Is IBS caused by infectious diarrhea?** *Nat Clin Pract Gastroenterol Hepatol* 2007, **4:**642-643.
3. Marshall JK, Thabane M, Garg AX, Clark WF, Salvadori M, Collins SM: **Incidence and epidemiology of irritable bowel syndrome after a large waterborne outbreak of bacterial dysentery.** *Gastroenterology* 2006, **131:**445-450.
4. Soyturk M, Akpinar H, Gurler O, Pozio E, Sari I, Akar S, *et al.*: **Irritable bowel syndrome in persons who acquired trichinellosis.** *Am J Gastroenterol* 2007, **102:**1064-1069.
5. Thabane M, Kottachchi DT, Marshall JK: **Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome.** *Aliment Pharmacol Ther* 2007, **26:**535-544.
6. D'Anchino M, Orlando D, De Feudis L: **Giardia lamblia infections become clinically evident by eliciting symptoms of irritable bowel syndrome.** *J Infect* 2002, **45:**169-172.
7. Grazioli B, Matera G, Laratta C, Schipani G, Guarnieri G, Spiniello E, *et al.*: **Giardia lamblia infection in patients with irritable bowel syndrome and dyspepsia: a prospective study.** *World J Gastroenterol* 2006, **12:**1941-1944.
8. Simren M, Mansson A, Langkilde AM, Svedlund J, Abrahamsson H, Bengtsson U, *et al.*: **Food-related gastrointestinal symptoms in the irritable bowel syndrome.** *Digestion* 2001, **63:**108-115.
9. Vandvik PO, Wilhelmsen I, Ihlebaek C, Farup PG: **Comorbidity of irritable bowel syndrome in general practice: a striking feature with clinical implications.** *Aliment Pharmacol Ther* 2004, **20:**1195-1203.
10. Ortega YR, Adam RD: **Giardia: Overview and update.** *Clinical Infectious Diseases* 1997, **25:**545-549.
11. Hanevik K, Hausken T, Morken MH, Strand EA, Morch K, Coll P, *et al.*: **Persisting symptoms and duodenal inflammation related to Giardia duodenalis infection.** *J Infect* 2007, **55:**524-530.
12. Morch K, Hanevik K, Robertson LJ, Strand EA, Langeland N: **Treatment-ladder and genetic characterisation of parasites in refractory giardiasis after an outbreak in Norway.** *J Infect* 2008, **56:**268-273.
13. Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA: **Functional bowel disorders and functional abdominal pain.** *Gut* 1999, **45(Suppl 2):**II43-II47.
14. Ljungstrom I, Castor B: **Immune response to Giardia lamblia in a water-borne outbreak of giardiasis in Sweden.** *J Med Microbiol* 1992, **36:**347-352.
15. Isaac-Renton JL, Cordeiro C, Sarafis K, Shahriari H: **Characterization of Giardia duodenalis isolates from a waterborne outbreak.** *J Infect Dis* 1993, **167:**431-440.
16. Istre GR, Dunlop TS, Gaspard GB, Hopkins RS: **Waterborne giardiasis at a mountain resort: evidence for acquired immunity.** *Am J Public Health* 1984, **74:**602-604.
17. Penrose AS, Wells EV, Aiello AE: **Infectious causation of chronic disease: examining the relationship between Giardia lamblia infection and irritable bowel syndrome.** *World J Gastroenterol* 2007, **13:**4574-4578.
18. Dunlop SP, Jenkins D, Spiller RC: **Distinctive clinical, psychological, and histological features of postinfective irritable bowel syndrome.** *Am J Gastroenterol* 2003, **98:**1578-1583.
19. Vandvik PO, Lydersen S, Farup PG: **Prevalence, comorbidity and impact of irritable bowel syndrome in Norway.** *Scand J Gastroenterol* 2006, **41:**650-656.
20. Farthing MJ: **Giardiasis.** *Gastroenterol Clin North Am* 1996, **25:**493-515.
21. Parry SD, Barton JR, Welfare MR: **Is lactose intolerance implicated in the development of post-infectious irritable bowel syndrome or functional diarrhoea in previously asymptomatic people?** *Eur J Gastroenterol Hepatol* 2002, **14:**1225-1230.
22. Nanda R, James R, Smith H, Dudley CR, Jewell DP: **Food intolerance and the irritable bowel syndrome.** *Gut* 1989, **30:**1099-1104.
23. Dainese R, Galliani EA, De Lazzari F, Di LV, Naccarato R: **Discrepancies between reported food intolerance and sensitization test findings in irritable bowel syndrome patients.** *Am J Gastroenterol* 1999, **94:**1892-1897.
24. Cevallos A, Carnaby S, James M, Farthing JG: **Small intestinal injury in a neonatal rat model of giardiasis is strain dependent.** *Gastroenterology* 1995, **109:**766-773.
25. Nygard K, Schimmer B, Sobstad O, Walde AK, Tveit I, Langeland N, *et al.*: **A large community outbreak of waterborne giardiasis-delayed detection in a non-endemic urban area.** *BMC Public Health* 2006, **6:**141.
26. Maxwell PR, Rink E, Kumar D, Mendall MA: **Antibiotics increase functional abdominal symptoms.** *Am J Gastroenterol* 2002, **97:**104-108.
27. Goka AK, Rolston DD, Mathan VI, Farthing MJ: **The relative merits of faecal and duodenal juice microscopy in the diagnosis of giardiasis.** *Trans R Soc Trop Med Hyg* 1990, **84:**66-67.

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IV

Immunophenotyping and *Giardia* specific immunity in post-giardiasis functional gastrointestinal disease and chronic fatigue syndrome

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38 word summary:
Patients with post-infectious functional gastrointestinal disorders and chronic fatigue syndrome five years after *Giardia* infection showed alterations in NK-cell and CD8-cell populations indicating ongoing immune dysfunction. Specific anti-*Giardia* cellular immune responses were similar to *Giardia* exposed healthy controls.

Key words: *Giardia lamblia*; functional gastrointestinal disorder; chronic fatigue syndrome; lymphocytes; immune response

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Abstract

Background: A *Giardia* outbreak was associated with development of post-infectious functional gastrointestinal disorders (PI-FGID) and chronic fatigue syndrome (PI-CFS). Markers of immune dysfunction have given conflicting results in CFS and FGID patient populations. The role of specific cellular immunity against the eliciting pathogen has not been assessed in PI-FGID or PI-CFS before. The aim of this study was to evaluate specific anti-*Giardia* cellular immunity and markers of immune dysfunction in these two post-infectious syndromes.

Methods: 48 patients, reporting chronic fatigue in a questionnaire study three years after the outbreak, were clinically evaluated five years after the outbreak and grouped according to Fukuda criteria for CFS (n=19) and idiopatic chronic fatigue (n=5) and Rome II criteria for FGIDs (n=54). 22 *Giardia* exposed non-fatigued individuals and 10 healthy unexposed individuals were recruited as controls. Peripheral blood lymphocyte subsets were analyzed by flow cytometry and *Giardia* specific immune responses were evaluated by flow cytometric and ^{3}H-thymidine proliferation assays.

Results: No differences in *Giardia* specific cellular immune responses were found between *Giardia* exposed controls and PI-FGID or PI-CFS patients. However, in peripheral blood we found significantly higher CD8 T-cell levels in PI-FGID, and significantly lower NK-cell levels in PI-CFS patients. Tendencies towards lower T-cell CD26 expression in PI-FGID and higher CD4 T-cell HLA-DR expression in PI-CFS were seen.

Conclusion: Patients with PI-CFS and/or PI-FGID 5 years after *Giardia lamblia* infection showed alterations in NK-cell and CD8-cell populations indicating ongoing immune dysfunction, but specific anti-*Giardia* cellular immune responses were similar to *Giardia* exposed no-fatigue controls.

INTRODUCTION

Infection with the protozoan intestinal parasite *Giardia lamblia* is common in developing countries and is often seen in travellers returning from endemic areas [1]. It is also a frequent cause of waterborne outbreaks in industrialized countries, but it is generally regarded as an uncomplicated infection for which there is effective antibiotic treatment. Although long term abdominal symptoms following acute giardiasis have been observed by clinicians in individual patients for decades, post giardiasis functional gastrointestinal disorders (FGIDs) [2] and chronic fatigue have only recently been described [3] after a waterborne outbreak in Bergen in 2004 [4].

FGIDs are a group of disorders characterized by recurring or chronic gastrointestinal symptoms without an identifiable disease process [5]. Irritable bowel syndrome (IBS) and functional dyspepsia (FD) are the most well described FGIDs. Fatigue is a frequent symptom in FGID patients [6]. One study found that 14% of IBS patients also have chronic fatigue syndrome CFS [7], while six studies report that 51% (median) of (CFS) patients also have IBS. FGID and CFS share the characteristics of female preponderance, both are diagnoses relying on symptom criteria alone, and in many cases the onset is preceded by an acute infection [8]. Researchers of both FGID and CFS focus on the same pathophysiologic mechanisms [9, 10], but rarely control for this co-morbidity.

When the onset of FGID or CFS is associated with an acute infection, it is often termed post-infectious CFS (PI-CFS) [11, 12] or FGID (PI-FGID) [13] or in the case of IBS, post-infectious IBS (PI-IBS) [10, 14]. Until recently few studies separated between infection related onset and a less defined onset in these disorders. A meta-analysis of PI-IBS estimates that the risk of having IBS one year after an acute gastroenteritis is approximately sixfold [15].

The role of specific cellular immunity against the eliciting microbe has not been evaluated in PI-CFS or PI-FGID. The present study, performed in a well-defined group of patients with clinically observed post-infectious FGID and CFS after a common eliciting infection, was done with two main aims;

1. to evaluate a wide selection of immune dysfunction markers reported in lymphocyte subsets and

2. to examine the possible role of host specific cellular immunity against the culprit infectious agent *G. lamblia*.

METHODS

Study population

In 2007, three years after the outbreak, all 1252 persons with microscopy confirmed *Giardia* infection during the outbreak received a questionnaire regarding fatigue and abdominal complaints [3]. Five years after the outbreak 253 persons reporting chronic fatigue in this study received a mailed invitation, and 53 of these chose to participate in the present study, see figure 1. These patients went through a clinical evaluation and were screened with a battery of routine blood tests and a magnetic resonance imaging (MRI) brain scan. Additionally, 22 sex and age matched controls were recruited among the respondents, and 10 healthy *Giardia* unexposed healthy controls were also included. All were HIV negative and were not taking immunomodulatory or antibiotic medications. Written consent was obtained from the participants. The study was approved by the Regional Committee for Ethics in Medical Research and the Norwegian Social Society Data Services in Bergen, Norway.

Sampling

Patient and control blood samples for routine exclusion of other diseases and for this study were taken between 08am and 09am after overnight fast and analyzed in parallel during the same period. Fecal samples were screened with microscopic examination and 18S PCR [16] of feces to rule out chronic giardiasis.

Questionnaires

Both patients and controls completed a Rome II questionnaire [5] enabling the classification of abdominal symptoms into IBS and FD or other FGID. An abdominal symptom scoring form was used to quantify abdominal symptoms [17]. Questions about abdominal complaints before the *Giardia* infection enabled identification of post-infectious FGIDs.

To evaluate severity of fatigue, patients and controls also completed the Fatigue Questionnaire [18], a validated set of 11 questions addressing different aspects of fatigue.

FGID and fatigue categorization

Patients and controls were classified according to the Rome II questionnaire with regard to FGID. Patients were clinically evaluated by specialists in internal medicine, psychiatry and neurology and were classified as PI-CFS or PI-idiopathic chronic fatigue (ICF) according to the 1994 Fukuda criteria [19], or as fatigue most plausibly due to other causes, or as recovered. In the PI-CFS group one patient was diagnosed with dysthymia and one with agoraphobia and in the PI-ICF group there were no psychiatric comorbidity. Patients who had co-morbidities that could plausibly explain their fatigue were termed “fatigue other cause”. Some individuals had fully recovered from the fatigue, which they had reported in the questionnaire two years previously, and were termed “fatigue recovered”.

Three different categories of patient groups were used in analyses of the relative importance of CFS and FGID co-morbidity on immunological variables; a fatigue category, a FGID category and a combined category (Table 1). In the fatigue category, exploratory analyses of lymphocyte subsets in the small group of patients with PI-ICF (n=5) showed similarities with the PI-CFS group. The two groups were therefore analyzed together unless otherwise clearly stated in the text.

Regarding the FGID division, a number of patients’ symptoms did not match either the FD or the IBS criteria. These would qualify for the less well-defined FGIDs like functional bloating, functional diarrhea, functional abdominal pain and unspecified functional bowel disorder. For simplicity they were termed “other FGID”, and in some analyses they were grouped together

with the IBS and FD groups forming an “all FGIDs” group. Three individuals had both FD and IBS and were grouped as IBS in the analysis. Some exposed non-fatigued controls also had FGID and were put together with the FGID group in some analyses. Seven of the participants had FGID-like abdominal symptoms also before their *Giardia* infection. In some analyses these were taken out in order to analyze PI-IBS and PI-FGID specifically.
In the analyses we also defined a combined category with one group of participants who had both FGID and CFS/ICF, one group who had FGID only, and one group with fatigue other cause with or without fatigue. These groups were compared to each other and to healthy controls with neither FGID nor fatigue.

Lymphocyte quantification

Lymphocyte subpopulation quantification was performed using the BDMultitest6-colorTBNK kit with BD Trucount Tubes for relative and absolute concentration determination (BD Biosciences, San Jose, CA, USA). The samples were prepared according to the manufacturer’s instructions and analyzed on a BD Canto II flow cytometer (BD Biosciences).

PBMC acquisition and immunophenotyping

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation from BD Vacutainer Na-citrate CPT tubes (BD, Franklin Lakes, NJ, USA). After harvesting, the PBMC were washed twice in PBS and were immunophenotyped as follows, or were cultured using the specific immunity assays described below. Cell suspensions (50µl) were stained 30 minutes in the dark using combinations of the following optimally titrated fluorescent dye-conjugated antibodies anti-CD8a-FITC, anti-CD27-FITC, anti-CD26-PE, anti-β7-PE, anti-CD45RO-PE, anti-CD4-PerCP/Cy5.5, anti-CD56-PerCP-Cy5.5, anti-CD19-PE/Cy-7, anti-CD45RA-PerCP-Cy5.5, anti-HLADR-PE/Cy7, anti-CD25-PE/Cy7 (BioLegend, SanDiego, CA, USA), anti-CD3-ECD (Beckman Coulter, Brea, CA, USA) and anti-CD127-PerCP-Cy5.5 (eBioScience, SanDiego, CA, USA). Appropriate isotype controls from the same manufacturer were used at equal concentrations. After staining, cells were washed

once, resuspended in PBS-paraformaldehyde solution (1%) and analyzed the same day using a Beckman Coulter Cytomics FC 500 MPL flow cytometer. In a typical acquisition $7x10^4$ lymphocytes (min 2.3 $x10^4$, max 1.7 $x10^5$) were collected. The collected data were analyzed with FlowJo 7.6 software (Tree Star Inc, Ashland, OR, USA).

Cellular immunity assays

PBMC were cultured for 6 days in the presence of control antigens and *Giardia* antigens obtained from the supernatant of sonicated *Giardia* genotype A and B reference strains trophozoites at a protein concentration of 10 μg/ml. Proliferative responses were measured in stimulated cultured cells after pulsing with ^{3}H-thymidine after 5 days, harvesting 18 hours later and analyzing incorporated radioactivity. T-cell activation markers were analysed by flow cytometric measurement of T-cell subsets with HLA-DR and CD45RO or CD25 and CD26 after 6 days in culture. Details of culture, control antigens and analyses are described elsewhere (Hanevik et al. 2011, in press).

Statistical analysis

Unless otherwise stated the data are presented as mean (standard deviation (SD)). Chi-squared tests were used for categorical comparisons between groups. Linear regression analysis was used for correlation between symptom scores and lymphocyte data. The significance level was p=0.05. Lymphocyte subset data and stimulation indices were analyzed using the Kruskal-Wallis test for all groups and then Mann Whitney U test to compare two groups. Due to multiple comparisons in lymphocyte subset analyses and a high number of variables, we chose a nominal significance level of 0.01 for subgroup comparisons. PASW 18 (SPSS Inc, Chicago, Ill, USA) was used for statistical analysis.

RESULTS

Data from 48 patients (mean age 42.3 years (11.5) range19-68, females 79%), 22 exposed controls (mean age 39.8 (10.5) range 26 -66, females 73%) and 10 unexposed controls (mean age 43.3 (14.3) range 22-63, females 76%) were analyzed in this study. All participated in lymphocyte quantification, but for logistical reasons not all patients and controls has all immunological tests done, numbers given in Figure 1. The numbers of patients and controls allocated into the fatigue, FGID and combined categories are shown in Table 1. There were no significant differences in age and male/female distributions between the groups in each category.

Lymphocyte quantification

Peripheral blood lymphocyte subset results for the three study categories are shown in Table 2. We found the CD4:CD8 ratio to be reduced and total CD8 T-cells to be increased in the FGID groups compared to the no-FGID control group. The PI-CFS/ICF patients had a lower percentage of natural killer (NK)-cells than the no fatigue control group (p=0.006).
The same pattern was seen in the combined category, with elevated CD8 in both the group with FGID&CFS/ICF and the FGID only group, and also a strong trend for differences in NK-cell percentage (p=0.044) and concentration (p=0.036) between these groups. The percentage and concentrations of CD3 and CD4 T-cells and CD19 B-cells were similar in all groups tested. A significant correlation was found between the fatigue and abdominal symptoms scores (R=0.421, p<0.001) and both correlated significantly with NK-cell levels (Figure 2) but not with CD8 T-cell levels.

Immunophenotyping

CD4 and CD8 T-cells were analysed for expression of activation markers HLA-DR, CD25, CD26, and CD27 and CD45RO. $CD56^+$ NK-cells were analysed for CD26. $CD19^+$ B-cells were analysed for activation markers CD25, CD26 and CD27. Additionally, we analysed

percentage of gut-homing integrin β7 positive T-cells, B-cells and $CD4^{+}CD25^{++}CD127^{neg}$ T-regulatory cells.

T-cells expressing activation markers HLA-DR and CD26 showed some differences between controls and patient groups. In particular, the percentage HLA-DR positive CD4 T-cells was higher in the FGID&CFS/ICF group (6.2 (1.9)) compared to the "no FGID/no fatigue" group (4.9 (2.2)) (p=0.02) and was significantly lower (p=0.002) in the recovered group compared to the PI-CFS group, see selected subset data given in Supplementary table 1.

The percentage of CD26 positive CD3 T-cells in the "all FGID" group was decreased (p=0.03) compared to the control group without FGID. The same difference was seen again in the combined category with 83.7% (6.2) CD26 positive CD3 T-cells in the "FGID, no fatigue" group and 77.1% (8.8) in the "no FGID/no fatigue" group (p=0.01).

We did not find any significant differences along the FGID or CFS categories regarding the percentage of $CD56^{dim}$ or $CD56^{bright}$ NK-cells or for CD26 positive subpopulations of these NK-cell subsets. Nor did we find any significant differences between groups for activation markers CD25, CD26 and CD27 in CD19 positive B-cells or in the percentage of gut-homing integrin β7 positive T-cells, B-cells or $CD4^{+}CD25^{++}CD127^{-}$ Treg cells.

Cellular immune response

Both the proliferation assay and flow cytometric markers showed a significant difference in *Giardia* specific cellular immunity between the patients and exposed controls compared to the unexposed controls. However, neither assay showed any significant differences between exposed controls and patients with post-giardiasis FGID or CFS. Data from proliferation assay and one flow assay for the two human infecting *Giardia* genotypes and control antigens are shown in Table 3. In additional assays and when analyzing CD4 and CD8 T-cell subsets separately we did not identify significant differences either (data not shown).

DISCUSSION

In this study we classified patients and controls with FGID according to Rome II criteria and by the Fukuda CDC criteria for CFS and ICF regarding fatigue. A range of peripheral blood lymphocyte subsets reported to be altered in CFS and FGID were analyzed and the specific cellular immune response against *Giardia* was investigated.

CD8 T-cells and CD4:CD8 ratio

In literature regarding CD4 and CD8 T-cell subsets and ratio in FGID we found two studies which did not find differences in T-cell subsets [20, 21] and two studies with lowered CD4/CD8 ratio in IBS patients; one due to high total CD8 cells [22] and one due to low CD4 cells [23]. While CD4 and CD8 T-cell numbers and percentages may fluctuate considerably within an individual over time, the CD4:CD8 ratio is found to be relatively stable [24]. We found the CD4:CD8 ratio to be low due to increased CD8 T-cell numbers in our PI-FGID study population. Similar peripheral blood T-cell patterns are reported in patients suffering from a number of viral diseases like mononucleosis, dengue, RSV and cytomegalovirus infection and herpes simplex recrudescence as well as in chronic toxoplasmosis infection [24, 25]. A low grade ongoing immune response against reactivated viruses is a hypothesis of CFS morbidity [26]. However, in our patient population this pattern was seen in PI-FGID and not to PI-CFS.

NK-cells in FGID and CFS

Peripheral blood $CD56^+$ NK-cells levels in IBS patients were similar to healthy controls in two studies [21, 27]. A post-prandial decrease in NK-cells in IBS patients relative to controls has also been described [28].

The majority of studies of NK-cells in heterogeneous CFS populations have not found differences in NK-cell levels [29, 30]. Two studies have found significantly reduced NK-cell levels in both PI-CFS and non-PI-CFS patients [12, 31]. In one of these non-PI-CFS patients

had significantly lower NK-cells levels than PI-CFS patients [31], and NK-cell levels returned to normal after successful recovery in the PI-CFS group [32]. In our study, fatigue scores correlated with NK-cell levels, and the patients who had recovered from chronic fatigue showed NK-cell levels comparable to the non-fatigue control group. These data suggest that decreased NK- cells are associated with ongoing fatigue in PI-CFS.

Activation markers and subsets

HLA-DR expressing CD8 T-cells in CFS have been increased in three published studies, while six other studies did not find a difference [29]. One study in IBS patients has shown elevated levels of gut homing T-cells expressing HLA-DR [20]. In the present study we found a trend towards increased HLA-DR expression in CD4 T-cells in the combined FGID & CFS/ICF group compared to controls and a significant reduction in the small group recovered from fatigue.

We could not replicate the elevated levels of CD26 expression in T and NK-cells as a robust marker of CFS reported by Fletcher et al [33]. Instead, there was a strong tendency towards lower $CD26^{+}$ T-cells in FGID patients.

A study in CFS patients recently reported higher levels of $CD4^{+}CD25^{++}FoxP3^{+}$ cells [30]. We found no difference in regulatory T-cells as measured by $CD4^{+}CD25^{++}CD127^{-}$ cells, similar to a finding done in FGID patients [34].

Specific immune response

Analysis of specific humoral immunity in PI-CFS after parvovirus B19 infection did not identify any distinguishing pattern [35]. Pathogen specific cellular immunity against the infection eliciting PI-CFS and PI-FGID has to our knowledge not been investigated before. The similar levels of long term cellular immunity against *G. lamblia* found in symptomatic patients and the exposed recovered control group indicates that T-cell dependent host response towards the eliciting organism is not a risk factor for development of PI-FGID or PI-IBS. This does not exclude the possibility of an initially stronger, qualitatively different or prolonged immune

response leading to the immune dysfunction markers seen by elevated CD8 and lowered NK-cell levels in our patient group.

Cautionary remarks

NK-cells are influenced by sleep and depression [36]. We did not control for poor sleep, but no patients in the CFS/ICF group had clinical depression. NK-cells are also known to fluctuate considerably with exercise, but the resting immune function evaluated in this study, is not very different in athletes compared to non-athletes [37]. The cytotoxic activity of NK-cell has been more consistently decreased in CFS studies, but may be a bystander effect as it has been shown to be decreased in chronic stress like unemployment, while NK-cell concentration was not [38].

Conclusion

Using two different methods for assessing the host cellular immune response against *Giardia* we found no difference between asymptomatic *Giardia* exposed controls and patients with FGID or CFS. In the patients who developed PI-FGID and PI-CFS after giardiasis, we found significantly increased CD8 T-cells in patients with FGID and reduced levels of NK-cells in CFS patients. There was a close correlation between fatigue and abdominal symptoms in the study population and severity of symptom scores correlated with NK-cell levels. The findings support an ongoing immune dysfunction that does not seem to be associated with an altered specific immune response to *Giardia*.

Authors' contributions

Laboratory work, data collection and analyses were done by KH, EK, SSø. *Giardia* antigens were prepared and supplied by SSv. Clinical evaluation of patients was done by KM, HN, ACR and JEB. TH and OB assisted in planning and analysis. NL supervised all parts of the study. All authors assisted in preparation of, and approved, the final manuscript.

Conflicts of interests

The authors declare that they have no competing interests.

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Reference List

(1) Okhuysen PC. Traveler's diarrhea due to intestinal protozoa. Clin Infect Dis **2001 Jul 1**; 33 (1) :110-4.

(2) Hanevik K, Dizdar V, Langeland N, Hausken T. Development of functional gastrointestinal disorders after Giardia lamblia infection. BMC Gastroenterol **2009**; 9:27.

(3) Wensaas KA, Langeland N, Hanevik K, Morch K, Eide GE, Rortveit G. Irritable bowel syndrome and chronic fatigue 3 years after acute giardiasis: historic cohort study. Gut **2011 Sep 12**.

(4) Nygard K, Schimmer B, Sobstad O, et al. A large community outbreak of waterborne giardiasis- delayed detection in a non-endemic urban area. BMC Public Health **2006 May 25**; 6 (1) :141.

(5) Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel disorders and functional abdominal pain. Gut **1999 Sep**; 45 Suppl 2:II43-II47.

(6) Simren M, Svedlund J, Posserud I, Bjornsson ES, Abrahamsson H. Predictors of subjective fatigue in chronic gastrointestinal disease. Aliment Pharmacol Ther **2008 Sep 1**; 28 (5) :638-47.

(7) Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology **2002 Apr**; 122 (4) :1140-56.

(8) Hickie I, Davenport T, Wakefield D, et al. Post-infective and chronic fatigue syndromes precipitated by viral and non-viral pathogens: prospective cohort study. BMJ **2006 Sep 16**; 333 (7568) :575.

(9) Devanur LD, Kerr JR. Chronic fatigue syndrome. J Clin Virol **2006 Nov**; 37 (3) :139-50.

(10) Spiller R, Garsed K. Postinfectious irritable bowel syndrome. Gastroenterology **2009 May**; 136 (6) :1979-88.

(11) Gow JW, Hagan S, Herzyk P, Cannon C, Behan PO, Chaudhuri A. A gene signature for post-infectious chronic fatigue syndrome. BMC Med Genomics **2009**; 2:38.

(12) Masuda A, Munemoto T, Yamanaka T, Takei M, Tei C. Psychosocial characteristics and immunological functions in patients with postinfectious chronic fatigue syndrome and noninfectious chronic fatigue syndrome. J Behav Med **2002 Oct**; 25 (5) :477-85.

(13) Mearin F. Postinfectious Functional Gastrointestinal Disorders. J Clin Gastroenterol **2011 Aug**; 45 Proceedings from the Pan-European Conference on Irritable Bowel Syndrome December 10, 2010 Vienna, Austria:S102-S105.

(14) Connor BA. Sequelae of traveler's diarrhea: focus on postinfectious irritable bowel syndrome. Clin Infect Dis **2005 Dec 1**; 41 Suppl 8:S577-S586.

(15) Thabane M, Kottachchi DT, Marshall JK. Systematic review and meta-analysis: The incidence and prognosis of post-infectious irritable bowel syndrome. Aliment Pharmacol Ther **2007 Aug 15**; 26 (4) :535-44.

(16) Verweij JJ, Schinkel J, Laeijendecker D, van Rooyen MA, van LL, Polderman AM. Real-time PCR for the detection of Giardia lamblia. Mol Cell Probes **2003 Oct**; 17 (5) :223-5.

(17) Kane SV, Sandborn WJ, Rufo PA, et al. Fecal lactoferrin is a sensitive and specific marker in identifying intestinal inflammation. Am J Gastroenterol **2003 Jun**; 98 (6) :1309-14.

(18) Chalder T, Berelowitz G, Pawlikowska T, et al. Development of a fatigue scale. J Psychosom Res **1993**; 37 (2) :147-53.

(19) Fukuda K, Straus SE, Hickie I, Sharpe MC, Dobbins JG, Komaroff A. The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group. Ann Intern Med **1994 Dec 15**; 121 (12) :953-9.

(20) Ohman L, Isaksson S, Lindmark AC, et al. T-cell activation in patients with irritable bowel syndrome. Am J Gastroenterol **2009 May**; 104 (5) :1205-12.

(21) Kindt S, Van OL, Broekaert D, et al. Immune dysfunction in patients with functional gastrointestinal disorders. Neurogastroenterol Motil **2009 Apr**; 21 (4) :389-98.

(22) Linghu EQ, Yang Y.S. Analysis of peripheral blood lymphocytes subgroup in irritable bowel syndrome. Chin J Dig **2002**; 22:423-5.

(23) Jin HB, Gu ZY, Zhao HW, Wang QY, Wu W, Li YM. [T lymphocyte subgroups and serum levels of trace elements in patients with diarrhea type of irritable bowel syndrome]. Zhejiang Da Xue Xue Bao Yi Xue Ban **2008 Nov**; 37 (6) :634-7.

(24) Laurence J. T-cell subsets in health, infectious disease, and idiopathic CD4+ T lymphocytopenia. Ann Intern Med **1993 Jul 1**; 119 (1) :55-62.

(25) Liu CC, Huang KJ, Lin YS, Yeh TM, Liu HS, Lei HY. Transient CD4/CD8 ratio inversion and aberrant immune activation during dengue virus infection. J Med Virol **2002 Oct**; 68 (2) :241-52.

(26) Ablashi DV. Viral studies of chronic fatigue syndrome. Clin Infect Dis **1994 Jan**; 18 Suppl 1:S130-S133.

(27) Motzer SA, Jarrett M, Heitkemper MM, Tsuji J. Natural killer cell function and psychological distress in women with and without irritable bowel syndrome. Biol Res Nurs **2002 Jul**; 4 (1) :31-42.

(28) Elsenbruch S, Holtmann G, Oezcan D, et al. Are there alterations of neuroendocrine and cellular immune responses to nutrients in women with irritable bowel syndrome? Am J Gastroenterol **2004 Apr**; 99 (4) :703-10.

(29) Natelson BH, Haghighi MH, Ponzio NM. Evidence for the presence of immune dysfunction in chronic fatigue syndrome. Clin Diagn Lab Immunol **2002 Jul**; 9 (4) :747-52.

(30) Brenu EW, van Driel ML, Staines DR, et al. Immunological abnormalities as potential biomarkers in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. J Transl Med **2011**; 9:81.

(31) Racciatti D, Dalessandro M, Delle DL, et al. Study of immune alterations in patients with chronic fatigue syndrome with different etiologies. Int J Immunopathol Pharmacol **2004 May**; 17 (2 Suppl):57-62.

(32) Masuda A, Nakayama T, Yamanaka T, Koga Y, Tei C. The prognosis after multidisciplinary treatment for patients with postinfectious chronic fatigue syndrome and noninfectious chronic fatigue syndrome. J Behav Med **2002 Oct**; 25 (5):487-97.

(33) Fletcher MA, Zeng XR, Maher K, et al. Biomarkers in chronic fatigue syndrome: evaluation of natural killer cell function and dipeptidyl peptidase IV/CD26. PLoS One **2010**; 5 (5):e10817.

(34) Holmen N, Isaksson S, Simren M, Sjovall H, Ohman L. CD4+CD25+ regulatory T cells in irritable bowel syndrome patients. Neurogastroenterol Motil **2007 Feb**; 19 (2):119-25.

(35) Kerr JR, Bracewell J, Laing I, et al. Chronic fatigue syndrome and arthralgia following parvovirus B19 infection. J Rheumatol **2002 Mar**; 29 (3):595-602.

(36) Irwin M, Lacher U, Caldwell C. Depression and reduced natural killer cytotoxicity: a longitudinal study of depressed patients and control subjects. Psychol Med **1992 Nov**; 22 (4):1045-50.

(37) Gleeson M, Bishop NC. The T cell and NK cell immune response to exercise. Ann Transplant **2005**; 10 (4):43-8.

(38) Cohen F, Kemeny ME, Zegans LS, Johnson P, Kearney KA, Stites DP. Immune function declines with unemployment and recovers after stressor termination. Psychosom Med **2007 Apr**; 69 (3):225-34.

(39) Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. Gastroenterology **2006 Apr**; 130 (5):1480-91.

Table 1. Sex ratio and mean age in the different clinical groups after allocation of patients and controls according to Rome II FGID groups [5] and Fukuda CDC criteria [19] for the three analysis categories used in the present study. Subgroups of interest within each group are indicated by indents.

				Breakdown into recruited groups		
	Age (mean (SD))	Females (%)	Total participants, n	Exposed patients, fatigue at 3 years, n	Exposed controls, no fatigue at 3 years, n	Unexposed, healthy controls, n
Fatigue category						
no-fatigue	40.9 (11.7)	72	32	0	22	10
CFS	45.5 (9.1)	79	19	19	0	0
ICF	37.8 (8.9)	80	5	5	0	0
Fatigue other cause	45.5 (15.2)	100	12	12	0	0
Fatigue recovered	36.0 (9.6)	58	12	12	0	0
FGID category						
no-FGID	41.8 (13.2)	78	26	5	11	10
All FGID	41.4 (10.8)	76	54	43	11	0
PI-FGID	40.8 (10.2)	76	45	36	9	0
IBS	41.7 (10.4)	80	30	27	3	0
PI-IBS	40.4 (10.4)	79	24	22	2	0
Other FGID	41.1 (11.5)	71	24	16	8	0
Combined category						
no-FGID/no-fatigue	39.9 (12.5)	74	23	2	11	10
FGID&CFS/ICF	44.3 (9.4)	78	23	23	0	0
CFS, no FGID	35.0	100	1	1	0	0
FGID, no fatigue	39.1 (10.1)	62	21	10	11	0
Fatigue other cause w/wo FGID	45.5 (11.5)	100	12	12	0	0

Abbreviations: CFS: chronic fatigue syndrome, FGID: functional gastrointestinal disorder, ICF: idiopathic chronic fatigue, IBS: irritable bowel syndrome, PI: post-infectious

Table 2. Peripheral blood CD4:CD8 T-cells ratio and CD3 T-cells, CD4 and CD8 T-cell subsets and CD56CD16 natural killer cells percentages and concentrations by analysis categories and relevant subgroups. All values are given as mean (SD). Data for the subgroups of PI-CFS, PI-FGID and PI-IBS are also given.

	CD4:CD8 ratio	CD3 (%)	CD3 10^6 cells/L	CD3CD4 (%)	CD3CD4 10^6 cells/L	CD3CD8 (%)	CD3CD8 10^6 cells/L	CD16CD56 6 (%)	CD16CD56 10^6 cells/L	CD19 (%)	CD19 10^6 cells/L
no-fatigue controls (n = 32)	*2.43 (0.97)*	*76.2 (7.1)*	*1471 (539)*	*51.1 (8.2)*	*990 (376)*	*22.9 (5.5)*	*440 (198)*	*11.7 (6.7)*	*210 (99)*	*11.8 (3.8)*	*226 (106)*
PI-CFS or PI-ICF (n=24)	2.05 (0.76)	79.3 (4.9)	1470 (529)	50.5 (7.0)	939 (366)	27.0 (7.0)	497 (207)	**7.5 (3.5)****	**130 (54)****	12.9 (4.5)	245 (144)
PI-CFS (n = 19)	2.01 (0.77)	79.3 (4.8)	1435 (452)	50.3 (6.9)	912 (296)	**27.5 (7.0)***	497 (200)	**8.0 (3.6)***	**138 (55)****	12.4 (4.2)	227 (101)
Fatigue other cause n=12	2.37 (1.14)	78.8 (6.5)	1786 (1047)	51.9 (8.1)	1132 (543)	26.1 (10.4)	635 (531)	8.7 (4.6)	186 (139)	12.1 (3.6)	247 (77)
Fatigue recovered n=12	**1.80 (0.82)***	76.8 (8.9)	1425 (431)	46.1 (10.3)	834 (318)	**28.8 (9.1)***	545 (225)	10.6 (9.0)	203 (188)	12.3 (4.1)	224 (80)
no-FGID (n=26)	*2.74 (1.06)*	*76.3 (7.6)*	*1434 (473)*	*53.3 (8.6)*	*1008 (363)*	*21.1 (5.4)*	*394 (167)*	*11.3 (7.1)*	*192 (80)*	*12.1 (3.9)*	*229 (107)*
All FGID n=54	**1.96 (0.75)****	78.3 (6.2)	1550 (688)	**48.9 (7.7)***	958 (415)	**27.6 (7.6)*****	**555 (311)****	9.1 (5.6)	176 (129)	12.3 (4.1)	237 (113)
PI-FGID n=45	**2.00 (0.78)****	78.3 (6.1)	1527 (706)	**49.2 (7.9)***	949 (418)	**27.1 (7.35)****	**537 (313)***	8.8 (5.4)	169 (122)	12.6 (4.3)	239 (118)
PI-IBS n=23	**1.97 (0.91)****	79.8 (4.9)	1621 (827)	49.5 (8.8)	988 (463)	**28.2 (7.1)****	**592 (376)***	**8.1 (5.0)***	157 (111)	11.8 (4.3)	227 (98)
no-FGID/no-fatigue controls (n=23)	*2.65 (1.05)*	*75.8 (8.1)*	*1402 (493)*	*52.4 (8.8)*	*974 (380)*	*21.5 (5.5)*	*389 (171)*	*11.8 (7.6)*	*194 (86)*	*12.1 (4.2)*	*226 (115)*
FGID&CFS/ICF n=23	**2.01 (0.75)***	79.5 (4.9)	1458 (538)	50.2 (7.0)	923 (366)	**27.3 (6.9)****	500 (211)	**7.4 (3.5)***	**127 (53)****	12.8 (4.6)	241 (146)
FGID, no fatigue n=21	**1.82 (0.65)****	77.0 (6.9)	1518 (531)	**46.9 (8.5)***	924 (356)	**27.9 (7.3)****	**548 (214)****	11.0 (7.0)	223 (156)	11.7 (3.6)	224 (81)
Fatigue other cause w/wo FGID n=12	2.37 (1.14)	78.8 (6.5)	1787 (1048)	51.9 (8.1)	1133 (543)	26.1 (10.4)	635 (531)	8.7 (4.6)	186 (140)	12.1 (3.6)	247 (77)

* $p < 0.05$ level compared to the control group (in italics) in each category

** Significant at $p<0.01$ level or below compared to the control group (in italics) in its category

*** Significant at $p<0.001$ level or below compared to the control group (in italics) in its category

Table 3. Immune responses against *Giardia* and control antigens as measured by ^{3}H-thymidine proliferation assay and CD25CD26 positive lymphocytes measured by flow-cytometry in the exposed controls and exposed patients with PI-CFS and PI-FGID and "fatigue other cause" groups, omitting individuals with FGID before the outbreak (n=3) and CFS without FGID (n=1). All responses expressed as median stimulation indicies (standard deviation). No significant differences were found, except between exposed and unexposed groups.

Antigen	Exposed, no-fatigue/ no FGID (n = 10)	Exposed, PI-CFS/ICF and FGID (n = 19)	Exposed, PI-FGID, no fatigue (n=15)	Exposed, fatigue other cause w/wo FGID (n=9)	Unexposed healthy controls (n=10)
Proliferation assay					
Giardia A cytosolic fraction	17.4 (11.6)	15.3 (19.9)	10.0 (51.6)	20.3 (10.9)	3.5 (6.2)*
Giardia B cytosolic fraction	14.2 (16.7)	13.3 (15.3)	9.3 (31.8)	23.3 (13.4)	3.5 (4.7)*
LPS *S.typhi* 1µg/ml	15.0 (11.6)	14.4 (31.2)	21.9 (22.0)	38.2 (34.3)	17.7 (10.5)
Tuberculin (PPD) 10µg/ml	33.1 (42.2)	34.0 (47.5)	30.2 (23.4)	54.9 (84.5)	27.7 (27.0)
aCD3aCD28 (pos ctr)	76.2 (37.6)	51.4 (33.0)	51.8 (54.2)	52.7 (44.1)	60.1 (39.8)
Flow cytometric assay					
Giardia A cytosolic fraction	112 (238)	53.1 (83.1)	68.5 (64.7)	43.3 (33.4)	12.5 (98.5)*
Giardia B cytosolic fraction	34.7 (133)	34.0 (50.4)	28.5 (29.6)	26.9 (27.9)	11.8 (19.2)*
LPS *S.typhi* 1µg/ml	110 (388)	40.0 (96.8))	46.4 (55.7)	36.9 (24.4)	32.8 (128)
Tuberculin (PPD) 10µg/ml	70.9 (175)	83.1 (120)	85.4 (77.4)	43.0 (56.2)	61.7 (113)

* Statistical difference between the combined exposed group and unexposed group with p-values <0.001 for the proliferation assay and <0.01 for the flow cytometry assay by the Mann-Whitney U test.

Supplementary table 1. Selected variables from the 46 subsets analyzed in the immunophenotyping assay. Values are mean (SD). The Kruskal-Wallis test p-value is given for the fatigue and combined classification as a measure of the overall significance between more than two groups. Specific p-values based on the secondary Mann-Whitney U test comparison between each group against the control group are given in the footnotes. Data for the clinically interesting subgroups of PI-CFS, PI-FGID and PI-IBS are also given.

	$CD3^+$ $HLADR^+$	$CD4^+$ $HLADR^+$	$CD8^+$ $HLADR^+$	$CD4^+$ $CD25^+$	$CD4^+$ $CD25^+$ $CD127^{neg}$	$CD3^+$ $CD26^+$	$CD56^+$ $CD26^+$	$CD3^+B7^+$	$CD3^{neg}$ $CD56^{bright}$	$CD3^{neg}$ $CD56^{dim}$
Kruskal-Walli (4 groups)	***0.175***	***0.022****	***0.603***	***0.827***	***0.890***	***0.508***	***0.455***	***0.961***	***0.312***	***0.159***
no-fatigue controls (n = 29)	*7.0 (4.3)*	*5.2 (2.4)*	*12.0 (9.0)*	*4.0 (1.8)*	*4.1 (1.7)*	*81.9 (7.0)*	*11.3 (6.1)*	*57.8 (9.3)*	*1.20 (0.7)*	*10.9 (5.8)*
PI-CFS or PI-ICF (n=20)	7.5 (2.7)	6.0 (2.0)	11.4 (5.4)	3.5 (1.9)	3.8 (1.9)	78.8 (10.2)	12.7 (6.3)	55.7 (9.9)	1.45 (0.90)	10.3 (4.7)
PI-CFS (n = 15)	8.1 (2.6)	**6.4 (2.0)**[a]	12.8 (5.3)	3.3 (1.9)	3.6 (1.8)	77.9 (10.4)	11.0 (4.5)	52.4 (8.4)	1.10 (0.52)	10.9 (5.0)
Fatigue other cause n=9	7.0 (3.2)	5.0 (1.4)	12.9 (8.3)	4.3 (1.5)	4.6 (1.5)	80.4 (8.9)	9.4 (5.2)	58.3 (11.0)	0.91 (0.51)	11.1 (7.3)
Fatigue recovered n=10	5.4 (2.3)	**3.9 (1.2)** [b]	9.3 (6.8)	3.8 (2.0)	4.0 (1.8)	77.0 (10.3)	10.6 (5.9)	57.3 (6.7)	1.16 (0.77)	11.0 (8.4)
no-FGID controls (n=22)	*6.4 (4.2)*	*4.8 (2.2)*	*11.3 (9.2)*	*3.7 (2.1)*	*3.9 (2.0)*	*83.4 (6.4)*	*9.3 (4.2)*	*57.2 (10.6)*	*0.98 (0.38)*	*11.7 (6.6)*
All FGID n=46	7.2 (3.1)	5.4 (2.0)	11.6 (6.8)	3.9 (1.6)	4.1 (1.5)	**78.5 (9.4)**[c]	12.3 (6.5)	57.1 (8.8)	1.35 (0.84)	10.3 (5.8)
PI-FGID n=38	6.8 (2.4)	5.4 (1.8)	11.1 (1.8)	3.7 (1.7)	3.9 (1.6)	79.1 (9.38)	12.4 (6.6)	57.1 (9.5)	1.42 (0.89)	10.7 (6.1)
PI-IBS n=19	6.3 (2.4)	5.3 (2.0)	8.8 (3.3)	4.1 (1.8)	4.3 (1.7)	78.5 (10.1)	12.3 (6.5)	55.3 (9.8)	1.41 (0.90)	11.3 (7.2)
Kruskal-Wallis (3 groups)	***0.118***	***0.039****	***0.614***	***0.901***	***0.974***	***0.036****	***0.467***	***0.822***	***0.130***	***0.130***
no-FGID/no-fatigue controls (n=21)	*6.5 (4.2)*	*4.9 (2.2)*	*11.5 (9.4)*	*4.0 (2.0)*	*4.0 (2.0)*	*83.7 (6.2)*	*10.4 (4.4)*	*58.3 (10.0)*	*0.96 (0.39)*	*10.7 (6.5)*
FGID and CFS/ICF n=19	7.7 (2.6)	**6.2 (1.9)**[d]	11.6 (5.4)	3.6 (1.8)	4.0 (1.8)	78.4 (10.4)	13.1 (6.2)	55.7 (9.9)	1.46 (0.92)	10.2 (4.7)
FGID, no fatigue n=18	6.7 (3.7)	4.9 (2.2)	11.0 (7.4)	3.9 (1.6)	4.1 (1.4)	**77.1 (8.8)**[e]	12.0 (7.5)	56.9 (7.0)	1.46 (0.85)	11.1 (6.6)

* denotes significant difference within the groups. [a] p=0.046 compared to no-fatigue. [b] p=0.002 for difference between CFS/ICF group and fully recovered patients. [c] p=0.030 compared to no-FGID controls. [d] p=0.021 compared to no-FGID/no-fatigue controls. [e] p=0.01 compared to no-FGID/no-fatigue controls. controls.

Figure legends

Figure 1. Study recruitment base and participants.

Participants were recruited based on a mailed questionnaire study regarding fatigue [18] and abdominal complaints [39] to all individuals with outbreak related laboratory confirmed giardiasis [3]. Five years after the outbreak, patients who reported chronic fatigue in this questionnaire were invited to participate in a thorough clinical evaluation and screening. Fifty three individuals agreed to participate. Five patients were excluded from this study after evaluation. Two control groups were recruited; 22 individuals with normal score (=11) in the questionnaire three years after, and 10 healthy individuals not affected by the outbreak (unexposed controls) and without particular abdominal symptoms or fatigue.

Figure 2. NK-cell levels and symptoms correlations

Correlation plots between the percentage of peripheral blood CD16CD56 NK-cells and fatigue symptom score (A) as recorded by the fatigue questionnaire [18] and total abdominal symptoms score (B) (including the six symptoms nausea, early satiety, bloating, abdominal pain, constipation and diarrhoea, where patients graded their symptoms on an ordinal scale from 0 to 10 with 0 = no symptoms and 10 = severe symptoms).

Figure 1.

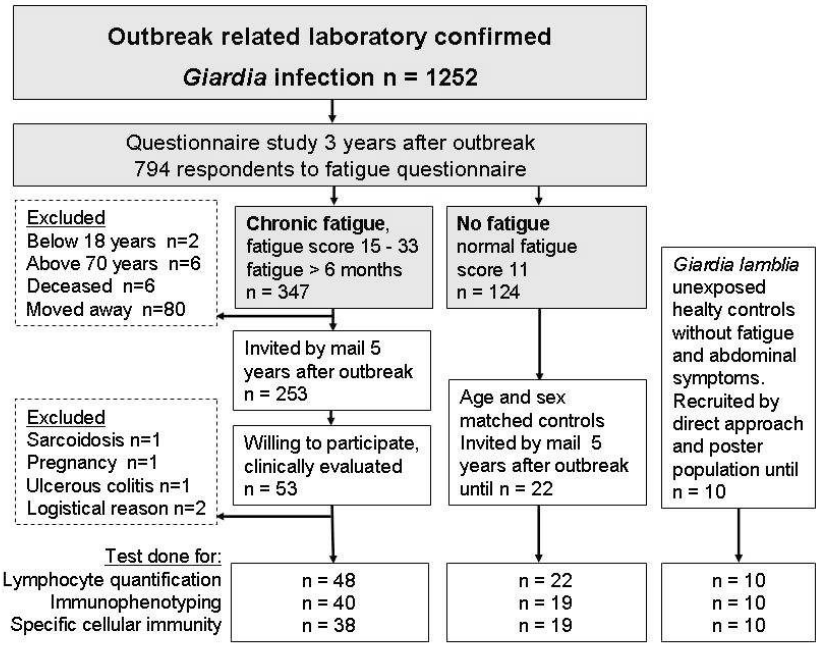
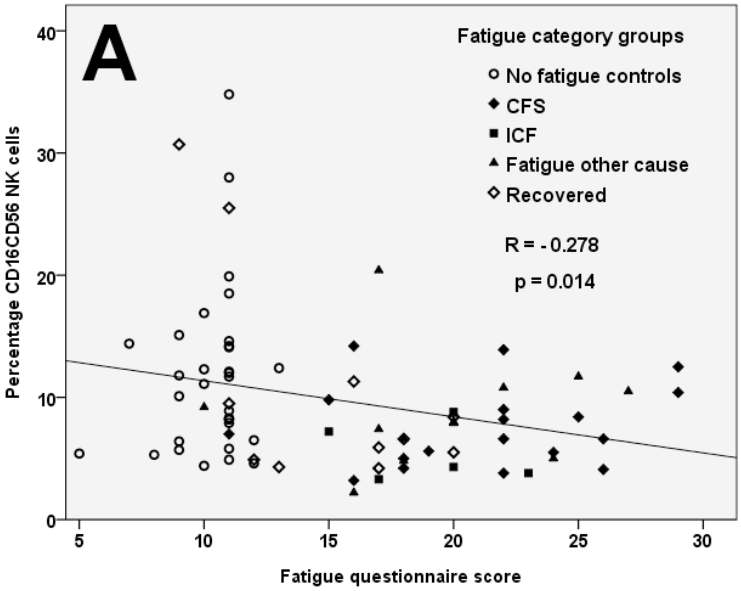
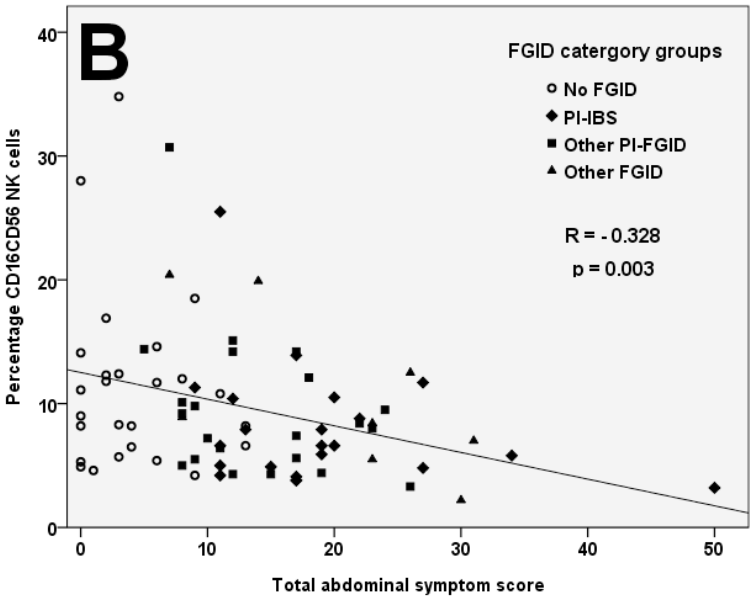

Outbreak related laboratory confirmed
Giardia infection n = 1252
Questionnaire study 3 years after outbreak
794 respondents to fatigue questionnaire
Excluded
Below 18 years n=2
Above 70 years n=6
Deceased n=6
Moved away n=80
Chronic fatigue, fatigue score 15 - 33 fatigue > 6 months n = 347
No fatigue normal fatigue score 11 n = 124
Giardia lamblia unexposed healty controls without fatigue and abdominal symptoms. Recruited by direct approach and poster population until n = 10
Invited by mail 5 years after outbreak n = 253
Age and sex matched controls Invited by mail 5 years after outbreak until n = 22
Excluded
Sarcoidosis n=1
Pregnancy n=1
Ulcerous colitis n=1
Logistical reason n=2
Willing to participate, clinically evaluated n = 53
Test done for:
Lymphocyte quantification
Immunophenotyping
Specific cellular immunity
n = 48
n = 40
n = 38
n = 22
n = 19
n = 19
n = 10
n = 10
n = 10

Figure 2.


A
Fatigue category groups
No fatigue controls
CFS
ICF
Fatigue other cause
Recovered
R = - 0.278
p = 0.014
Percentage CD16CD56 NK cells
Fatigue questionnaire score
0
10
20
30
40
5
10
15
20
25
30


B
FGID catergory groups
No FGID
PI-IBS
Other PI-FGID
Other FGID
R = - 0.328
p = 0.003
Percentage CD16CD56 NK cells
Total abdominal symptom score
0
10
20
30
40
0
10
20
30
40
50

Appendix

Appendix I

Rome II - Functional gastrointestinal disorders

A. Esophageal disorders	A1. Globus
	A2. Rumination syndrome
	A3. Functional chest pain of presumed esophageal origin
	A4. Functional heartburn
	A5. Functional dysphagia
	A6. Unspecified functional esophageal disorder
B. Gastroduodenal disorders	B1. Functional dyspepsia
	B1a. Ulcer-like dyspepsia
	B1b. Dysmotility-like dyspepsia
	B1c. Unspecified (non-specific) dyspepsia
	B2. Aerophagia
	B3. Functional vomiting
C. Bowel disorders	C1. Irritable bowel syndrome
	C2. Functional abdominal bloating
	C3. Functional constipation
	C4. Functional diarrhea
	C5. Unspecified functional bowel disorder
D. Functional abdominal pain	D1. Functional abdominal pain syndrome
	D2. Unspecified functional abdominal pain
E. Biliary disorders	E1. Gall bladder dysfunction
	E2. Sphincter of Oddi dysfunction
F. Anorectal disorders	F1. Functional fecal incontinence
	F2. Functional anorectal pain
	F2a. Levator ani syndrome
	F2b. Proctalgia fugax
	F3. Pelvic floor dyssynergia
G. Functional pediatric disorders	G1. Vomiting
	G1a. Infant regurgitation
	G1b. Infant rumination syndrome
	G1c. Cyclic vomiting syndrome
	G2. Abdominal pain
	G2a. Functional dyspepsia
	G2b. Irritable bowel syndrome
	G2c. Functional abdominal pain
	G2d. Abdominal migraine
	G2e. Aerophagia
	G3. Functional diarrhea
	G4. Disorders of defecation
	G4a. Infant dyschezia
	G4b. Functional constipation
	G4c. Functional fecal retention
	G4d. Non-retentive fecal soiling

Modified from Drossman DA. The functional gastrointestinal disorders and the Rome II process. Gut **1999**