Giardiasis

with emphasis on treatment and post-infectious manifestations

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Water, is taught by thirst.
Land - by the Oceans passed.
Transport - by throe -
Peace - by its battles told -
Love, by Memorial Mold -
Birds, by the Snow.

Emily Dickinson, 1830-1886

CONTENT

I. Acknowledgements

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II. List of papers

1. Mørch K, Hanevik K, Robertson LJ, Strand EA, Langeland N: Treatment ladder and genetic characterisation of parasites in refractory giardiasis after an outbreak in Norway. Journal of Infection 2008; 56: 268 - 273.

2. Hanevik K, Mørch K, Eide GE, Langeland N, Hausken T: Effects of albendazole/metronidazole or tetracycline/folate treatments on persisting symptoms after *Giardia* infection: A randomised open clinical trial. Scand J Infect Dis. 2008; 40 (6-7): 517 - 22.

3. Mørch 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; 103: 530 - 532.

4. Mørch K, Hanevik K, Rortveit G, Wensaas KA, Eide GE, Hausken T, Langeland N: Severity of *Giardia* infection associated with post-infectious fatigue and abdominal symptoms two years after. BMC Infectious Diseases 2009, 9:206.

III. Abbreviations

Bid = Two times daily

E. coli = *Escherichia coli*

EIA = Enzyme immunoassay

ELISA = Enzyme-linked immunosorbent assay

ETEC = Enterotoxigenic *E. coli*

G. = *Giardia*

HIV = Human immunodeficiency virus

Ig = Immunoglobuline

IFA = Immunofluorescence assay

IIF = Indirect immunofluorescence

PCR = Polymerase chain reaction

MSIS = Norwegian Surveillance System for Communicable Diseases

Spp = Species

Syn = Synonymous

Tid = Three times daily

VSP = Variant surface protein

WHO = World Health Organisation

IV. Background

Giardiasis

1. Parasitology

Giardia duodenalis (syn. *G. lamblia, G. intestinalis*) is a single cell parasite, inhabiting the small intestine. Like *Plasmodium* species causing malaria by infecting red blood cells, the genus *Giardia* belongs to the family protozoans [1]. The name *lamblia* has its origin from Vilem Lambl who described the trophozoite in humans in 1859, and the cyst form was discovered by Grassi twenty years later; however, Antony van Leeuwenhoek described the parasite in his own stool as early as in the 17th century [2].

Six different *Giardia* species, characterised by differences in morphology and host specificity, have been described since 1952 [1, 3]; *G. duodenalis* infects humans as well as other primates, dogs, cats, livestock, rodents and some other wild animals, *G. agilis* infects amphibians, *G. psittaci* and *G. ardeae* birds and *G. microti* and *G muris* rodents. A revision of taxonomy is under debate, since genetic studies and sequencing of the *Giardia* genome have revealed several genotypic groupings/assemblages that could be classified as new species, including assemblage A and B in *G. duodenalis,* which are proposed to be classified as *G. duodenalis* (= assemblage A) and *G. enterica* (= assemblage B) [1, 4]. In addition, the following assemblages infecting animals have been proposed to be classified as species: Assemblage C/D (infecting dogs and other canids): *G. canis,* assemblage F (cats): *G. cati,* assemblage E (cattle and other hoofed livestock): *G. bovis,* and assemblage G (rats) *G. simondi*. The mechanism for host specificity is not known [1].

Giardia has three morphologic forms; cysts, excyzoites and trophozoites [5]. Cysts are responsible for faecal-oral transmission, and are able to survive for a long period in the environment, especially in cold water in which experimental studies have shown survival for up to two months [6]. In the upper part of the small intestine, they release excyzoites containing four nuclei, which attach to the intestinal wall and rapidly divide into four trophozoites [5]. Trophozoites cause disease in the small intestine where they multiply by simple binary fission, though there is some evidence also of sexual reproduction [4]. The trophozoites have a characteristic duplication of organelles; four pairs of flagella enabling them to move, two identical nuclei, two median bodies and a ventral sucking disc which

enables it to attach to the intestinal surface (Figure 1) [1]. Trophozoites may be found in fresh faeces, but usually encyst, triggered by bile salts [7] or cholesterol depletion and micelle destruction [8], before being excreted in the stool.

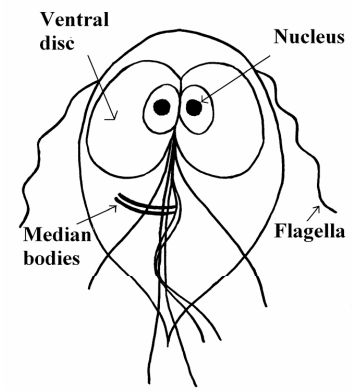

Figure 1. *Giardia* trophozoite showing ventral disc, flagellae, nuclei and median bodies.

2. Epidemiology

Diarrhoea is probably the most common infectious disease worldwide, and following lower respiratory infections the second leading cause of death due to infections; in 2004 WHO reported an incidence of 4.6 billion episodes and 2.2 million deaths due to diarrhoea per year, of these 1.8 million deaths in developing countries [9]. The most common etiologic agents are species among the viruses rotavirus, calicivirus, astrovirus and enteric adenovirus; the bacteria *E. coli*, *Shigella*, *Salmonella*, *Campylobacter* and *Vibrio cholera,* and the parasites *Giardia, Entamoeba histolytica, Cryptosporidium, Cyclospora and Isospora* [10]. Infections transmitted faecal-orally are more easily spread under conditions associated with poverty; such as decreased access to clean water, inappropriate sewage disposal, poor hygiene, crowding, close contact to farm animals and low educational level. A predominance of bacteria and parasites as diarrhoeal pathogens in the South, and viruses in the North, reflects this situation [10].

A prevalence of 200 million cases of giardiasis in tropical countries has been estimated by WHO [11]. However, decreased access to reliable diagnostic tools in socioeconomic underdeveloped areas makes it difficult to assess the aetiology of diarrhoea in clinical

practice, and reporting systems are insufficient. Such estimates are also limited by the fact that few case-control studies have been performed in developing countries, and the clinical studies that are available show that prevalence varies greatly between and within countries.

Serologic studies show a high infection rate, and that people are commonly infected with *Giardia* during childhood, in developing countries. In a national serologic survey in Mexico, 55% (1914/3461) of the samples were positive for anti-*Giardia* IgG, and seropositivity increased with age [12].
In a study comparing populations in developing and developed areas, anti-*Giardia* IgG was found in 44% (27/62), 48% (16/33) and 46% (12/26) of adults in an Apache Indian reservation in Arizona, in Panama and Peru respectively, while significantly fewer (18%, 7/41) adults in Baltimore were seropositive [13]. In Baltimore, seropositivity was low throughout childhood, children in Panama achieved adult levels of antibodies between nine and 20 years, Apache Indians by eight years of age, whereas 46% of Peruvian children were seropositive already by the age of six months [13].
Similar high prevalence was found in a cohort with low socioeconomic standard in Egypt, where 37% were anti-*Giardia* IgG positive [14].

Despite such high prevalence, giardiasis is not commonly associated with acute diarrhoea in hospital based studies. In different reviews, rotavirus and *E. coli* spp. are the most common causes of acute diarrhoea in both developing and developed countries [10, 15, 16]. In a study from India among children hospitalised due to diarrhoea, and children admitted to hospital for causes other than diarrhoea included as controls (n = 158 cases, n = 99 controls), the following pathogens were detected among cases and controls respectively: Rotavirus 43% and 10%, enterophatogenic *E. coli* 16% and 2%, norovirus 16% and 7%, and *Cryptosporidium spp.* 15% and 2%, while other agents were less prevalent, among these *Giardia* only 0.6% and 0% [17]. This study also demonstrated that molecular techniques increased the detection rate and changed the picture, compared to conventional diagnostic methods.
Such low prevalence among hospitalised children is supported by other case-control studies, using children admitted for reasons other than diarrhoea as controls. In a study from Nigeria (n = 215 cases, n = 100 controls) *Giardia* was identified in only one case, and in none of the controls [18]. In a large study from Bangladesh (n = 2534 cases, n = 1229 controls), *Giardia* was inversely associated with diarrhoea (8% versus 18%) [19]. A significantly higher

prevalence among controls (23%) than cases (14%) was also found in a study from Thailand [20].

These findings emphasises that when interpreting the clinical relevance of *Giardia*, it must be taken into account that identifying the parasite may be confounding rather than causal, since many cases are asymptomatic. In an uncontrolled study of 529 hospitalised children with diarrhoea from a rural area in Mozambique, *Giardia* was detected in 3%; however, 36% of the 74% cases who had malaria, which commonly causes diarrhoea, also had an enteropathogen (not specified) isolated. Both low level malaria parasitaemia and enteropathogens may be either without clinical relevance, or etiologic agents, in patients with diarrhoea. This illustrates the importance of case-control studies.

A review of 33 mainly population based cross-sectional prevalence studies from Asia showed that prevalence varied greatly, also within countries [21]. Studies from Nepal, which is considered one of the poorest countries in the world, reported prevalence between 2% (elderly home) and 73% (children), and Thailand, which generally has a higher socioeconomic status, reported prevalence between 1% in a study from North Thailand [22] and 37% in a study among orphans in Pathum Thani Province [23].

These serologic and clinical studies show that giardiasis is highly prevalent in many areas, but may not lead to severe acute diarrhoea with dehydration needing hospitalisation in most cases. The impact of giardiasis on the high mortality of diarrhoeal infections globally is not known, however the fact that *Giardia* in 2004 was included in the WHO's "Neglected disease initiative" may hopefully strengthen the focus on this poverty related disease [24].

In Western countries, *Giardia* infection usually occurs after travel to endemic areas, during waterborne outbreaks, or through person to person spread in institutions or between risk groups such as homosexual men.

Common causes of acute travellers diarrhoea are enterotoxigenic *E. coli* (ETEC), *Campylobacter*, *Shigella* and *Salmonella* spp, while persistent diarrhoea more often are caused by protozoans (*Giardia*, *Cryptosporidium*, *Entamoeba histolytica, Cyclospora, Isospora and microsporidia spp.)* [25].

Among 17 228 travellers visiting tropical medicine clinics in Europe, 4% had acute diarrhoea due to *Giardia*, most commonly after travel to South-Central Asia (11%), in a recent report from the GeoSentinel Surveillance Network [26]. In a study among 328 travellers and foreign

residents with diarrhoea in Nepal, *Giardia* was diagnosed in 12%, and was more likely to occur in cases with diarrhoea lasting more than two weeks (27%) than in acute diarrhoea [27].

In Norway, laboratory confirmed cases of giardiasis are notifiable to the Norwegian Surveillance System for Communicable Diseases (MSIS). About 200-300 reported *Giardia* cases have been infected abroad each year since 1990 (Table 1) [28]. Of diarrhoeal infections in Norway registered by MSIS in 2008, 270 were caused by *Giardia*, 2875 by *Campylobacter*, four by *Diphtheria*, 1941 by *Salmonella*, 134 by *Shigella* and 50 by *Yersinia* spp. [28].

Approximately 50 cases each year of *Giardia* infections acquired in Norway, have been registered by MSIS since 1980 (Table 1) [28]. However, in 2004 as many as 621 cases were registered, due to a large waterborne outbreak in Bergen in the period from August to December [29]. Leaking sewage pipes into the city's water source, Svartediket (Figure 2), during a period of heavy rainfall, combined with insufficient water treatment, was identified as the source of the outbreak, and approximately 1 300 laboratory confirmed cases were registered. Based on the excessive number of metronidazole prescriptions made in the period, and taking into account that half of cases may be asymptomatic, more than 5000 cases have probably been infected during this outbreak [29]. This is the largest waterborne outbreak, and the first *Giardia* outbreak, registered in Norway. A total of 72 waterborne outbreaks, infecting 10 616 cases, have been registered in the period 1988 to 2002, 26% of these were caused by *Campylobacter*, 18% by norovirus and 46% had unknown aetiology [30].

Waterborne outbreaks are the most important route of *Giardia* transmission in Western countries. Several factors contribute to this; experimental studies have shown that cysts may survive for up to two months in cold water [6], conventional water treatment methods may not eliminate the parasites [24], and contamination of a community water supply has the potential to affect a great number of people. Before the Bergen outbreak in 2004, the largest outbreak registered in Europe had been at the ski resort Sälen in Sweden in 1986, affecting >1400 people [31].

Waterborne outbreaks of protozoan parasites reported worldwide in the period 1955 to 2003 have recently been extensively reviewed, and *Giardia* accounted for 132 (41%) of these [32]. Only outbreaks in Western countries have been reported, probably due to resource limitations and insufficient outbreak investigations in developing countries.

Zoonotic transmission has been reported in giardiasis [33], however, the impact of such transmission is not clear. Beavers were the likely source of an outbreak in USA in 1986 [34], but animal sources in outbreaks are not commonly reported [32].

Clinical case definitions are useful if parasitological testing must be avoided when laboratories experience capacity problems during large outbreaks. During a waterborne outbreak in Colorado, a *Giardia* case definition included illness lasting seven days or more combined with two or more of the symptoms diarrhoea, flatulence, foul-smelling stools, nausea, abdominal cramps or excessive tiredness [35]. The sensitivity and specificity of this definition was 88% and 73% respectively.

Figure 2. Svartediket.

Smaller outbreaks due to other routes of transmission have been well described from Western countries, although rarely. Raw vegetables and fruits contaminated with cysts from water or from an infected food handler are typical sources in food-borne infections [36-42]. Shellfish may accumulate pathogens from infected water and be a potential source of infection, and *Giardia* cysts have been identified in both oysters and mussels [43, 44]. Several outbreaks have been reported from child day care centres [45-48]. Transmission may also occur in swimming and wading pools [42, 49, 50]. Other groups at risk include homosexual men [51-55] and persons living in institutions [47, 56].

Table 1. *Giardia* cases in Norway reported to MSIS and place of infection in the period 1980-2009.

Place of infection	1980	1990	2000	2003	2004	2005	2006	2007	2008	2009
Norway	-	7	37	25	706	162	63	55	42	37
Unknown	13	66	19	43	621	58	35	37	26	60
Abroad	61	255	287	254	253	208	196	198	202	156
Total	74	328	343	322	1580	428	294	290	270	253

3. Pathogenesis

Both parasite and host factors seem to be involved in the pathophysiological processes causing diarrhoea, maldigestion and malabsorption in giardiasis, although incompletely understood.

In vitro studies on human samples have shown that *Giardia* attach by its adhesive ventral disc to the microvillus brush border of the intestinal epithelium, and cause barrier dysfunction by disrupting tight junctions and inducing epithelial apoptosis [57-59]. Further have experimental studies shown that activated CD8 T lymphocytes produce cytokines responsible for shortening of epithelial microvilli, which lead to malabsorption of electrolytes, nutrients and water as well as inhibition of the digestive enzymes lipase, protease and disaccharidase [60]. Disaccharidase insufficiency, and consequently failure in splitting and absorbing milk lactose, causes osmotic diarrhoea characteristic for temporary lactose intolerance commonly seen in giardiasis. Bacterial overgrowth in the small intestine may also play a part in the pathogenesis of the disease [61].

In clinical studies, inflammation and villous shortening in duodenal biopsies varies from 4% to 87% [62, 63], and why there is such a high variability in mucosal reactions, as well as in clinical manifestations, is not known. Genotypes and mixed infections have been proposed to be responsible for disease variability [64], but results from studies of the association between genotypes and severity of disease are not conclusive. An experimental study by Nash et al showed an association between strain variation and infectivity [65], and it seems that infection with a genotype less prevalent in a community induce more severe symptoms, however, both assemblage A and B have been associated with different symptom patterns in studies from different populations [19, 66-76].

The host defence mechanisms are complex. Experimental studies recently reviewed [77-79] have shown that natural barrier mechanisms (mucus, peristalsis, proteases, lipases, bile salts,

intestinal microbiota and paneth cells), innate immune responses (nitric oxide, reactive oxygen species, lactoferrin, defensins, phagocytes, mast cells and dendritic cells) and adaptive immune responses, both cell-mediated as described above and humoral, are involved although incompletely understood. Although *Giardia* is not an invasive parasite, it induces a humoral immune reaction with production of immunoglobulines (IgG, IgM and IgA) [31, 65, 79, 80]. Clinical studies support that adaptive immune response play a role; children in endemic areas and non-immune travellers seem to have a higher risk for symptomatic disease than those who have been exposed for a longer period of time [81], and hypo-gammaglobulinemia is associated with chronic infection [82]. Proteins on the surface of the parasite, variant surface proteins (VSPs), are major immuno-reactive proteins [80], and are also responsible for an important defence mechanism by enabling the parasite to undergo variation of its surface proteins and thereby evade the host immune response [83, 84].

HIV infection does not seem to be associated with more severe disease [85, 86]. Interestingly HIV infection stimulates the production of CD8 T-lymphocytes in the gut [87], and these lymphocytes are probably essential in the immune reaction against the *Giardia* parasite, as described above.

4. Clinical presentation

Experimental studies create optimal situations to study the course of an infection, although ethical considerations obviously limit the use of this method. Despite the questionable method, results from two experimental studies in humans have been reported, and are commonly used as references to the natural course of giardiasis. In 1953, Rendtorff reported results from four experiments in a controlled study of prison volunteers experimentally infected with *Giardia* cysts [88]. Of all cases receiving cysts, 53% (21/40) became infected. Risk for infection was associated with infectious dose: All 13 cases who received from 100 to 1 million cysts, compared to 36% (8/22) who received 10 or 25 cysts, became infected, while only one cyst was not infectious in any cases. Persistent infection after at least 129 and 132 days was found in 15% of infected cases (2/14), while 85% (12/14) spontaneously cleared the infection within 5-41 days (mean 18, median 13 days). Asymptomatic infection was found in 40% (6/15), while frequent and loose stools lasting from two to four days was observed in 60% (9/15) in these experiments [88].

The variation in infectivity and clinical presentation reported by Rendtorff was observed in another experimental study in 1987 [65]. Nash et al infected 15 healthy volunteers with two different *Giardia* strains, GS/M and Isr. Of cases infected with GS/M, 100% (10/10) became infected, and 50% of these became ill after a prepatent period (the time from inoculation until parasites are detected in stool) of 7.5 days (mean). Severity of symptoms varied; one volunteer had diarrhoea, flatulence, abdominal pain, anorexia, vomiting, abdominal cramps, headache, malaise and abdominal gurgling for six days, three volunteers had loose stools and milder symptoms, while one volunteer had fever and headache but no diarrhoea. Interestingly none of the volunteers inoculated with the Isr strain became infected; suggesting that degree of infectivity may be strain dependent.

Both of these experimental studies are limited by few cases, and that they included adult males only. However, symptoms during acute infection in larger cohorts in developed countries have been studied during outbreaks, and these reports support that symptoms are variable and that the majority of infected cases remain asymptomatic.
Among laboratory confirmed cases during an outbreak in New Hampshire in 1980, the following symptoms were recorded (n = 213): Diarrhoea 86%, abdominal cramps 81%, anorexia 65%, flatulence 58%, abdominal distension 55% and weight loss 53%. Duration of symptoms was 10 days (mean) and 13% were hospitalised. A community survey revealed that 76% of the city residents had an asymptomatic and self limiting infection during this outbreak [89].
During the Bergen outbreak in 2004, 137 laboratory confirmed cases were interviewed during the early phase of the epidemic, and among these 90% reported diarrhoea, nausea, stomach pain, flatulence and foul smelling stools, 83% reported weight loss (mean 5 kg, range 1-23 kg), 36% vomiting, 17% reported fever and 7% were hospitalised [29].

Giardiasis seems to be self limiting in most cases, but a striking feature of the parasite is its ability to induce chronic infection, symptomatic or asymptomatic, if not treated. Rendtorff reported chronic infection in 15% (2/14) of experimentally infected and untreated cases [88]. A Nordic meta-analysis reported *Giardia* infection in 6% of symptomatic cases (one or more of the symptoms vomiting, gastroenteritis, diarrhoea and abdominal pain/cramps/discomfort), and 3% of asymptomatic cases, in the population, supporting that chronic giardiasis is prevalent in non-endemic countries [90].

Chronic infection may present with symptoms similar to irritable bowel syndrome (IBS); a condition characterised by abdominal pain or discomfort, associated with altered bowel habits, lasting for more than 12 weeks [91]. Among 137 cases with symptoms of dyspepsia or IBS who satisfied the Rome II criteria [91], 7% had giardiasis in one report from Italy [92].

Malabsorption in chronic giardiasis may cause chronic diarrhoea and steatorrhea, weight loss and nutrient and vitamin deficiencies [93].
Vitamin B12 malabsorption, and anaemia due to Vitamin B_{12} and folate deficiencies, has been reported [94-96]. Vitamin A deficiency, a potentially severe condition since such deficiency is an important cause of blindness in developing countries, has also been documented in giardiasis; a significant improvement of vitamin A absorption was demonstrated after anti-*Giardia* treatment in one study [97].
Osmotic diarrhoea due to lactose malabsorption is common in giardiasis, and may persist for weeks after eradication of the parasite [98].
Fat and carhohydrate malabsorption has been documented in controlled clinical studies [96], but the role of amino acid malabsorption is unclear. Although there have been casuistic reports on severe protein loss and hypoalbuminaemia [99], no association between giardiasis and protein loosing enteropathy was found in a study in Gambian children [100].
In a case-control study from India malabsorption syndrome was caused by *Giardia* in 24% (12/50) of adult cases compared to 8% (4/50) of healthy controls [101]. In children the difference was not significant (16% versus 6%) which also illustrate the problem in interpreting the finding of *Giardia* in patients from endemic areas since the infection may be asymptomatic.

In developing countries where frequent re-infections are common [102], giardiasis contributes together with other infectious agents to malnutrition. Considering its high prevalence and the parasites' ability to induce chronic infection and malabsorption, one would suspect an impact on growth in children. Since first reported in 1921, several studies have shown an association between severe giardiasis and such impairment [103-105]. More than one episode of giardiasis per year during infancy was associated with poor cognitive function at 9 years of age in 239 Peruvian children [106]. However, in another longitudinal study of 220 Peruvian children, no significant association between *Giardia* and nutritional status or diarrhoea was found [107].

Also studies in asymptomatic children have shown diverging impact on growth. In one study from Brazil (n = 597) asymptomatic giardiasis was significantly associated with impeded growth [108], while studies from day care centres in western countries have not found this association [109], suggesting a difference in disease susceptibility between healthy and malnourished children.

Rare extra-intestinal manifestations have been reported, probably due to immune mechanisms since *Giardia* is not invasive. These reports have been of giardiasis associated with reactive arthritis and synovitis [110, 111], urticaria and pruritis [112], uveitis [113] and allergy [114, 115].

5. Laboratory diagnostic methods

Giardiasis is diagnosed by examining stool samples by light microscopy, immunofluorescence assay (IFA), enzyme immunoassay (EIA) or polymerase chain reaction (PCR) methods. Analyses of serum or duodenal aspirates may also be performed.

Light microscopy is a labour intensive method, but has the advantage of detecting additional parasites if present. Stool is concentrated and examined directly (wet-mount preparation) or permanently stained (trichrome) for cysts or trophozoites (detection of trophozoites requires fresh stool). Sensitivity is poor when only a single sample is analysed, particularly if there are few cysts or quality of microscopy is insufficient, but sporadic samples in an infected person may also be negative due to intermittent excretion of cysts; in one report of 91 cases all with three samples analysed, one, two or all three stool samples were microscopy positive in only 23%, 22% and 55% respectively [116]. Sensitivity increases when multiple samples collected from separate defecations are analysed; sensitivity of 73%, 81% and 85% from analysis of one, two or three samples respectively have been reported (n=73) [117].

Sensitivity of microscopy increases when samples are stained with a specific anti-*Giardia* antibody coupled to a fluorescent compound (IFA). Cysts show fluorescence when examined under ultraviolet light in a fluorescence microscope. High sensitivity (92%-100%) and specificity (100%) has been reported with this method [118-120].

EIA also uses antibodies to detect *Giardia* specific antigen in stool, and these tests are less time consuming since the result is read after only few minutes. Several commercial rapid antigen tests are available, and high sensitivity (80%-99%) and specificity (>99%) has been reported [118, 121-124]. However, EIA was significantly less sensitive (61%) than microscopy in a study from the Bergen outbreak [125]. Reduced sensitivity of EIAs was associated with low cyst numbers and single samples in these studies.

PCR is a sensitive and specific method also when there are few cysts [119, 124, 126]. In one study comparing PCR and IFA, sensitivity was 97% and 92% respectively (no significant difference) and specificity was 100% for both [119]. PCR is mainly used in research, and during the Bergen outbreak PCR was used to genotype and sub-genotype the *Giardia* parasite and thereby elucidate host and parasite factors [127], but the method has limited use in routine diagnosis.

Detection of trophozoites in duodenal aspirate was previously considered a sensitive test when stool microscopy was negative, but studies supporting this were based on few cases, as shown in a review by Goka et al [117]. Larger studies have shown very low benefit from this test. In one study only 44% (32/73) of *Giardia* positive cases had positive duodenal aspirates, compared to 85% stool microscopy positive [117]. This finding was supported by a study from the Bergen outbreak were only 10% (4/40) of *Giardia* positive cases had trophozoites in duodenal biopsies [62].

Both indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA) may be used in detecting specific anti-*Giardia* antibodies [128, 129]. Serologic methods have been used in epidemiologic studies as described previously, but not widely in routine diagnostic laboratories. In endemic areas, detection of *Giardia* antibodies may be due to previous exposure, as reported in a study from Bangladesh where no association between sero-positivity and positive stool microscopy was found [130]. However, in areas with no previous exposure, serologic tests are more specific in diagnosing acute infection. In a study among 352 exposed cases during the outbreak in Sälen in Sweden, IgG and/or IgA antibodies were detected in 68% of *Giardia* positive cases, in 22% of *Giardia* negative cases and only among 10% of healthy controls [31]. A significant increase in sensitivity was observed when serum was collected more than three weeks after infection. Also in a controlled study from

Cuba, serology was strongly associated with positive microscopy, although sero-positivity increased with age which might have been due to previous exposure [131].

6. Treatment

Six classes of drugs are effective against the *Giardia* parasite: Quinacrine has been used since the 1930s, nitroimidazoles, furazolidone and paromomycin since the 1960s, benzimidazoles since the 1980s and the last 10 years nitazoxanide has been reported to be effective against giardiasis [132].

Results from controlled clinical studies of efficacy, based on parasitological response, are presented in table 2 (modified from table in press by our group [133]). Recommendations of effective dosages based on these studies, as well as mechanisms of action and reported adverse effects, are presented in table 3 (modified from tables published and submitted by our group [133, 134]). Pharmacological aspects in anti-*Giardia* drugs have also recently been reviewed by others [132, 135, 136].

Efficacy reported in the clinical studies are not directly comparable due to variations in the populations studied (age, endemicity), time until follow-up, number of stool samples examined, dosage of drugs and duration of treatment. In studies from endemic areas, relapse rates may be overestimated if duration of follow-up is too long, due to high risk of re-infections, as reported in a study from Peru where 98% of children became re-infected within 6 months after effective treatment [102]. However, relapses may be underestimated if follow-up time is too short. In one study from a non-endemic area, 43% (6/14) of relapses were diagnosed after more than four weeks [137]. Per protocol rather than intention to treat analyses were presented in most of these studies, which potentially also may overestimate efficacy.

Metronidazole has for many years been the drug of choice for giardiasis. However, single dose treatment with tinidazole, ornidazole or secnidazole (nitroimidazoles with long half lives) seems to have similar efficacy and fewer side effects. Short course treatment with metronidazole and other classes of anti-*Giardia* drugs are less effective. Nitazoxanide and benzimidazoles are well tolerated, and have the advantage of also being active against helminth infections which are endemic in developing countries [138-142], although benzimidazole treatment has low efficacy in some studies. Quinacrine is highly effective in most studies, but is not recommended as first line treatment due to potentially severe side

effects. The aminoglycoside paromomycin seems to be less effective than other drugs, although few reports are available.

Available documentation of risks during pregnancy varies for different anti-*Giardia* drugs. Of the nitroimidazoles, metronidazole has been best studied. Metronidazole has shown carcinogenic and teratogenic effect in animal studies, and although carcinogenic effect has not been reported in humans, it raises concern about safety during pregnancy [143-146]. In a meta-analysis including 1336 women exposed during first trimester [147], and in two studies including 228 and 1041 pregnant women respectively [148, 149], no association with birth defects were found. However, malformations possibly associated with metronidazole during first trimester has been reported, although rarely [146, 150]. Based on these reports, metronidazole during pregnancy is controversial, although often recommended during second and third trimester, weighted against indication and availability of alternative drugs, since the possible risk is low [146]. Nitroimidazoles should be avoided during first trimester.
Benzimidazoles have been widely used and recommended by WHO after first trimester in treatment programs among pregnant women, in order to reduce hookworm induced anaemia [151, 152]. In controlled studies evaluating birth defects following these programs, significant risk for teratogenic effects were not found [153-155]. However, the dosages used in anti-helminth programs are lower than recommended dosages for giardiasis, and experimental studies have suggested teratogenic effects [156]. One controlled clinical study found higher risk of major birth defects (odds ratio 1.66), although not significant, among women who had used mebendazole during first trimester [155]. Based on clinical reports on safety during second and third trimester, benzimidazoles could be used during this period, but should be avoided during first trimester.
Due to mutagenic effects in animal studies, and lack of clinical studies in humans on teratogenic effects from furazolidone, quinacrine and nitazoxanide, these drugs should be avoided throughout pregnancy [157, 158].
Paromomycin is the only anti-*Giardia* drug not contraindicated during first trimester [159], since it is poorly absorbed and therefore has negligible systemic effect.

Studies on treatment refractory giardiasis are limited. In one study, albendazole in combination with metronidazole was effective in nine out of 10 metronidazole resistant cases [160]. Quinacrine combined with metronidazole or tinidazole was effective in five of six treatment refractory cases in one study, four of these were immunosuppressed [161]. Such

synergistic effect of combining metronidazole and quinacrine has also been reported *in vitro* [162]. One report showed that nitazoxanide was effective in a patient suffering from HIV and metronidazole/albendazole resistant giardiasis, and the *Giardia* isolate from the patient showed the same resistance pattern *in vitro* and in a mouse model [163].

Clinical resistance has been demonstrated for all drugs as described above, however, several factors may influence clinical response other than drug resistance, like inadequate immune response, compliance problems, reduced quality of drugs or impaired absorption due to vomiting and diarrhoea.

Laboratory studies have the advantage of eliminating these factors, and *in vitro* resistance has been reported in all classes of drugs [164, 165]. Different laboratory methods have been used; *in vivo* animal studies and *in vitro* tests of viability, or molecular characteristics, of cultured human *Giardia* isolates or laboratory induced resistant clones. Although mechanisms of drug resistance are not completely understood, molecular studies have elucidated some characteristics. The enzyme pyruvat:ferredoxin oxidoreductase (PFOR) was downregulated in metronidazole resistant but not in furazolidone resistant *Giardia* strains, which is consistent with the role this enzyme has in activating metronidazole, while furazolidone probably is activated by nicotinamide adenine dinucleotide (NADH) and not by PFOR [166]. In a molecular study of genes potentially involved in resistance, laboratory induced nitazoxanide resistant and metronidazole resistant *Giardia* clones were compared to *Giardia* WB C6 wild type, and in addition to slightly reduced expression of pyruvat oxidoreductase (POR) and nitroreductase (NR), a gene encoding a major variant surface protein (VSP) was significantly reduced in both resistant clones, indicating that the parasites ability to evade host immune response by variable expression of surface proteins may be a target for these drugs [167]. The nitazoxanide resistant strain showed cross resistance to metronidazole, while the metronidazole resistant strain was sensitive to nitazoxanide in this study [167]. Cross resistance found *in vitro* between other anti-*Giardia* drugs has also been reported; furazolidone resistant strains induced more easily quinacrine resistance in one study [168], and albendazole resistance was more readily induced in a furazolidone resistant strain in one report [169].

Drug susceptibility testing is not routinely used, although relatively simple systems for resistance testing are commercially available and could be used in surveillance of drug resistance in giardiasis [170].

Other drugs have been studied experimentally, and have recently also been reviewed by others [132, 171]. The following compounds have shown anti-*Giardia* effect *in vitro* or in animal studies: Bithionol, dichlorophene and hexachlorophene [172], pyrimethamine and chloroquine [173], the tricyclic antidepressant drug chlorimipramine [174], sodium fusidate which also has the advantage of not being terotegenic [175], ronidazole, satranidazole, fexinidazole, flunidazole and nimorazole (5-nitroimidazoles), nitrofurantoin and niridazole [176], mefloquine, doxycycline and rifampin [177], ivermectin [178], fenbendazole [179], ciprofloxacin [180], bismuth subcitrate [181], thiosemicarbazone [182], new benzimidazoles [183-185], ethyl-phenylcarbamates [186], menadione which also killed cysts [187], the naturally derived saturated fatty acid dodecanoid (lauric) acid [188], nocodazole and colchicine [189], silymarin [190], new thiazolides [191], disulfiram [192] and azithromycin [193].

Controlled clinical trials of experimental drugs against *Giardia* have shown some promising results. Bacitracin zinc had anti-*Giardia* effect both *in vitro* [194] and *in vivo*; one clinical study reported 95% (20/21) efficacy [195]. In a study from Cuba, chloroquine 10mg bid for five days cured 86% [196], and chloroquine has also shown anti-*Giardia* effect *in vitro* [173, 197]. D-propranolol has demonstrated inhibition of growth and motility of the protozoan *in vitro* [198], and clinical effect in a metronidazole resistant case has been reported [199]. The bee glue preparation propolis inhibited growth *in vitro* in one report [200], and in a clinical study treatment for 20 days cured 80% [201]. Ozone have shown anti-*Giardia* effect *in vitro* in different studies [202, 203], and in a clinical trial ozonized sunflower oil cured 64% [204].

Table 2. Clinical studies evaluating anti-*Giardia* treatment (modified from table in press [133]).

Place/ ref.	Population		Follow up (d)[1]	5-nitroimidazoles								Benzimidazoles				Amino-glycoside		Acridine		Nitrofuran		5-nitro-thiazolyl	
	Age	N		Metronidazole		Tinidazole		Ornidazole		Secnidazole		Albendazole		Mebendazole		Paromomycin		Quinacrine		Furazolidone		Nitazoxanide	
				Dose/ Duration[2]	Ef %	Dose/ Duration	Ef %	Dose/ Dur.	Ef %	Dose/ Dur.	Ef %	Dose/ Duration	Ef %	Dose/ Duration	Ef %	Dose/ Dur.	Ef %	Dose/ Dur.	Ef %	Dose/ Duration	Ef %	Dose/ Duration	Ef %
Leningrad [137]	A	60	56	200 tid/7 200tid/7x2 2400 sd 2400 sd/ 2	73 93 60 80																		
Asia [205]	C	120	30	200 tid/7[3] 2000 sd[3]	60 52	2000sd	86	2000sd	95									100tid/5	100				
Iran [206]	C	52	21	20[4]/10	96					30[5]sd	100												
Egypt [207]	A+C	80	21	500/10	95	2000sd	90	1000sd	97														
India [208]	A+C	75	16	50[5]sd/1	54	50[5] sd	98																
Leningrad [209]	A	45	56			150bid/7 2000 sd	74 92																
Bangladesh [210]	A+C	63	28	60[5] sd 50[5]sd/3	56 93	50[5] sd 50[5] sd	94 100																
Venezuela [211]	C	70	15							30[5]sd	98												
Leningrad [212]	A	100	56			1500 sd	90	1500sd	90														
Turkey[213]	C	175	14	20[4]/7	89			25[5] sd 20[5] sd 12bid/5	97 94 100														
Israel [214]	C	75	14	20[4]/7	100			40[5]sd	92														
Brazil [215]	C	267	21			50[5] sd	90			30[5]sd	91												
Cuba [196]	C	165	10			50[5]sd	91					400sd/5	62										
Bangladesh [216]	C	103 114 116 115	10	125tid/5	97							600 sd 800 sd 400sd/3 400sd/5	62 75 81 95										
India [217]	C	150	2	22.5[4] / 5	97							400 sd	97										
Pakistan [218]	A	68	17	400tid/ 5	84							1200 sd 400bid/3	55 70										
Turkey [219]	C	107	14	20[4]/7	89							10[5]sd/5	90										
Turkey [220]	A	57	10	500tid/5	100							400sd/5	96										
Thailand [221]	C	113	2			50[5] sd	96					400sd/3	50										
Cuba [196]	C	165	10			50[5] sd	91					400sd/5	62										
Austria [222][6]	Ns	20	14									10-15[5]/3	10										
Cuba [223]	C	146	7							30[5]sd	79			200tid/3	78								

Thailand [224]	C	84				50[5] sd	93				400 sd[7] 800 sd	74 50										
Turkey [225]	C	48		15[5] sd/7	93			40[5] sd	100		100tid/1 100tid/7	42 58										
Iran [226]	C	100	7	15[4]/7	90						200tid/5	86										
Iraq [227]	A+C	40	5-56								200tid/1	95										
Spain [228]	A	23	28	250tid/7	89						200tid/1	14										
Italy [229][6]	C	10	Ns								200tid/5	0										
Cuba [230]	C	122	7			50[5] sd	82				200tid/1	64										
Cuba [231]	C	122	7								200tid/5	79			6[4]/5	84						
Iran [232]	C	160	28	125sd-tid/5 250tid/5	100										8[4]/5	100						
USA [233]	C	45	42-84												6[4]/10	77	8[4]/10	89				
Brazil [234]	A+C	172	21	125bid-tid/7 250bid/7	87	150bid/7	97										8[4]/7	72				
USA [235]	C	22	28														8[4]/10 8[4]/ 5	92 20				
Mexico [236]	C	82	3	63-250tid/10	96												17-67qd/10	92				
Cuba [201]	C	256	21	25[4]/7	80								35[4]/7	92								
USA [237]	A+C	15	70										15[4]/5	40								
Mexico [238]	C	82	7								100bid/3	80							100bid/3	78		
Cuba [239]	C	137	10			50[5] sd	91												7.5[5]bid/3	78		
Egypt [240]	A	22[8]	7-10																500bid/3	91		
Mexico [141]	C	32[9]	11																100bid/3	56		
Peru [241][10]	C	110	2-7	125-250 bid/5	75														100-200bid/3	71		
Mexico [242]	C	22	10																Ns	69/79/81[11]		
Mexico [243]	A+C	87	11																100bid/3	71		

Abbreviations: D, days; Ef, efficacy; dur, duration; A, adults; C, children; sd, single dose; bid, twice daily; tid, three times daily; qd, four times daily; Ns, not stated.
[1]Response assessed by stool microscopy. Duration of follow-up in days.
[2]Dosages in milligram (mg), duration in days. Single dose was given for one day, if other is not stated.
[3]The dosage was adjusted to the body surface of the patients from the adult dosage given in this table.
[4]mg/kg/day divided into three doses.
[5]mg/kg.
[6]Uncontrolled study.
[7]Albendazol 400mg in combination with Praziquantel 20mg/kg sd.
[8]Placebo controlled study, with 11 cases in the nitazoxanide group.
[9]Of 275 cases with intestinal parasitic infections, 32 *Giardia* cases received nitazoxanide, and 19 received mebendazole, quinfamide or both.
[10]Results from intention to treat analyses.
[11]Efficacy reported for first, second and third treatment.

Table 3. Drugs active against *Giardia* infection; mechanisms of action, recommended dosages and side effects (modified from [133, 134]).

Drug	Mechanism	Adverse events	Recommended dosage[1]		Other
			Adults	Children	
Five-nitroimidazole compounds					
Metronidazole	Reductive activation of nitro group, by ferredoxin and the enzyme PFOR. Anti-parasitic effect: -Bind to DNA -Produce toxic radicals -Inhibit trophozoite respiration	GI discomfort, metallic taste, disulphiram-like effects. Headache, vertigo, insomnia, irritability, neuropathy, seizures. Rash. Reddish-brown urine. Transient elevation of transaminases. Leukopenia. Pancreatitis, hepatitis, cholangitis (rare). Metronidazole less tolerated than the other 5-nitromidazole compounds.	200 mg tid x 7 d 500 mg sd[3] x 10d 500 mg tid x 5 d	15 – 20[2] x7 d 22.5[2] x 5 d	First line treatment. Metronidazole efficacy low when shorter course than 5 days, the other compounds effective as single dose due to longer half life.
Tinidazole			1.5 – 2 g sd	50[4] sd (syrup available)	
Ornidazole			1 – 2 g sd	20 – 40[4] sd	
Secnidazole			2 g sd	30[4] x 1 d	
Nitrofuran derivatives					
Furazolidone	Possibly reductive activation by the enzyme NADH oxidase, and production of toxic nitro radicals which damage the parasites functional organelles including its DNA.	Nausea, vomiting, diarrhoea. Haemolytic anemia in neonates and in G6PD-deficiency. Disulphiram-like activity. Interaction with MAO inhibitors. Brownish urine.	100 mg qd x10 d	6[5] x 10 d (syrup available)	Should not be given to neonates or breastfeeding women, due to risk of haemolytic anemia.
Benzimidazoles					
Albendazole	Inhibits cytoskeleton polymerization and impaire glucose uptake by binding to the parasites β-tubulin cytoskeleton.	Usually well tolerated. Nausea, vomiting, diarrhoea, epigastric pain.	400 mg sd x 5d	10[4] x 5 d	Also effective against helminths. Albendazole effective in treatment refractory cases in combination with metronidazole.
Mebendazole		Usually well tolerated. Transient abdominal pain.	100 - 200 mg bid - tid x 1 – 5 d (Optimal dose and duration unclear)		
Acridine derivatives					
Quinacrine	Not fully understood. Possibly inhibition of nuclein acid cyntesis by binding to DNA, or decreased oxygen consumption due to interference with the enzyme NADH oxidase.	Potentially severe side effects. Vomiting, bitter taste, nausea, headache. Yellow discoloration of skin, urine or sclerae (reversible). Urticaria, exfoliative dermatitis, exacerbation of psoriasis. Haemolysis in G6PD-deficiency. Psychosis.	100 mg tid x 5 d	8[2] x 5 d	Effective in treatment refractory cases, alone or in combination with other drugs.
Amoniglycosides					
Paromomycin	Interaction with 50S and 30 S ribosomal subunits leads to misreading of mRNA, and thereby inhibits the parasites protein synthesis.	Usually well tolerated. Gastrointestinal discomfort.	500 mg tid x 10 d	25[2] x 10d	Regarded as safe in pregnancy.
5-nitrothiazolyl derivatives					
Nitazoxanide	Not fully understood. Anti-parasitic effect after reductive activation. Inhibition of parasite nitroreductase G1NR-1.	Usually well tolerated. Abdominal pain, diarrhoea, vomiting, headache, yellowish urine.	500 mg bid x 3d	7.5[4] bid x 3 (syrup available)	Also effective against helminths and some bacterial enteric infections. Effective in metronidazole resistant infection.

Abbreviations: GI, gastrointestinal; Sd, single dose; PFOR, Pyruvate ferredoxin oxidoreductase; G6PD, Glucose-6-phosphat-dehydrogenase; NADH, Nicotinamide adenine dinucleotide; DNA, Deoxyribonucleic acid; mRNA, messenger ribonucleic acid; bid, twice daily; tid, three times daily; qd, four times daily; d, days; g, gram.

[1]Based on results from clinical studies (Table 2).

[2]mg/kg/day, divided into three doses.

[3]One day duration of sd if not other is stated.

[4]mg/kg.

[5]mg/kg/day, divided into four doses.

V. Aims of the study

To evaluate efficacy of a treatment ladder, and genetic characteristics, in treatment refractory giardiasis after an outbreak in Bergen, Norway in 2004.

To investigate if cases with persistent abdominal symptoms after *Giardia* infection, and no detectable *Giardia* parasites in stool, suffered from chronic, cryptic giardiasis.

To investigate the prevalence of fatigue and abdominal symptoms, and factors associated with such symptoms, two years after the *Giardia* outbreak.

VI. Summary of papers

A. Paper 1

Metronidazole is the only drug licensed against *Giardia* infection in Norway. The objectives of this study were to evaluate the efficacy of three different anti-*Giardia* treatment regimens in cases who had not responded to metronidazole treatment, and to compare genetic characteristics of the parasites.
This was a clinical observational study among cases who had been infected with *Giardia* during the outbreak in Bergen in autumn 2004, and who experienced chronic, treatment refractory infection. Among 1268 laboratory confirmed cases registered during the outbreak in August-December 2004, 120 cases were referred to our out patient clinic due to protracted abdominal symptoms. Of these, 42 cases still had *Giardia* cysts in stool samples after mean 2.2 (range 1-3) courses of metronidazole, and 38 of these were treated according to a standardised treatment ladder in the period between January 2005 and December 2006.

All patients were treated with albendazole 400 mg bid in combination with metronidazole 250 mg bid for one week. Those who did not respond to this regimen were treated with paromomycin 500 mg tid for one week. Those who failed on both these regimens were treated with quinacrine 100 mg tid in combination with metronidazole 750 mg tid for three weeks. Treatment efficacy was based on parasitological response defined as seven microscopy negative stool samples up to four weeks after the end of treatment. Clinical symptoms and adverse events were evaluated at baseline and four weeks after treatment.
Giardia isolates were available from 45% of cases (17/38) for characterisation by PCR and sequencing at the gdh and β-giardin genes respectively.

Albendazole in combination with metronidazole was effective in 79% (30/38), paromomycin was effective in 50% (3/6), and quinacrine in combination with metronidazole was effective in 100% (3/3) of the cases. Discoloration of skin, confusion, nightmares, dizziness and nausea were recorded in the quinacrine/metronidazole group, and mild hair loss was reported by one patient in the albendazole/metronidazole group. Except for the episode of mild hair loss, no unexpected or severe side effects were recorded in any of the groups.
Sequencing of PCR products revealed that all cases had *Giardia* cysts of genotype gd-ber3 at the gdh gene and BG-ber2 at the β-giardin gene, while previously published sequence profiles from the peak of the outbreak were more heterogenous.

In this study, albendazole and quinacrine, both in combinations with metronidazole, were effective against metronidazole refractory giardiasis, while paromomycin seemed to be less effective. Particular sub-genotypes may be associated with treatment refractory infection in this cohort.

B. Paper 2

The aim of this study was to evaluate if *Giardia* negative patients, referred to our outpatient clinic due to protracted abdominal symptoms after the Bergen outbreak in 2004, suffered from cryptic and metronidazole refractory chronic giardiasis.

This was a prospective randomised open clinical study. The included patients had been exposed to contaminated water and had clinical giardiasis during the outbreak, and after treatment with one to three courses of metronidazole for five to ten days, all cases had more than three microscopy negative stool samples and one negative faecal antigen test.
Based on reports on synergistic anti-*Giardia* effect of metronidazole and albendazole combination treatment as described in paper 1, patients in one arm were treated with albendazole 400 mg bid and metronidazole 250 mg tid for seven days (A/M). Based on the hypothesis that an illness similar to post-infectious tropical sprue may be one possible explanation for protracted abdominal symptoms and weight loss, patients in the other arm received tetracycline 250 mg tid and folic acid 5 mg once daily for 28 days (T/F).
Abdominal symptoms were reported by the patients on a written questionnaire at baseline, at the end of treatment, one month after treatment and finally one year after treatment. The primary endpoint was global improvement of symptoms one month and one year after treatment. Secondary endpoints were improvement of nausea, bloating, abdominal pain, diarrhoea, constipation and anorexia recorded on a scale from zero to ten, and changes in blood inflammation and malabsorption parameters.
Symptom scores were analysed regarding time and treatment using mixed linear modelling.

A total of 25 cases were included in the study. Blood tests taken at baseline and after one month were not significantly changed. At the end of treatment, total symptom score improved in both groups, although significantly only in the T/F group, while bloating decreased significantly in both groups at this point.

One month after treatment, 23% (3/13) in the T/F group and 8% (1/12) in the A/M group reported global symptom improvement. However, after one year total symptom scores were unchanged from baseline in both groups.

C. Paper 3

The objective of this study was to evaluate the prevalence of fatigue and abdominal symptoms among cases who had been infected during the Bergen outbreak in 2004, two years after.

Inclusion criteria was laboratory confirmed giardiasis during the Bergen outbreak. All 1262 *Giardia*–positive cases registered in the period of October 2004 to June 2005, received a mailed questionnaire in August 2006 (Appendix).
The following questions regarding fatigue and abdominal symptoms, respectively, were used in the statistical analyses: "Do you have abdominal symptoms now that you did not have prior to the *Giardia* infection?" (no/unsure/yes, dichotomized into no/unsure vs. yes) and "Do you have problems with fatigue?" (less or same as usual/more than usual/much more than usual). The last two answer options were defined as fatigue.
The association between fatigue and abdominal symptoms, gender and age, was investigated by simple and multiple ordinal logistic regression analyses using SPSS.

Among the 1017 (81%) respondents, 64% were women and the median age was 31 years, compared to 61% and median 30 years among all 1262 cases.
Fatigue and abdominal symptoms was reported by 41% (419/1017) and 38% (389/1017), respectively, and 25% (253/1017) reported both symptoms.
Increasing age was significantly associated with fatigue ($p < 0.001$) in all analyses. Female gender was significantly associated with fatigue in the simple ($p = 0.038$) but not in the multiple regression analyses. Neither age nor gender was significantly associated with abdominal symptoms. A significant association between fatigue and abdominal symptoms ($p < 0.001$) were found in all analyses, and neither gender nor age interacted with this association.

A high level of post-infectious fatigue and abdominal symptoms, not previously reported in giardiasis, were found in this study.

D. Paper 4

This paper describes risk factors associated with post-*Giardia* fatigue and abdominal symptoms two years after the Bergen outbreak.

Inclusion criteria and questionnaire (Appendix) used were the same as described in paper 3, and in this study the data were further analysed with respect to risk factors.
Number of treatment courses, delayed education and sick leave were used as indices of protracted and severe *Giardia* infection in the statistical analyses, and the sub-cohort of treatment resistant cases described in paper 1 were also included in these analyses. Previous abdominal problems, symptoms during infection, age and gender were also evaluated as possible risk factors. Simple and multiple ordinal logistic regression analyses were used to investigate the association between these possible risk factors (explanatory variables) and degree of abdominal symptoms and fatigue (response variables).

More than one course of anti-*Giardia* treatment and delayed education, were significantly associated with both fatigue and abdominal symptoms in all analyses. In the multiple regression analysis, female gender, bloating at the time of infection and treatment refractory infection were associated with abdominal symptoms. Age, previous abdominal problems without seeking health care, malaise at the time of infection and sick leave were associated with fatigue in the multiple regression analysis.

Indices of protracted and severe *Giardia* infection were associated with post-infectious fatigue and abdominal symptoms in this study.

VII. Main results and discussion

Paper 1. Treatment-ladder and genetic characterisation of parasites in refractory giardiasis after an outbreak in Norway

The paper describes high efficacy of combination treatment of albendazole or quinacrine in combination with metronidazole, respectively, and less efficacy of paromomycin, in metronidazole refractory chronic *Giardia* infection. Synergistic effect of metronidazole and albendazole has been reported from a randomised trial of metronidazole resistant cases [160], and our study is in line with this finding. Randomised trials on quinacrine and paromomycin in treatment refractory giardiasis have not been performed, although a case series among resistant cases successfully treated with quinacrine combined with nitroimidazole support our findings [161]. This study was an observational study using a treatment-ladder, while a randomised study design would have strengthened our findings. Metronidazole is the only anti-*Giardia* drug licensed in Norway, and delays in availability were different for the different drugs. A randomised study would have delayed treatment in patients with bothersome symptoms, and was therefore not performed for ethical reasons.
The three regimens can not be compared, since patients treated in the late steps in the ladder had clinically more multi-resistant parasites than patients treated in the first step. The efficacy of paromomycin and quinacrine/metronidazole should also be interpreted with care due to low number of cases.

In order to study genetic characteristics of the parasites, two gene sequences were analysed and the parasites were identical at these sequences, which was different from the picture during the peak of the outbreak, where 10 different sub-genotypes were described [244]. This leads to the hypothesis that the sub-genotypes found in our study may have been responsible for more virulent or resistant infection. The genes involved in metronidazole resistance are not fully characterised. Results from molecular studies show that such resistance may be mediated by altered gene expression, possibly involving reduced expression of the genes encoding for PFOR and VSPs [167], however, this remains to be further investigated. The sub-genotypes characterised in the present study do not fully explain metronidazole resistance, since these sub-genotypes were also found in cases diagnosed subsequent to the outbreak who responded to metronidazole treatment [127]. However, if these strains were more virulent, resistant or infective than other strains, they may have persisted longer in the environment, and thereby infected cases subsequent to the outbreak and also induced chronic infection. The host-parasite interactions in giardiasis are not fully understood, as previously

discussed, and other factors explaining chronic infection in this cohort are probably also involved. This is underlined by the fact that one of the more resistant cases in this study, who finally responded to quinacrine/metronidazole, had clinically significant IgA deficiency, an immunological disorder known to predispose to chronic giardiasis [82].

Paper 2. Effects of albendazole/metronidazole or tetracycline/folate treatments on persisting symptoms after *Giardia* infection: A randomized open clinical trial

After the Bergen outbreak all patients referred to our out patient clinic with chronic infection, diagnosed by detection of cysts in stool, were successfully treated as described above. However, approximately half the cases referred due to protracted abdominal symptoms did not have detectable parasites in stool. Chronic infection and fluctuation in cyst excretion is well described in giardiasis [88], and could be a possible explanation for symptoms in these cases.

Diarrhoea and weight loss were common during the outbreak [29], which are characteristic symptoms of malabsorption. Important differential diagnoses to malabsorption in addition to giardiasis are *Cryptosporidium* infection, celiac disease, lymphoma and tropical sprue [93]. *Cryptosporidium* had been excluded by negative faecal antigen tests, and anti-endomysial and anti-tissue transglutaminase antibodies as well as duodenal biopsies did not reveal celiac disease or lymphoma as etiologic agents [62]. Tropical sprue is a syndrome of undefined aetiology, characterised by small intestinal mucosa damage and often severe malabsorption of vitamine B12, folate and fat, following an episode of acute diarrhoea [93]. The name refers to that the condition is geographically restricted to specific areas in the tropics; it is much more common in Asia than in South America and Africa. Antibiotics may be effective, which support an infectious aetiology. The cases in our cohort had not travelled abroad, but the hypothesis was that a similar condition, involving bacterial agents, could explain the symptoms.

This study described that treatment directed against chronic giardiasis and tropical sprue, among cases with persistent abdominal symptoms following giardiasis, had no effect on symptoms one year after treatment.

The temporary symptom reduction reported may have been due to anti-inflammatory effect, or effect on gut microbial flora, of both treatments. The difference in temporary symptom reduction seen between the groups may be explained by the longer duration of T/F treatment.

It is not known if reporting of symptoms were influenced by cases expecting to improve during treatment, which would have been elucidated if a placebo group had been included. The reason for not including a placebo group was mainly that extending to three arms would have been more resource and time consuming in a clinical setting, and would have delayed treatment in the symptomatic group.
Taking these limitations into account, the lack of change in symptoms after one year, leads to the conclusion that chronic giardiasis, or a tropical sprue-like illness, did not explain symptoms in this cohort.

Paper 3. High rate of fatigue and abdominal symptoms 2 years after an outbreak of giardiasis

During the Bergen outbreak, protracted symptoms after infection, among *Giardia* negative patients, were reported both in primary health care and among cases referred to our outpatient clinic [62, 245]. Of those referred, all *Giardia* positive cases had been successfully treated as described in paper 1, while the majority were *Giardia* negative but still complained of IBS-like symptoms and fatigue [62]. Chronic infection could hypothetically explain protracted symptoms [88], but this was excluded among referred cases as described in paper 2. Extensive work-up among all referred cases, including stool culture and microscopy, endoscopy and blood tests, did not reveal the cause of IBS-like symptoms and fatigue, and on this background the present study of post-infectious symptoms among all cases was initiated.

We reported fatigue in 41% and IBS-like symptoms in 38% of patients two years after the outbreak, among all laboratory confirmed cases. Such complications are not previously reported after giardiasis, but post-infectious fatigue and IBS are well described complications after other infections [246, 247], which support this finding. However, the prevalence of such symptoms is high in the general population, and both age and gender are possible risk factors; in Norwegian population studies, fatigue has been reported by 22% [248], and IBS by 10% [249]. It is therefore an important methodological limitation in the present study that an age and sex matched control group was not included.
Another limitation is the recording of symptoms, especially fatigue which was recorded by one question only (Appendix). Validated questionnaires have been developed for chronic fatigue [250], and Rome diagnostic criteria are widely used for IBS [91]. Use of validated questionnaires would have strengthened our findings. However, these forms are extensive, and the high response rate (81%), which is a strength of our study, is probably partly

explained by the short two-page questionnaire used. It is likely that abdominal symptoms recorded in our study are similar to IBS, since the questions used have been used in recording severity of IBS previously [251], and 81% in a sub-group of cases from the Bergen outbreak fulfilled the Rome II criteria for IBS [115].

The inclusion criteria may have created a selection bias. Only laboratory confirmed cases were included, to ensure that only *Giardia* cases were investigated. However, this selects cases who visited their doctor due to symptoms during giardiasis, and could exclude cases with milder symptoms and cases with a less care seeking behaviour. In a large case-control study (n = 4388) among fatigue syndrome and IBS patients, both these patient groups consulted their GP more often, and received more often sickness certificates, than controls within three years prior to diagnosis [252]. Many cases will have experienced a self-limiting infection, and if they hesitated in visiting their doctor they may have become well without treatment. More women were registered during and subsequent to the outbreak [253], and this could reflect that women seek medical care earlier, and that more men experienced a self-limiting infection, although water drinking habits has also been suggested to explain this difference.
Co-morbidities were not registered in our study, and it is possible that patients with co-morbidities would visit their doctors earlier than healthy, young individuals. A strong association between psychiatric disorders and chronic fatigue has been reported in population based studies [254], and other conditions prior to diagnosis have also been found more frequently in chronic fatigue compared to controls [252]. These possible confounding factors could have been elucidated if we had recorded information on co-morbidity and previous health in our study.

The possibility that any economical loss could be compensated by the health system creates a risk for over-reporting of symptoms. This could have been elucidated by using application for compensation as explanatory variable in the analyses, but this was not recorded in this study. Finally, recall bias is an obvious limitation, which also has been discussed in the paper.

Taking these limitations into account, our finding of a high level of fatigue and IBS-like symptoms, which often lead to pronounced symptoms and reduced quality of life among young individuals, should lead to further investigations on post-infectious complications in giardiasis.

Paper 4. Severity of *Giardia* infection associated with post-infectious fatigue and abdominal symptoms

Severity of infection has been reported as an important risk factor for both post-infectious fatigue and IBS following different infections [246, 255]. To study if this was the case also in giardiasis, we defined factors that hypothetically could indicate severity (number of treatment courses, treatment refractory infection, delay in education progress and sick leave), and used these as explanatory variables in the statistical analyses. We found a significant association between several of these indices of severity and fatigue and abdominal symptoms.

The factors defined as indices of severity have several limitations. Several treatment courses may have been a sign of treatment refractory and protracted infection, but also a sign of PI-IBS if stools were not controlled at follow-up, which often was the case during the outbreak due to reduced laboratory capacity. However, it is likely that patients experienced a change in symptom pattern when the parasites were cleared, especially disappearance of the foul smell, and then additional treatment courses may not have been requested. A further limitation of several treatment courses as an index of protracted infection is that some cases have been infected for several months before they received treatment, due to late detection of the outbreak [29], while those who became ill when the outbreak was well known from the media may have received treatment immediately. Nevertheless, the finding of several treatment courses as a risk factor was supported by that laboratory confirmed treatment refractory and chronic infection in a sub-group (paper 1) was associated with post-infectious abdominal symptoms as well. These findings suggest that treatment resistant parasites may have been more virulent and caused more severe infection during this outbreak.

Both delays in education progress and sick leave may have been caused by post-infectious complications rather than protracted and severe infection, and this has been discussed. However, it is probable that cases were unable to work or study due to severe symptoms during infection, while the post-infectious complications developed more slowly or fluctuated, which also is our clinical impression from referred cases. If sick leave or delayed education progress were due to giardiasis, they are good indices of severity of infection, since many cases experience mild symptoms during infection which would not influence their work [88].

Some questions were designed in a way that did not include all respondents (Appendix), and it is not clear whether these limitations in the questionnaire have influenced the outcome of

the multiple regression analyses. The category “not recovered” was included in the categorical variable “Treatment courses” to avoid loosing cases, although this category could reflect the response variables. However, when the analyses were performed without this variable, other risk factors remained significant.

Frequent re-infections in endemic regions [102], and the fact that giardiasis most often is self-limiting [88], have led to recommendations of not to treat in many cases [132]. However, if our findings are causal, early detection and treatment of *Giardia* infection could be important also in order to avoid protracted infection, and thereby prevent post-infectious fatigue and abdominal symptoms.

VIII. Proposals for future studies

- Molecular studies on resistance in treatment refractory cases.

- Prospective, randomised studies on combination treatment in refractory giardiasis.

- Controlled, long term follow up studies using validated questionnaires on prevalence of post-infectious fatigue and IBS.

- Prospective, controlled studies on fatigue and IBS after severe versus mild or asymptomatic infections, using validated questionnaires, clinical evaluation and intestinal biopsies evaluating mucosa damage.

- Clinical, epidemiologic and molecular studies in collaboration with centres in endemic countries to evaluate manifestations among cases experiencing frequent re-infections and co-infections compared to non-immune cases in western countries.

IX. Conclusions

Metronidazole in combination with albendazole or quinacrine, were effective and safe treatments in a cohort of metronidazole refractory giardiases, while paromomycin was effective in only 50%; however, efficacy from the different treatment regimens can not be compared due to limitations in the design of the study.

The *Giardia* parasites causing metronidazole refractory infection were closely related compared to the sub-genotypes at the beginning of the outbreak, indicating that parasite factors may have been partly responsible for treatment resistance during this outbreak.

Persistent abdominal symptoms in a cohort of *Giardia*-negative cases after the outbreak were not due to chronic *Giardia* infection or to a tropical sprue-like infection.

Post-infectious fatigue and IBS-like symptoms, not previously described in giardiasis, were found in 41% and 38%, respectively, among all laboratory confirmed cases during the Bergen outbreak two years after clearing the *Giardia* infection.

A strong association between fatigue and IBS-like symptoms suggest that these symptoms may be manifestations of the same post-infectious condition following giardiasis.

Indices of severe and protracted *Giardia* infection were associated with post-infectious fatigue and IBS-like symptoms two years after.

X. References

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XI. Paper I-IV