Persistent Symptoms in Treated Coeliac Disease
PILVI LAURIKKA

Persistent Symptoms in Treated Coeliac Disease

ACADEMIC DISSERTATION
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Words are, in my not-so-humble opinion, our most inexhaustible source of magic.

_J.K. Rowling_

To my family and friends.
ABSTRACT

Coeliac disease is a chronic immune-mediated disease leading to the destruction of small-intestinal mucosa in genetically susceptible individuals. The disease may manifest as various gastrointestinal or extraintestinal symptoms. The only available treatment is a lifelong and strict gluten-free diet. This means the exclusion of wheat, rye, barley, and products with added gluten from the diet.

Initiation of dietary treatment usually alleviates the symptoms rapidly, followed by slower recovery of the intestinal mucosa. However, the long-term response of the gluten-free diet is not well-known. Clinical experience suggests that a considerable proportion of treated coeliac disease patients may suffer from persistent gastrointestinal symptoms. Globally, the most frequent cause of poor clinical response is ongoing gluten consumption. Other reasons include concomitant diseases such as microscopic colitis, functional gastrointestinal disorders, and, rarely, refractory coeliac disease or malignancy. Nevertheless, in many patients there is no identifiable reason for symptoms. Epithelial stress and alteration in the intestinal microbiota, innate immunity and epithelial integrity have been suggested to play a role in active coeliac disease. However, these issues have not been investigated in treated coeliac disease patients.

The aim of this dissertation was to investigate the prevalence and severity of gastrointestinal symptoms in well-defined cohorts of untreated and treated coeliac disease patients, and to compare the results with other common gastrointestinal diseases. A further aim was to elucidate the mechanisms of persistent gastrointestinal symptoms by comparing the clinical background, composition of intestinal microbiota and diet, and markers of small-bowel mucosal immune activation and epithelial integrity in treated coeliac patients with and without gastrointestinal symptoms. In addition, predictors for reduced quality of life were investigated.

The dissertation is composed of four separate studies. Study I investigated the prevalence and severity of symptoms in 856 coeliac disease patients with different durations of gluten-free diet and in healthy controls. In Study II, factors associated with persistent gastrointestinal symptoms and reduced quality of life were investigated in 596 long-term treated coeliac patients and compared with
healthy controls. In Study III, the composition of the duodenal microbiota was compared in 34 treated coeliac patients with and without persistent symptoms. Further, in Study IV, dietary factors and markers of epithelial stress, innate immunity and epithelial integrity were compared in 47 treated coeliac disease patients with and without persistent symptoms.

In Study I, symptoms were alleviated after patients began dietary treatment, yet short-term treated patients had excessive diarrhoea. Almost a quarter of the long-term treated patients had persistent gastrointestinal symptoms compared with healthy controls, reflux being the most frequent symptom. The symptoms of coeliac disease patients were variable and considered mild to moderate compared with other gastrointestinal diseases.

Study II showed that a long diagnostic delay, severe symptoms before diagnosis and the presence of thyroid disease, other gastrointestinal disease, food intolerance or any coeliac disease-associated comorbidity increased the risk of persistent symptoms. Furthermore, a long diagnostic delay, current gastrointestinal symptoms and psychiatric comorbidity increased the risk of a reduced health-related quality of life.

In Study III, treated coeliac disease patients with ongoing gastrointestinal symptoms were shown to have different intestinal microbiota composition and a reduced bacterial richness compared with asymptomatic patients. Additionally, in Study IV, the treated coeliac patients with persistent symptoms were shown to have a significantly lower intake of fibre than asymptomatic patients. Symptomatic patients also had lower densities of CD3+ intraepithelial lymphocytes in their intestinal mucosa. No differences were observed in the other markers of epithelial stress, innate immunity, or epithelial integrity.

The results of this dissertation show that persistent gastrointestinal symptoms are common in coeliac disease patients on a long-term gluten-free diet. Some symptoms may require long-term dietary treatment before alleviation, which should be taken into account by physicians treating coeliac disease patients. The findings also support the hypothesis that a low fibre intake and alterations in the intestinal microbiota may play a role in poor symptom response. The causality of these issues with respect to symptoms, as well as possible common mechanisms with irritable bowel syndrome, should be further investigated. Based on the present study, it is possible that an altered innate immune activation or epithelial integrity does not have a role in the development of persistent symptoms. However, findings in inflammatory cells suggest that the role of intraepithelial lymphocytes in coeliac disease may be more complex than previously thought.
Keliakia on pitkäaikainen immuunivälitteinen sairaus, joka johtaa ohutsuolen limakalvovaurioon geneettisesti alttiilla henkilöillä. Sairaus voi ilmentyä monipuolisina vatsaoireina tai suoliston ulkopuolisina oireina. Toistaiseksi ainoa hoito on elinikäinen ja tiukka gluteeniton ruokavalio. Tämä tarkoittaa vehnän, rukiin ja ohran sekä lisättävät gluteenia sisältävien tuotteiden poistamista ruokavaliosta.


Tämän väitöskirjatyön tavoitteena oli tutkia pitkäaikaisen gluteenittoman ruokavalion yleisyyttä ja vakavuutta hyvin määritellyissä ja poikkeavissa keliakikoissa sekä suoliston mikrobiston koostumuksesta, synnynnäisestä immuuniteetista ja epiteelin stressitekijöistä sekä suoliston mikrobiston, synnynnäisestä immuuniteetista ja epiteelin yhteneväsyyden muutoksista, joiden tarkoituksena on selvittää, miten keliakiaa ja pitkäaikaisvakavaisuus riippuu elinikäisestä ja poikkeavasta ruokavaiheesta. Väitöskirjatyö koostuu neljästä erilaisesta osatyöstä. Osatyössä I tutkittiin oireiden esiintyvyyttä ja vakavuutta 856 eri hoidon vaiheessa olevalla keliakikoilla sekä terveillä verkeilla. Osatyössä II tutkittiin pitkäaikaisvastaa.
Oireisiin ja huonontuneeseen elämänlaatuun liittyviä tekijöitä 596 pitkään hoidetuilla keliaakikoilla sekä terveillä verrokeilla. Osatyössä III verrattiin pohjukaissuolen mikrobiston koostumusta 34 oireisella ja oireettomalla, hoidolla olevalla keliaakikolla. Lisäksi osatyössä IV verrattiin päivittäistä kuidun saantia sekä epiteelilistressiin, synnynnäiseen immunitettiin ja epiteelin yhteneväisyyteen liittyviä merkkiaineita 47 oireisella ja oireettomalla, hoidetuilla keliaakikolla.

Osatyössä I oireet helpottivat ruokavaliohoidon aloittamisen jälkeen, joskin lyhyen aikaa hoidetut potilaat käräivät vielä ripulista. Liki neljänneksellä pitkään hoidetuista keliaakikoista oli pitkittyneitä vatsaoireita, ja refluksi oli oireista yleisin. Keliaakikoiden oireet olivat monimuotoisia ja vakavan alkaen lieviä tai kohtalaisia verrattuna muihin ruoansulatuskanavan sairauksiin.

Osatyössä II pitkä diagnoositteen viive, vakavat oireet ennen keliakiadiagnososia sekä samanaikainen kilpirauhassairaus, muu ruoansulatuskanavan sairaus, ruokarajoite tai mikä tahansa muu keliaakiaan liittyvä liitännäissairaus lisäsivät riskiä pitkittyneille oireille. Lisäksi pitkä diagnoositteen viive, vatsaoireet tutkimushetkellä sekä samanaikainen psykiatrinen sairaus altistivat huonolle elämänlaadulle.

Osatyössä III jatkuvista oireista kärsivillä hoidetuilla keliaakikoilla oli erilainen ohutsuolen mikrobikoostumus verrattuna oireettomien potilaisiin. Lisäksi osatyöön IV hoidolla olevat oireiset potilaat käyttivät merkittävästi vähemmän kuitua kuin oireettomat potilaat. Oireista kärsivillä potilailla oli myös vähemmän CD3+ epiteelinalaisia lymfosyyttejä ohutsuolen limakalvolla. Muissa epiteelilistressiin, synnynnäiseen immunitettiin tai epiteelin yhteneväisyyteen liittyvissä merkkiaineissa ei havaittu eroja.

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ABBREVIATIONS

AGA  anti-gliadin antibody
APC  antigen presenting cell
ARA  anti-reticulin antibody
AT2  angiotensin 2
BMD  bone mineral density
CI   confidence interval
DC   dendritic cell
DGP  deamidated gliadin peptide
DH   dermatitis herpetiformis
EATL enteropathy-associated T-cell lymphoma
EmA  endomysial antibody
ESPGAN European Society of Paediatric Gastroenterology and Nutrition
FODMAP fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FOXP3 forkhead P3
GALT gut-associated lymphoid tissue
GER  gastro-oesophageal reflux
GSRS Gastrointestinal Symptom Rating Scale
H&E  haematoxylin-eosin
HIV  human immunodeficiency virus
HLA  human leucocyte antigen
HSP  heat shock protein
IBD  inflammatory bowel disease
IBS  irritable bowel syndrome
IEL  intraepithelial lymphocyte
Ig   immunoglobulin
IL   interleukin
INF-γ interferon γ
MDS  multidimensional scaling
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MICA/B</td>
<td>MHC class I polypeptide-related sequence A/B</td>
</tr>
<tr>
<td>NHL</td>
<td>non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NKG2C</td>
<td>natural killer group 2, member C</td>
</tr>
<tr>
<td>NKG2D</td>
<td>natural killer group 2, member D</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroid anti-inflammatory drug</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic units</td>
</tr>
<tr>
<td>PCR</td>
<td>polymeric chain reaction</td>
</tr>
<tr>
<td>PGWB</td>
<td>Psychological General Well-Being</td>
</tr>
<tr>
<td>PPI</td>
<td>proton-pump inhibitor</td>
</tr>
<tr>
<td>RCD</td>
<td>refractory coeliac disease</td>
</tr>
<tr>
<td>RDA</td>
<td>redundancy analysis</td>
</tr>
<tr>
<td>SIBO</td>
<td>small-intestinal bacterial overgrowth</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SOP</td>
<td>standard operating procedure</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TG2</td>
<td>transglutaminase 2</td>
</tr>
<tr>
<td>TG2-ab</td>
<td>transglutaminase 2 autoantibody</td>
</tr>
<tr>
<td>Th</td>
<td>helper T-cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TNHL</td>
<td>T-cell associated non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T-cell</td>
</tr>
<tr>
<td>VH/CrD</td>
<td>villous height crypt depth ratio</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by the Roman numerals I–IV:


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INTRODUCTION

Coeliac disease is an immune-mediated disease triggered by dietary gluten in genetically susceptible individuals (Green and Cellier 2007). The disease is characterized by a T-cell-mediated immune response and destruction of the small-intestinal mucosa, developing gradually from increased intraepithelial lymphocytosis to crypt hyperplasia and finally to subtotal or total villous atrophy (Oberhuber et al. 1999). Many steps of the pathogenesis remain unclear, but the disease is thought to develop with synergistic innate and adaptive immune responses (Jabri and Sollid 2009, Setty et al. 2015). The small-intestinal mucosal biopsy taken upon upper gastrointestinal endoscopy is still the cornerstone of coeliac disease diagnosis in a majority of patients. Serological tests measuring antibodies against transglutaminase-2 (TG2-ab) and endomysium (EmA) are also widely used, in addition to endoscopy (Husby et al. 2012, Baj et al. 2013).

For now, the only available treatment for coeliac disease is a lifelong and strict gluten-free diet. This means the total exclusion of wheat, rye, barley and products with added gluten from the diet (See et al. 2015). In a majority of patients, symptoms are alleviated within weeks or months after beginning dietary treatment (Murray et al. 2004). Mucosal recovery may take a remarkably longer time or, in adults, even remain incomplete (Wahab et al. 2002, Tuire et al. 2012). Although symptoms of untreated coeliac disease are often presumed to be caused by small-intestinal mucosal damage, there is no straightforward association between the degree of mucosal lesion and presence of symptoms (Murray et al. 2008, Kurppa et al. 2009). It has also been observed that symptom relief and recovery from mucosal damage happen at different speeds (Pekki et al. 2015).

In contrast to the well-known short-term effects of a gluten-free diet, the long-term response to dietary treatment is not as well known. Clinical experience suggests that there might be a considerable proportion of coeliac disease patients who continue to have symptoms despite good dietary adherence (Midhagen and Hallert 2002, Pulido et al. 2013). Globally, the most common reason for persistent symptoms in treated coeliac disease is ongoing advertent or inadvertent gluten consumption (Abdulkarim et al. 2002, Leffler et al. 2007, Dewar et al. 2012, Stasi et al. 2016). Other plausible reasons include concomitant gastrointestinal diseases
such as inflammatory bowel disease (IBD), microscopic colitis, functional gastrointestinal disease, or rarely, true refractory coeliac disease or malignancy (Rubio-Tapia and Murray 2010a, Stewart et al. 2011, Sainsbury et al. 2013b). However, in many patients there is no identifiable reason for persistent symptoms.

Intestinal microbiota and alterations in innate immunity and epithelial integrity have been studied in the context of many gastrointestinal disorders, including inflammatory bowel disease (Frank et al. 2011), untreated coeliac disease (Nistal et al. 2012, Setty et al. 2015), and irritable bowel syndrome (Rajilić-Stojanović et al. 2011, Martínez et al. 2012, Martínez et al. 2013). Changes in the microbiota, innate immunity, and epithelial integrity might also contribute the development of persistent gastrointestinal symptoms in treated coeliac disease patients. However, research in the field is scarce, and further evidence is urgently needed. Suffering from continuing symptoms despite a strict and socially restrictive diet may, at worst, discourage patients from maintaining a strictly gluten-free diet (See et al. 2015) and increase coeliac-disease associated complications. Elucidating the mechanisms behind these persistent gastrointestinal symptoms may lay the foundation for new therapeutic interventions to ease the daily life of coeliac disease patients.
1 Definition of coeliac disease

Coeliac disease is a common immune-mediated disease of the small intestine triggered by dietary gluten in genetically susceptible individuals (Green and Cellier 2007). It shares the T-cell-mediated inflammatory response similar to many autoimmune diseases. However, in coeliac disease there is a known exogenous trigger whose removal from the diet leads to complete remission of the disease. Although the main trigger of coeliac disease has been established, many steps of the disease pathogenesis remain unresolved. Development of coeliac disease requires the presence of heterodimeric human leukocyte antigen (HLA) class II genes HLA-DQ2 or HLA-DQ8 genotype (Sollid and Thorsby 1993), but presence of the genotype alone is not enough to cause the disease in the presence of gluten. The disease develops step by step from increased intraepithelial lymphocytosis to crypt hyperplasia and finally to subtotal or total villous atrophy of the small-intestinal mucosa. Today, coeliac disease is seen to develop as a result of synergistic adaptive and innate immune reactions (Setty et al. 2015). Besides small-bowel mucosal manifestations, coeliac disease may affect many organ systems outside the intestine such as the skin and the nervous system (Leffler et al. 2015a).
2 Epidemiology of coeliac disease

Previously, when the various manifestations of coeliac disease were poorly known, the disease was considered a rare disorder occurring in small children (Davidson and Fountain 1950). In the late 1980s, it was realized that the clinical presentation of coeliac disease had become milder and the onset of the disease had shifted towards older age groups (Mäki et al. 1988). In population-based studies, the prevalence of coeliac disease in many Western countries has been estimated to be about 1%, both in children (Mäki et al. 2003, Korponay-Szabó et al. 2007) and in adults (Fasano et al. 2003, West et al. 2003). In Finland, the prevalence is even higher and increases with age: approximately 2% in adults (Mustalahti et al. 2010) and 2.7% in the elderly population (Vilppula et al. 2009). Currently the clinical prevalence, meaning patients diagnosed with coeliac disease, is about 0.7% in the Finnish population (Ilus et al. 2014). Especially the elderly remain underdiagnosed (Vilppula et al. 2008), but the reported percentage of diagnosed coeliac disease patients in the population of Finland is nevertheless among the highest worldwide.

The increasing prevalence of coeliac disease is partly explained by development of diagnostics and increased awareness of the disease, but, as shown by Lohi and colleagues, the true prevalence has also almost doubled over the past two decades (Lohi et al. 2007). Similar results were subsequently confirmed in the United States (Rubio-Tapia et al. 2009a). Reasons for this increase are not well-known, but the same phenomenon has been shown in many other autoimmune diseases (Okada et al. 2010). Furthermore, there are differences in the prevalence of coeliac disease between countries, both within Europe (Mustalahti et al. 2010) and globally. The disease is, for example, rare in Asian populations (Cummins and Roberts-Thompson 2009), whereas the world’s highest prevalence has been detected in Saharawi children in Northern Africa (Catassi et al. 1999). However, significant differences in prevalence have been detected even in adjacent countries with similar genetic backgrounds (Kondrashova et al. 2008, Simre et al. 2016). These differences and rapid changes in prevalence (Lohi et al. 2007) cannot be explained by genetics. Thus,
environmental factors probably also play a considerable role in the development of coeliac disease.
3 Pathogenesis of coeliac disease

The adaptive anti-gluten immunity is the best-understood part of coeliac disease pathogenesis and was earlier thought to be the main process leading to the enteropathy. However, there is evidence that CD4+ T-cells are not directly responsible for mucosal destruction, but instead create the inflammatory environment that allows cytotoxic CD8+ intraepithelial lymphocytes (IELs) to induce tissue damage (Jabri and Sollid 2009). Today, coeliac disease is suggested to develop with a combination of adaptive and innate immune reactions. The associations of adaptive and innate immunity are still obscure, yet it is probable that these reactions happen synergistically, leading to a loss of oral tolerance to gluten and licensing of cytotoxic IELs to kill intestinal epithelial cells (Jabri and Sollid 2009, Setty et al. 2015).

3.1 Genetic background

The prevalence of coeliac disease in first-degree relatives of coeliac disease patients varies between 3% and 10% (Mäki et al. 1991, Fasano et al. 2003, Kurppa et al. 2012a, Singh et al. 2015). The significant role of genetics is further confirmed by the high concordance rate of up to 90% between monozygotic twins (Hervonen et al. 2000, Greco et al. 2002). In the 1970s, the connection was further defined to exist with certain HLA molecules (Stokes et al. 1972). HLA genes are polymorphic genes located in the chromosome 6p21.3, in a gene cluster called the major histocompatibility complex. HLA genes are shown to be associated with over a hundred disorders, mostly autoimmune diseases (Shiina et al. 2009). Subsequently, the HLA genes affecting coeliac disease were further specified to be alleles encoding HLA DQ2 and DQ8 molecules (Sollid et al. 1989). More than 90% of coeliac disease patients carry the HLA DQ2 (DQA1*0501/DQB1*0201) haplotype (Sollid and Thorsby 1993). Of the remaining coeliac disease patients, 5% have the HLA DQ8 (DQA1*0301/DQB1*0302) haplotype, and practically all the others have at least one of the two genes encoding DQ2 αβ-heterodimer (DQB1*0201 or DQA1*0501; Karell et al. 2003).
There is a correlation between the number of predisposing HLA alleles in the individual and the risk of coeliac disease (Vader et al. 2003, Liu et al. 2014). The greatest risk for coeliac disease is in DQ2 homozygotes, which lead to an almost fivefold increase in the risk of coeliac disease compared with DQ2 heterozygotes (Murray et al. 2007). The homozygous DQ2 haplotype is also related to more severe phenotype and an increased risk of complications through the gene dose effect; in DQ2 homozygotes, antigen-presenting cells (APCs) are able to present a larger number of gluten peptides and thus induce stronger T-cell activation (Vader et al. 2003). The predisposing HLA alleles are necessary but not sufficient to cause the development of coeliac disease, as about one-third of the general population present with HLA DQ2, but only minority of them develop coeliac disease, even in the presence of dietary gluten (Sollid et al. 1989). Approximately 57 genetic variants located in 39 risk loci outside the HLA region have been identified as associated with coeliac disease, all as part of inflammatory and immune responses (Hunt et al. 2008, Dubois et al. 2010, Trynka et al. 2011, Gutierrez-Achury et al. 2015).

3.2 Environmental factors

3.2.1 Dietary gluten

Dietary gluten is the main exogenous driver of coeliac disease. The term gluten is used as a general term for insoluble prolamines: gliadin in wheat, hordein in rye and secalin in barley (Platt and Kasarda 1971). They are structurally similar to each other and are thought to induce similar immunologic reactions in coeliac disease. In contrast, the prolamin avenin in oats differs somewhat in structure compared with prolamines of the other above-mentioned cereals and is thought to be non-toxic to coeliac disease patients (Arentz-Hansen et al. 2004). Dietary gluten consists of α-, γ- and ω-gliadins and glutenins (Howdle 2006). The toxicity of gluten in coeliac disease has mostly been found to be related to gliadin peptides, although more recent evidence suggests that also glutenins are toxic for coeliac disease patients (Dewar et al. 2006). The properties of gliadin enhance the baking properties of dough, and thus breeding has favoured the cereals with high gluten concentrations (Molberg et al. 2003). Besides, gluten is added industrially to many food products to improve their structure.
The role of age at the first introduction of gluten, as well as the amount of gluten in the diet, in the risk of coeliac disease has been investigated. In Sweden, the prevalence of coeliac disease suddenly increased in the 1980s, which seemed to be associated with infant feeding with gluten-containing products (Ivarsson et al. 2000). Later, age at the introduction of gluten was not found to be associated with an increased risk of coeliac disease (Lionetti et al. 2014, Vrienzinga et al. 2014, Aronsson et al. 2016, Simre et al. 2016). However, large amounts of ingested gluten in children under two years of age may be associated with coeliac disease (Ivarsson et al. 2002, Aronsson et al. 2016).

3.2.2 Other environmental factors

Other environmental factors besides gluten have also been suggested to play a role in the coeliac disease development by inducing epithelial stress and activating immune responses. The composition of intestinal microbiota is closely related to intestinal homeostasis, and oral tolerance to food antigens and its connections with coeliac disease are under investigation (Chapter 7.4.2 below). Certain viral (Lähdeaho et al. 1993, Bouziat et al. 2017, Kemppainen et al. 2017) and bacterial (De Palma et al. 2010a) infections promote the helper T-cell (Th) 1 type immunity response and could increase the risk of coeliac disease. However, the role of viruses is probably more complex, as cytomegalo, Epstein-Barr and other herpes viruses may even be protective in children (Jansen et al. 2016). It was suggested earlier that children born by caesarean section have higher risk of coeliac disease (Decker et al. 2010), which would have supported the role of intestinal microbes in the disease development. However, the effect of delivery mode is controversial, as a more recent study showed no association between caesarean section and risk of coeliac disease (Lionetti et al. 2017). The use of antibiotics may also increase the risk of coeliac disease, although the causality is unsure (Mårild et al. 2013).

The increasing prevalence of many autoimmune diseases in the developed countries has led to the so called “hygienic hypothesis”, which may play a role in the development of coeliac disease. According to this hypothesis, a decreased amount of microbial exposure is thought to contribute to the development of autoimmune disorders, leading the immune responses to be targeted against self-antigens (Bodansky et al. 1992, Lohi et al. 2007, Whyte et al. 2014). The role of breast-feeding in the development of coeliac disease has also been investigated,
but it had no effect on the risk of coeliac disease in the most recent multicentre studies (Lionetti et al. 2014, Vrienzinga et al. 2014).

3.3 Pathogenetic mechanisms

3.3.1 Gut immunology and loss of oral tolerance

Gut-associated lymphoid tissue (GALT) is part of the mucosa-associated lymphoid tissue, and is often considered as its own immunological compartment. The lymphoid cells in this compartment are distributed in three functional regions: as organized lymphoid tissue in Payer’s patches and mesenteric lymph nodes and as single lymphoid cells in the lamina propria and the epithelial layer (Wawrzyniak et al. 2017). Lymphocytes of the lamina propria are mostly effector cells of adaptive immunity, whereas IELs contribute the innate immunity. In healthy intestinal epithelial tissue, the majority of IELs are CD3+, of which CD8+ T-cell receptor (TCR) αβ+ IELs constitute the greatest share (Figure 1). Many of these cells express also inhibitory and activating natural killer (NK) receptors.

![Intraepithelial lymphocytes diagram](Figure 1. Normal phenotype of intraepithelial lymphocytes in the intestinal epithelium. Adapted from Leon 2011.)
A balanced activation of inflammatory immune responses towards exogenous pathogens and the inhibition of over-activation of these reactions to gain immunologic tolerance is crucial for normal tissue function and homeostasis. The GALT plays a key role in this process, for it constantly confronts environmental antigens, inducing an immune response leading either to oral tolerance or an inflammatory reaction (Husby et al. 1994). Dendritic cells (DC) and regulatory T-cells (Tregs), such as forkhead P3 (FOXP3)+ T-cells, contribute the oral tolerance by secretion of transforming growth factor β (TGF-β) and other anti-inflammatory cytokines (Coombes et al. 2007, Worthington et al. 2011).

In coeliac disease, oral tolerance to dietary gluten is lost, and specific inflammatory CD4+ T-cells are seen in the intestinal mucosa (Molberg et al. 1997). Incidences leading to this are not clear, but it is possible that a micro-environment rich in pro-inflammatory cytokines such as interleukins (IL) 15 and 21 change the phenotype of DCs to promote the differentiation of inflammatory T-cells and make the CD8+ and CD4+ T-cells resistant to regulatory signals (Peluso et al. 2007, Ben Ahmed et al. 2009, Jabri and Sollid 2009). In active coeliac disease, an increase in both TCR-αβ+ and γδ+ IELs is seen, along with a decrease in the CD3- IELs (Leon 2011). The number of TCR-αβ+ IELs has shown to correlate with coeliac disease activity and is diminished by dietary treatment (Kutlu et al. 1993). However, although TCR-γδ+ IELs are shown to be more specific to coeliac disease, their role in the disease development is more obscure. They have been shown to be present at all stages of the disease, also in patients in long-term dietary treatment (Camarero et al. 2000, Koskinen et al. 2010, Calleja et al. 2011). Some studies suggest that the TCR-γδ+ IELs may have regulatory or even protective functions in coeliac disease patients (Bhagat et al. 2008).

3.3.2 Role of epithelial stress in coeliac disease

During stressful situations in the intestine, induced for example by infections or other exogenous agents, intestinal epithelial cells express various stress markers, such as heat shock proteins (HSP), MICA/B and HLA-E, and simultaneously induce the production of inflammatory mediators such as IL-15. In patients with active coeliac disease, gliadin peptides and possibly other co-acting environmental factors induce epithelial stress (Iltanen et al. 1999a, Allegretti et al. 2013). The production of IL-15 in intestinal epithelial cells and DCs upregulates the expression of activating NK-receptors CD94/NKG2C and
NKG2D in the cytotoxic IELs (Meresse et al. 2004), which lowers the threshold for T-cell activation (Bauer et al. 1999). MICA/B and HLA-E are ligands for CD94/NKG2C and NKG2D receptors, and binding of these activating NK receptors to their ligands leads to the cytotoxic killing and lysis of the intestinal epithelial cells.

3.3.3 Pathogenetic cascade leading to coeliac disease

Due to the high concentration of amino acids proline and glutamine, gluten peptides are relatively resistant to degradation by gastrointestinal enzymes (Shan et al. 2002), leaving long immunogenic gliadin peptides in the small-intestinal lumen. To confront the gut lymphoid cells, the peptides have to pass the intestinal epithelium. In a healthy bowel, permeability of the intestinal mucosa is low because of tight junctions between the epithelial cells. In coeliac disease, ingested gluten fragments induce the enterocytes lining the gut to release zonulin, a mediator that loosens tight junctions between the enterocytes, thus increasing intestinal permeability (Drago et al. 2006). Some transcellular mechanisms enabling gliadin passage through the epithelial layer may also exist (Matysiak-Budnik et al. 2008). In consequence, gluten peptides pass through the epithelium into the submucosal layer, where they further induce upregulation of IL15 and the killing of intestinal epithelial cells through NKG2C and NKG2D and their ligands (Chapter 3.3.2 and Figure 2).

Activation of adaptive anti-gluten immunity is initiated when gluten peptides are attached to HLA DQ2 or DQ8 molecules of APCs and are then presented to specific CD4+ T-cells in the lamina propria (Molberg et al. 1998). Usually gluten peptides have low affinity for HLA DQ molecules due to the lack of negative charge. However, in the lamina propria, transglutaminase 2 (TG2) enzyme deamidates specific glutamine residues of gliadin to negatively charged glutamate, increasing the affinity of gliadin to HLA DQ2 and DQ8 molecules and enabling the formation of HLA DQ-gliadin complexes (Dieterich 1997, Molberg et al. 1998). The presentation of gliadin to CD4+ T-cells by APCs activates a pro-inflammatory Th1 response in CD4+ T-cells (Figure 2). Activated CD4+ T-cells secrete inflammatory mediators, including interferon γ (INF-γ), IL-21 (Bodd et al. 2010) and tumour necrosis factor α (TNF-α), which contributes to the crypt hyperplasia and activation and migration of CD8+ cytotoxic T-cells (Bajaj-Elliott et al. 1998). Side by side with direct cytotoxic reactions, an increased release of matrix metalloproteinases leads to destruction of small-bowel mucosal structure.
IL-15 secreted by stressed epithelial cells also contributes to the inflammatory Th1 response (Figure 2). Additionally, the Th2 response of CD4+ T cells activates the differentiation of B-cells into plasma cells that produce anti-gliadin and anti-TG2 antibodies (Sollid et al. 1997).

Figure 2. Pathogenetic cascade of coeliac disease (adapted from Galipeau and Verdu 2014). Dietary gluten and possibly other additional triggers induce epithelial stress in the intestinal epithelial cells (1). Increased permeability and transcellular transport of gliadin peptides through the epithelium leads to deamidation by TG2 and attachment of gluten peptides to DQ2/DQ8 molecule (2). APC presents gluten peptide to CD4+ T-cells (3), leading to a gluten-specific T-cell-mediated immune reaction (4). Destruction of small-intestinal mucosa is mediated by cytotoxic IELs, by activating NK receptors and their ligands HLA-E and MICA/B (5). This is enhanced by epithelial stress and the secretion of IL-15. Adaptive immune reactions also lead to the differentiation of B-cells to gluten-specific plasma cells and the secretion of TG2-ab (6). These antibodies may play a role, for example, in the extraintestinal manifestations of coeliac disease. TG2: transglutaminase 2; APC: antigen-presenting cell; DC: dendritic cell; MLN: mesenteric lymph node; PP: Payer’s patch; LP: lamina propria; Treg: regulatory T-cell; IL: interleukin; INF: interferon; TNF: tumour necrosis factor; IEL: intraepithelial lymphocyte; TG2-ab: transglutaminase 2 antibodies.
3.3.4 The role of coeliac disease autoantibodies

It was determined relatively early on that there were deposits of immunoglobulin (Ig) A in the intestinal mucosa of coeliac patients (Shiner and Ballard 1972). Later, these deposits were shown to be directed against TG2 and found also in many extraintestinal tissues (Korponay-Szabo et al. 2004, Salmi et al. 2010). TG2 as an autoantigen of coeliac disease was recognized in 1997 (Dieterich et al. 1997). Subsequently, TG2 auto-antibody (TG2-ab) tests were developed (Chapter 5.3). It is known that TG2- abs are produced by specific plasma cells in the lamina propria of the intestinal mucosa (Marzari et al. 2001, Di Niro et al. 2012).

The significance of TG2-ab in coeliac disease pathogenesis is still not fully understood. Evidence suggest that TG2- abs may play a role in promoting epithelial alterations characteristic of coeliac disease and increasing intestinal permeability to allow the entrance of gliadin peptides across the epithelial barrier (Rauhavirta et al. 2011). However, in animal models, the presence of TG2-ab alone was not sufficient to cause villous atrophy, so other immune reactions are likely needed in the development of full-blown coeliac disease, also in humans (Freitag et al. 2004, Kalliokoski et al. 2015). The role of autoantibodies has been most often speculated in the development of extraintestinal manifestations of coeliac disease (Chapter 4.2), as antibody deposits have been detected in various tissues outside the intestines (Hadjivassiliou et al. 2006a, Simon-Vecsei et al. 2012). In addition to TG2-ab, TG3 antibody deposits are characteristic in dermatitis herpetiformis (DH; Sardy et al. 2002), even though these patients usually also have TG2 antibodies in their sera and TG2 deposits in the intestinal epithelium (Dieterich et al. 1999, Salmi et al. 2014). In addition, antibodies targeted against TG6 have been observed as related to gluten ataxia (Chapter 4.2; Hadjivassiliou et al. 2008).
4 Clinical features of coeliac disease

4.1 Gastrointestinal manifestations

Historically, chronic diarrhoea, steatorrhoea and malnutrition were considered classical manifestations of coeliac disease (Visakorpi and Mäki 1994). Deficiencies of fat-soluble vitamins A, D, E and K and micronutrients such as vitamin B12, folic acid, iron and calcium often accompanied these signs, resulting in anaemia, low bone mineral density (BMD), weight loss, growth retardation or failure to thrive (Visakorpi and Mäki 1994). Due to increased knowledge and serological tests, the classic phenotype of coeliac disease has become rarer. Instead, patients nowadays often suffer only from mild abdominal symptoms, including loose stools, abdominal discomfort or flatulence, or have even no gastrointestinal symptoms at all (Collin et al. 2007, Volta et al. 2014, Agardh et al. 2015). In addition, severe signs of malabsorption are rare, and deficiencies are not always clinically detectable (Tikkakoski et al. 2007). Nevertheless, osteoporosis and iron deficiency anaemia are still relatively common, especially in patients with a long diagnostic delay (Tikkakoski et al. 2007, Sanseviero et al. 2016, Kamycheva et al. 2017).

4.2 Extraintestinal manifestations

Extraintestinal symptoms comprise a remarkable portion of the clinical manifestations of coeliac disease (Table 1). They are suggested to be partly a consequence of adaptive anti-gluten immunity (see Chapter 3.3) and partly secondary changes to enteropathy (Leffler et al. 2015). DH is the best known extraintestinal manifestation of coeliac disease (Table 1). It is present in about 25% of coeliac patients and is characterized by itching blisters, particularly on the elbows, knees, buttocks and scalp. It is diagnosed by using the immunofluorescence method to detect IgA deposits in the uninvolved skin next to the rash (Collin and Reunala 2003). Besides this skin manifestation, almost all DH patients also suffer from villous atrophy in the small-bowel mucosa, although
only some have gastrointestinal or malabsorptive symptoms (Savilahti et al. 1992, Salmi et al. 2014).

Table 1. Extraintestinal manifestations of coeliac disease.

<table>
<thead>
<tr>
<th>Manifestation</th>
<th>Recovery on GFD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatitis herpetiformis</td>
<td>Yes</td>
<td>Collin and Reunala 2003</td>
</tr>
<tr>
<td>Neurological manifestations(^a)</td>
<td>Sometimes</td>
<td>Hadjivassiliou et al. 1998, Luostarinen et al. 1999, Chin et al. 2003</td>
</tr>
<tr>
<td>Psychiatric conditions(^b)</td>
<td>Contradictory</td>
<td>Hallert and Åström 1982, Addolorato et al. 2001, Smith and Gerdes 2012</td>
</tr>
<tr>
<td>Elevated liver enzymes</td>
<td>Yes</td>
<td>Korpimäki et al. 2011, Castillo et al. 2015, Kaukinen et al. 2002a</td>
</tr>
<tr>
<td>Problems in reproductive health(^c)</td>
<td>Sometimes</td>
<td>Santonicola et al. 2011, Tersigni et al. 2014, Moleski et al. 2015</td>
</tr>
</tbody>
</table>

\(^a\)Most commonly gluten ataxia and peripheral neuropathy.  
\(^b\)Anxiety, depression.  
\(^c\)E.g. miscarriages, intrauterine growth restrictions, preterm labour, late menarche, early menopause.

Neurological symptoms may affect up to one-fifth of coeliac disease patients (Luostarinen et al. 1999, Briani et al. 2008, Table 1), only some of which have gastrointestinal manifestations (Hadjivassiliou et al. 2003). Response to a gluten-free diet in these conditions is variable; neural damage may be irreversible (Luostarinen et al. 1999, Hadjivassiliou et al. 2006b). Psychiatric conditions such as anxiety and depression have also been connected with coeliac disease (Table 1). In a meta-analysis, the gluten-free diet often failed to relieve these symptoms, suggesting unspecific reasons behind the symptoms (Smith and Gerdes 2012). However, dietary treatment may have beneficial effects for certain patients (Hallert and Åström 1982, Addolorato et al. 2001, Smith and Gerdes 2012).
Liver abnormalities from subclinical elevation of transaminases to severe hepatic injury have been reported in untreated coeliac disease (Table 1). The increased liver enzyme values usually decrease after commencement of a gluten-free diet (Korpimäki et al. 2011, Castillo et al. 2015, Äärelä et al. 2016). On rare occasions, other parenchymal organs, including spleen, kidney, pancreas and heart, have also been shown to be affected by coeliac disease (Leffler et al. 2015). In addition, untreated coeliac disease may cause problems with reproductive health (Table 1), yet the issue is somewhat unclear (Collin et al. 1996, Tata et al. 2005). At least the risks of miscarriages and preterm labour seem to be reduced by a gluten-free diet (Tersigni et al. 2014).

Reduced BMD is common both in children and in adults with untreated coeliac disease (Table 1), and may be present in up to 50% at diagnosis (Volta et al. 2014). It is often more severe in patients with gastrointestinal symptoms, but may occur also in screen-detected and totally asymptomatic patients (Mazure et al. 1994, Mustalahti et al. 1999). Reduced BMD is partly caused by malabsorption of calcium and vitamin D followed by secondary hyperparathyroidism (Corazza et al. 1995, Kemppainen et al. 1999), and is thus pronounced in newly diagnosed and poorly treated patients (Kemppainen et al. 1999). However, the phenomenon is probably multifactorial: for example, an increased amount of circulating cytokines and an altered balance of bone turnover may contribute to the development of osteoporosis (Fornari et al. 1998, Fiore et al. 2006). The presence of neutralizing antibodies against osteoprogeterin, a receptor participating in the regulation of BMD, has also been hypothesized (Riches et al. 2009), yet research results are contradictory (Larussa et al. 2012).

4.3 Complications

Drawing a line between a manifestation and a complication of coeliac disease is not simple: for example anaemia and osteoporosis can be seen either as manifestations of the disease or secondary changes following malabsorption. Nevertheless, the osteoporosis seen in coeliac disease may lead to fractures as a complication of the disease. In some population-based studies, the fracture risk in coeliac patients has been similar to that in the general population (Thomason et al. 2003), but in contrast, a recent meta-analysis showed an increased risk of any fracture up to 30% and hip fracture up to 60% (Heikkilä 2015). However, BMD
usually increases after beginning a gluten-free diet (Kemppainen et al. 1999, Tau et al. 2006).

Especially earlier, malignancies were seen as a feared complication of coeliac disease. The overall risk of malignancies was suggested to be two-fold and of non-Hodgkin lymphoma (NHL) up to 43-fold compared with the healthy population (Holmes et al. 1989). However, such high numbers are probably caused by detection bias, as only clinically detected coeliac disease patients were evaluated, while asymptomatic patients remained undiagnosed. Thus, in diagnosed patients, the clinical picture was usually more severe and the diagnostic delay long.

In a recent meta-analysis evaluating both clinically diagnosed and screen-detected patients, the risk of cancer in general was not higher than in the general population, whereas the risk of NHL and especially T-cell associated NHL (TNHL) was moderately elevated (Tio et al. 2012). Coeliac patients are also shown to have elevated rate of gastrointestinal malignancies (Green et al. 2003) and, on the other hand, a decreased risk of breast cancer (West et al. 2004, Goldacre et al. 2008). Knowledge about the reasons for an increased cancer risk with coeliac disease is limited, but variable consequences of chronic inflammation and a constant activation of the immune system have been suggested (Green and Jabri 2002). The increased risk of TNHL is probably mostly due to enteropathy-associated T-cell lymphoma (EATL), which is a TNHL with low prevalence but a poor prognosis. It is associated with a rare and severe refractory coeliac disease (RCD) type II (Chapter 7.3.4). In general, a gluten-free diet is thought to protect against future malignancies, at least when the diagnostic delay is not long (Silano et al. 2008). However, the effects of a gluten-free diet are relatively complex to evaluate, since there is a possibility of ascertainment bias due to an incidental finding of coeliac disease when investigating symptoms related to malignancy, and vice versa.

An increased mortality has been detected with coeliac disease (Corraro et al. 2001, Viljamaa et al. 2006), especially in patients with malabsorptive symptoms (Corraro et al. 2001) or refractory coeliac disease (Biagi et al. 2014). However, in more recent studies, the mortality associated with undetected coeliac disease has been comparable with that of the general population (Lohi et al. 2009, Godfrey et al. 2010, Canavan et al. 2011, Chounq et al. 2017).
4.4 Associated diseases and risk groups

Several conditions with an increased prevalence of coeliac disease have been recognized (Table 2). First-degree relatives of coeliac disease patients have an increased risk of coeliac disease (Mäki et al. 1991, Fasano et al. 2003, Kurppa et al. 2012a, Singh et al. 2015). Another well-known risk group are patients with certain autoimmune diseases, in particular type 1 diabetes and autoimmune thyroid disease (Table 2). The prevalence of all autoimmune diseases in coeliac disease is estimated to be 15–30%: significantly more than in the general population (Sategna Guidetti et al. 2001, Viljamaa et al. 2005a, Neuhausen et al. 2008). The increased risk of concomitant autoimmune diseases is thought to be explained by common genetic background and pathogenic mechanisms (Collin et al. 1994, Viljamaa et al. 2005a). It is still uncertain whether the length of gluten exposure before a coeliac disease diagnosis or beginning a gluten-free diet affects the future risk of concomitant autoimmune diseases in patients (Sategna-Guidetti et al. 2001, Viljamaa et al. 2005a). In addition, patients with Down’s or Turner’s syndrome have an increased risk of coeliac disease (Table 2). Active screening for coeliac disease could be recommended in these at-risk groups (Husby et al. 2012).

4.5 Quality of life in untreated coeliac disease

A considerable disease burden and a reduced quality of life are often associated with untreated coeliac disease (Green et al. 2001). Untreated patients also have more outpatient visits to primary care facilities than is the situation after one year of treatment (Ukkola et al. 2012, Mattila et al. 2013). This seems to be especially emphasized in symptomatic patients (Mustalahti et al. 2002, Johnston et al. 2004, Castellas et al. 2008, Tontini et al. 2010), whereas screen-detected patients usually have a quality of life comparable with that of healthy individuals (Mustalahti et al. 2002, Johnston et al. 2004). Furthermore, women with untreated coeliac disease seem to be more susceptible to a poor quality of life than men (Casellas et al. 2008, Tontini et al. 2010). In DH patients, quality of life is also significantly reduced at diagnosis, especially in those with combined gastrointestinal symptoms (Pasternack et al. 2017). Notably, a reduced quality of life seems to be associated with a long diagnostic delay, meaning time from first symptoms to coeliac disease diagnosis (Norström et al. 2011).
Table 2. The associated risk groups of coeliac disease.

<table>
<thead>
<tr>
<th>Associated condition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coeliac disease in first degree family member</td>
<td>Kurppa et al. 2012a, Singh et al. 2015</td>
</tr>
<tr>
<td>Type I diabetes mellitus</td>
<td>Mäki et al. 1984a, Ludvigsson et al. 2006,</td>
</tr>
<tr>
<td></td>
<td>Salardi et al. 2008</td>
</tr>
<tr>
<td>Autoimmune thyroid disease</td>
<td>Collin et al. 1994, Viljamaa et al. 2005b</td>
</tr>
<tr>
<td>Sjögren’s syndrome</td>
<td>Iltanen et al. 1999b, Szodoray et al. 2004</td>
</tr>
<tr>
<td>Addison’s disease</td>
<td>Collin et al. 1994, Viljamaa et al. 2005b</td>
</tr>
<tr>
<td>Autoimmune liver diseases</td>
<td>Kingham and Parker 1998, Villalta et al. 2005,</td>
</tr>
<tr>
<td></td>
<td>van Gerven et al. 2014</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>Collin et al. 2002, Viljamaa et al. 2005a</td>
</tr>
<tr>
<td>Selective IgA deficiency</td>
<td>Collin et al. 1992, Cataldo et al. 1998</td>
</tr>
<tr>
<td>Down’s syndrome</td>
<td>Gale et al. 1997</td>
</tr>
<tr>
<td>Turner’s syndrome</td>
<td>Bonamico et al. 2002</td>
</tr>
</tbody>
</table>
5 Diagnosis of coeliac disease

5.1 Diagnostic criteria

The first international diagnostic criteria for coeliac disease were set by the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) at the Interlaken meeting in 1969 (Meeuwisse 1970). The criteria included the presence of at least subtotal villous atrophy while on a gluten-containing diet, improvement of mucosal structure on a gluten-free diet and, again, deterioration of mucosal structure during a gluten challenge. These criteria were revised by ESPGAN in 1990 (Walker-Smith et al. 1990), and in symptomatic patients with small-bowel mucosal lesion a complete clinical recovery was considered sufficient for diagnosis. A control biopsy was still recommended in asymptomatic patients and a gluten-challenge in equivocal cases and in children under two years old. Coeliac disease autoantibodies were considered to give weight to the diagnosis. The most recent European paediatric guidelines allow making a coeliac disease diagnosis without biopsy in a subgroup of children with a combination of TG2-ab titers over ten times the upper limit of normal positive endomysial antibodies (EmA) taken from a different blood sample combined with an HLA DQ2/DQ8 haplotype (Husby et al. 2012). However, these guidelines have not yet been implemented in Finland.

5.2 Small-bowel mucosal histology

A small-bowel mucosal biopsy is still the gold standard in the diagnosis of coeliac disease in most patients. The biopsies are taken from duodenal mucosa with endoscopic forceps during an upper gastrointestinal endoscopy. The classification of small-bowel mucosal lesions seen in the biopsy was created by Marsh (Marsh 1992) and later modified by Oberhuber (Table 3). In addition, reduced villous height crypt depth ratio (VH/CrD) has shown to be more specific for coeliac disease, as it also takes crypt depth into account (Shiner and Doniach 1960,
Kuitunen et al. 1982). Values for the normal ratio have been under some debate, but a cut-off value of 2.0 is widely used (Taavela et al. 2013).

Table 3. The Marsh-Oberhuber classification\(^a\) for staging small-bowel mucosal damage in coeliac disease.

<table>
<thead>
<tr>
<th>Stage</th>
<th>IEL density</th>
<th>Villous architecture</th>
<th>Crypt architecture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Increased</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Increased</td>
<td>Normal</td>
<td>Hyperplasia</td>
</tr>
<tr>
<td>3a</td>
<td>Increased</td>
<td>Partial villous atrophy</td>
<td>Hyperplasia</td>
</tr>
<tr>
<td>3b</td>
<td>Increased</td>
<td>Subtotal villous atrophy</td>
<td>Hyperplasia</td>
</tr>
<tr>
<td>3c</td>
<td>Increased</td>
<td>Total villous atrophy</td>
<td>Hyperplasia</td>
</tr>
</tbody>
</table>

\(^a\)Oberhuber et al. 1999.

There are several pitfalls in the interpretation of small-bowel mucosal samples. Firstly, duodenal villous atrophy and lymphocytic duodenitis are not entirely pathognomonic for coeliac disease, but these may be present, for example, in some other autoimmune and inflammatory diseases of the gut: infections such as giardiasis, \textit{Helicobacter pylori} and human immunodeficiency virus (HIV), as well as food allergies and intestinal lymphoma (Green and Cellier 2007). Secondly, coeliac disease lesions may be patchy, and, consequently, a minimum of four forceps biopsies are recommended to ensure diagnostic accuracy (Bonamico et al. 2004, Lebowl et al. 2011). Thirdly, proper orientation of the specimen is crucial for reliable interpretation. Incorrect orientation may even result in missing a coeliac disease diagnosis (Arguelles-Grande et al. 2012, Taavela et al. 2013). There may also be interobserver variation in the interpretation (Arguelles-Grande et al. 2012, Picarelli et al. 2014). Nevertheless, excellent results may be achieved with validated procedures (Taavela et al. 2013). There are also contradictory results with respect to the optimal site for biopsy, as some studies suggest that villous atrophy may be found only in the proximal duodenum, the so-called
anatomical bulb (Weir et al. 2010, Evans et al. 2011). Accordingly, many guidelines recommend bulb biopsies as part of the diagnostics (Husby et al. 2012, Baj et al. 2013). However, there are several potential challenges in the interpretation of bulb biopsies, and the issue remains controversial (Taavela et al. 2016).

5.3 Serology

The first coeliac disease autoantibodies were targeted against reticulin fibres in connective tissue (ARA) and were discovered in the 1970s (Seah et al. 1971). They were measured by indirect immunofluorescence using rodent tissues as antigens. The sensitivity of this test was relatively good in children (Mäki et al. 1984b), but rather low in adults (Seah et al. 1973). Nevertheless, they were widely used due to their specificity of 90–100% (Mäki et al. 1984b). Antibodies against wheat gliadin (AGA) were found in the 1970s and measured using an enzyme-linked immunosorbent assay. AGAs were widely used until it was realized that they were not specific for coeliac disease, but could be present also in other gastrointestinal diseases such as food allergies and inflammatory bowel disease (Lindberg et al. 1985, Hill et al. 1991). The sensitivity and specificity also showed a remarkable 30%–100% variation (Mäki et al. 1991, Hill et al. 1991, Kaukinen et al. 2007). Consequently, AGAs disappeared from clinical practice.

EmA were also found in the 1980s (Chorzelski et al. 1984). They are measured using an indirect immunofluorescence assay with monkey oesophagus, or more recently, human umbilical cord as a substrate. In a recent meta-analysis, the sensitivity and specificity of EmA detection varied between 76–97% and 98–100%, respectively (Schyum and Rumessen 2013). The method is relatively laborious as it requires microscopic evaluation. Nevertheless, due to its accuracy, EmA testing has maintained its position as the gold standard method for coeliac disease antibody detection (Husby et al. 2012). TG2 was recognized as an autoantigen for coeliac disease in the 1990s, and enzyme-linked immunosorbent assay (ELISA) tests were subsequently developed. The TG2-ab test is practical and inexpensive compared to EmA detection. IgA class TG2-ab testing has proved more sensitive (76–97%), yet less specific (90–98%) than EmA testing (Schyum and Rumessen 2013). In addition, an ELISA test to detect antibodies against deamidated gliadin peptides (DGPs) has been developed. The accuracy of the test is superior to conventional AGA testing (Kaukinen et al. 2007) and almost
achieves the accuracy level of TG2 antibody testing (Kurppa et al. 2011). Combining the test with EmA or TG2 testing further increases the accuracy (Schyum and Rumessen 2013). These tests may provide a useful tool for diagnostics in the early stages of coeliac disease (Kurppa et al. 2011), but their clinical use has yet to be established. In all diagnostics, IgA class antibody tests are routinely used, but IgG class antibodies can be measured in the case of known or suspected IgA deficiency (Baj et al. 2013).

5.4 Screening

Serological tests can be used to screen non-invasively for coeliac disease. Although coeliac disease fulfils most of the mass screening criteria proposed by the World Health Organization (Holland et al. 2006), there is no consensus about the cost effectiveness of mass screening (Shamir et al. 2006, Green et al. 2008, Hershcovici et al. 2010). In addition, the benefit-risk balance is not clear, especially in totally asymptomatic patients (Ludvigsson et al. 2015, Bibbins-Domingo et al. 2017), and it is possible that the perception of health may be worsened in asymptomatic patients after diagnosis (Ukkola et al. 2011). Thus, for the time being, active case finding in the risk groups for coeliac disease is recommended (Berti et al. 2006, Husby et al. 2012, Ludvigsson et al. 2015), as there is more evidence of the benefits of screening in these groups (Kurppa et al. 2014). The current guidelines for coeliac disease diagnostics recommend using EmA and/or IgA class TG2-ab testing as a primary method for serological testing (Husby et al. 2012, Baj et al. 2013). It is recommended that IgA deficiency be ruled out and, when needed, that IgG antibody testing be used instead (Husby et al. 2012). However, whether there is a need for routine serum IgA testing is still somewhat controversial, as IgA deficiency is rare, and patients with only a partial IgA deficiency are usually able to produce IgA class TG2-abs (Chow et al. 2012).
6 Treatment of coeliac disease

6.1 Dietary treatment

The gluten-free diet was established as a treatment for coeliac disease in the early 1960s (Collins and Isselbacher 1964), and it remains the only available treatment, at least for now. It means a total and lifelong exclusion of wheat, rye, barley, and products with added gluten from the diet (See et al. 2015). The tolerated amount of gluten traces in the diet is debated but probably varies between individuals (Lähdeaho et al. 2011). It is generally thought that an amount of 10–30 milligrams of gluten daily is safe (Catassi et al. 2007), whereas about 100 milligrams of gluten is usually enough to cause mucosal damage (Catassi et al. 1993, Collin et al. 2004) for coeliac patients. The international Codex Alimentarius standard defines a gluten-free product as one containing less than 20 milligrams of gluten per kilogram.

The prolammin avenin in oats contains less proline and glutamine than the prolamines in wheat, rye and barley, which are toxic to coeliacs (Arentz-Hansen et al. 2004). Some studies have wakened suspicions of a possible immune activation from the intake of oats (Lundin et al. 2003, Arentz-Hansen et al. 2004, Peräaho et al. 2004). However, according to current knowledge, oats are safe to use in coeliac disease when they are free of gluten traces (Janatuinen et al. 1995, Aaltonen et al. 2017, Pinto-Sánchez et al. 2017). There have also been some doubts about possible trace amounts of gluten in industrially purified wheat starch, but this starch has also proven to be safe for coeliacs (Peräaho et al. 2003).

As a downside, a gluten-free diet often contains less fibre and protein and higher amount of sugar and fat than an unrestricted diet (Thompson et al. 2005, Wild et al. 2010). It may also predispose patients to deficiencies of B1, B2 and B6 vitamins and minerals such as iron, calcium, magnesium, selenium and zinc (Martin et al. 2013, Thompson et al. 2005, Wild et al. 2010). A gluten-free diet is also remarkably higher in cost than an unrestricted diet (Missbach et al. 2015), and the availability of gluten-free products is limited in many countries (Singh and Whelan 2011). In addition, the diet has a profound impact on a patient’s social life, as a gluten-free diet makes travelling and eating outside home complicated.
(Zarkadas et al. 2013, Shah et al. 2014). Thus, patients may feel grief stemming from the loss of their former diet and a change in social identity (Hallert et al. 2002, Rose and Howard 2014). As its worst, problems with a gluten-free diet may discourage patients from following a strict dietary treatment and lead to a socially restricted life.

6.2 Clinical and histological recovery

In the majority of coeliac disease patients, gastrointestinal symptoms are alleviated within weeks after beginning a gluten-free diet (Murray et al. 2004), followed by a slower recovery of the intestinal mucosa. In adults, complete histological recovery may take years, and sometimes it remains incomplete (Tursi et al. 2006, Tuire et al. 2012). Slower histological recovery is often associated with a long diagnostic delay, old age and severe mucosal findings at the time of diagnosis (Tursi et al. 2006, Mahadev et al. 2017). The reduction in serum autoantibody titers is also slow and poorly predicts adherence to a gluten-free diet, especially in the first years of treatment (Gidrewicz et al. 2017, Tursi et al. 2003). However, coeliac patients following a strict gluten-free diet seem to manage equally well after one year of treatment, despite the differences in the speed of their intestinal recovery (Pekki et al. 2015). In addition to its beneficial effects on gastrointestinal symptoms, a gluten-free diet also alleviates extraintestinal symptoms (Jericho et al. 2016, Valdimarsson et al. 1994, Vilppula et al. 2011), improves BMD (Kemppainen et al. 1999) and removes the increased risk of mortality, malignancies and gynaecological complications that are the result of insufficiently treated coeliac disease (West et al. 2004, Tersigni et al. 2014).

6.3 Effects of treatment on quality of life

In general, a gluten-free diet improves the health-related quality of life in patients with coeliac disease (Green et al. 2001, Johnston et al. 2004, Casellas et al. 2008, Pasternack et al. 2017). The most evident factor improving quality of life is the alleviation of symptoms on a gluten-free diet. In a majority of the studies, dietary treatment does not impair the quality of life in either screen-detected or asymptomatic patients (Mustalahti et al. 2002, Johnston et al. 2004, Viljamaa et
al. 2005b, Paavola et al. 2012, Kurppa et al. 2014), although there are some contradictory results (Ukkola et al. 2011).

Nevertheless, there is evidence that quality of life remains worsened in many treated coeliac disease patients compared with the population in general. Shah and colleagues showed that although coeliac disease patients have an excellent overall health status compared with that of patients with many other chronic conditions, the treatment burden is similar or even greater (Shah et al. 2014). In some studies, a reduced quality of life was detected, especially in patients with poor adherence (Usai et al. 2002, Häuser et al. 2007, Nachman et al. 2010), whereas in some studies also the strictly adherent patients failed to attain a quality of life comparable with that of healthy individuals (Casellas et al. 2008, Paavola et al. 2012). The gender differences seen in untreated coeliac disease (Chapter 4.5) seem to be present also in treated coeliac disease, as women seem to have a worse quality of life than men during dietary treatment (Hallert et al. 2002). In addition, the presence of concomitant psychiatric co-morbidity predicts a reduced quality of life in treated coeliac patients (Häuser et al. 2007). There are probably several reasons for the impairment in quality of life. One explanation may be the social restrictions that accompany a gluten-free diet (See et al. 2015). Although many coeliac disease patients adapt to their disease over time, it seems that there is still a great need for training of healthcare professionals and food-industry workers to ease the life of coeliac patients (Zarkadas et al. 2013).

### 6.4 New emerging therapies

The difficulties in maintaining a strict gluten-free diet provide some impetus for developing treatment options beyond a gluten-free diet. Thus far, there have been several Phase 1 and Phase 2 studies looking at such options (see the current situation at Clinicaltrials.gov). Of these, the most promising group are the gluten-specific proteases, glutenases that degrade gliadin peptides into non-immunogenic fragments before they cross the intestinal epithelium (Lähdeaho et al. 2014). In addition, the polymer BL-7010, intended to bind the gluten before reaching the epithelium, is under investigation (McCarville et al. 2014). A tight-junction modulator, larazotide acetate, has been investigated in patients on a gluten-free diet in Phase 2 studies, but it shows contradictory results (Leffler et al. 2012, Kelly et al. 2013). A desensitizing vaccine to restore oral tolerance to gluten is under development and is thus far the only drug aimed at replacing the
gluten-free diet (Goel et al. 2017). Other strategies under development include TG2 inhibition (Rauhavirta et al. 2013) and HLADQ2 blocking (Rossi 2015).

As coeliac disease already has an effective dietary treatment that benefits most patients, the primary aim of these studies is to develop drugs that could be used alongside a gluten-free diet rather than replacing it. Defining satisfactory outcome measures for novel therapies is also needed (Hindryckx et al. 2016). Small-intestinal biopsy is the gold standard for assessing treatment response to a gluten-free diet, and it has been suggested that the same criteria should be applied to novel treatments (Mäki 2014, Hindryckx et al. 2016) until proper surrogate markers to detect minor mucosal damage have been found. Thus, before entering the market, a new treatment should be proven to prevent or attenuate small-intestinal mucosal injury. As a secondary endpoint, measuring patient-reported outcomes such as symptom relief is important, and its significance should be emphasized in Phase 3 and Phase 4 trials, once a treatment has already proven safe and effective (Mäki 2014).
7 Persistent gastrointestinal symptoms in treated coeliac disease

7.1 Definition of persistent symptoms

There are no exact criteria for persistent gastrointestinal symptoms in coeliac disease. However, they are most commonly defined as a failure of expected symptomatic response to a gluten-free diet, either initially or recurrently after maintaining dietary treatment (Abdulkarim et al. 2002, Dewar et al. 2012). The term “non-responsive coeliac disease” is often used to describe this situation. However, in this context, non-responsive coeliac disease is not a diagnosis and does not define the aetiology of symptoms, but is rather a clinical description of a state that requires systematic investigation (Dewar et al. 2012). In addition, there is no consensus as to how long a patient must maintain a gluten-free diet and remain symptomatic until his or her symptoms can be termed persistent, as mucosal recovery often takes a remarkably long time (Wahab et al. 2002). Although neither the degree nor extent of mucosal damage seems to correlate well with the presence of gastrointestinal symptoms (Murray et al. 2008, Kurppa et al. 2009, Pekki et al. 2015), a strict diet of at least 6–12 months is recommended before beginning further investigations due to symptoms (Abdulkarim et al. 2002, Rubio-Tapia and Murray 2010a, Dewar et al. 2012).

7.2 Prevalence of persistent symptoms

Due to the lack of strict definition and standardized methods for symptom assessment, the prevalence of persistent symptoms in treated coeliac disease is difficult to evaluate. The majority of the studies investigating the issue are conducted at tertiary care facilities acting as referral centres for complicated clinical dilemmas (Abdulkarim et al. 2002, Leffler et al. 2007, Dewar et al. 2012, Stasi et al. 2016); studies investigating the issue in the general coeliac population are scarce. Thus, it is possible that patients that are not actively seeking medical help for their symptoms remain unrecognized, and patients with severe symptoms
are overrepresented. Nevertheless, all previous studies agree that there is a remarkable proportion of treated coeliac patients suffering from symptoms. In these studies, at least one-fifth of patients has suffered from persistent symptoms (Pink and Creamer 1967, Stasi et al. 2016). In a large survey made in the Canadian coeliac population, an insufficient recovery from some gastrointestinal symptoms was reported in 28–53% of the cases, constipation being the symptom with the least tendency for recovery (Cranney et al. 2007). A more recent Canadian study conducted by Pulido and colleagues (Pulido et al. 2013) reported similar results. Interestingly, patients treated for more than five years had were less symptomatic than those treated for less than one year or 1–5 years (Pulido et al. 2013). Midhagen and colleagues (Midhagen et al. 2003) also reported significantly more indigestion, diarrhoea, constipation and abdominal pain compared with the general population, even though no estimate of symptom prevalence was given.

### 7.3 Aetiology of persistent symptoms

A systematic diagnostic approach is needed when investigating coeliac disease patients whose symptoms persist despite a gluten-free diet (Abdulkarim et al. 2002, Rubio-Tapia et al. 2011, Dewar et al. 2012). It has been evaluated that some identifiable cause for persistent symptoms can be found in over 90% of cases (Abdulkarim et al. 2002, Dewar et al. 2012, Stasi et al. 2016). However, as the majority of studies have been conducted at tertiary care centres, it is possible that the situation would be different in a primary care setting.

When evaluating a coeliac patient with persistent symptoms, the first thing to ensure is the accuracy of the initial diagnosis. The challenges of interpreting intestinal biopsy results (Taavela et al. 2013) and the previously used ARA and AGA tests (Chapter 5.3) may sometimes have led to an incorrect diagnosis. In patients with initially negative TG2 and EmA results and in whom IgA deficiency have been excluded, the significance of a careful assessment of the duodenal biopsy sample is emphasized. In these patients, HLA haplotype testing may also be of help due to the almost 100% negative predictive value of the test (Kaukinen et al. 2002b, Rubio-Tapia and Murray 2010a). Even if the initial diagnosis seems accurate, investigations often require repeating the upper gastrointestinal endoscopy, since the existence or absence of persistent villous atrophy greatly determines the direction of further investigations (Rubio-Tapia et al. 2011). The most common aetiologic possibilities in patients with confirmed coeliac disease
diagnosis are discussed in the following chapters (7.3.1–7.3.4), which deal with the situations with and without the presence of duodenal villous atrophy (Table 4). Furthermore, the prevalence of aetiological conditions for persistent symptoms detected in the previous studies is summarized in Table 5.

Table 4. Summary* of the differential diagnostics of persistent symptoms in adults with treated coeliac disease and how they relate to the presence of persistent villous atrophy.

<table>
<thead>
<tr>
<th>Aetiological factor</th>
<th>Villous atrophy</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ongoing gluten consumption (advertent and inadvertent)</td>
<td>Often</td>
<td>Most common reason: lack of sensitive methods to detect</td>
</tr>
<tr>
<td>Lactose intolerance</td>
<td>No</td>
<td>Frequent; may be secondary to active coeliac disease</td>
</tr>
<tr>
<td>Inflammatory bowel disorder</td>
<td>Rarely</td>
<td>In watery or bloody diarrhoea</td>
</tr>
<tr>
<td>Microscopic colitis</td>
<td>No</td>
<td>In watery diarrhoea</td>
</tr>
<tr>
<td>Infectious</td>
<td>Rarely</td>
<td>E.g. giardiasis, HIV, Whipple’s disease</td>
</tr>
<tr>
<td>SIBO</td>
<td>Rarely</td>
<td>Frequent; lack of sufficient diagnostic methods</td>
</tr>
<tr>
<td>Exocrine pancreatic insufficiency</td>
<td>No</td>
<td>Steatorrhoea</td>
</tr>
<tr>
<td>Functional gastrointestinal disorder (e.g. IBS)</td>
<td>No</td>
<td>Common; other reasons should be excluded</td>
</tr>
<tr>
<td>Medication-induced</td>
<td>Yes</td>
<td>Olmesartan, NSAIDs, PPIs</td>
</tr>
<tr>
<td>Malignancies</td>
<td>No</td>
<td>Prevalence increases with age</td>
</tr>
<tr>
<td>Refractory coeliac disease</td>
<td>Yes</td>
<td>Malabsorptive symptoms</td>
</tr>
<tr>
<td>Other reason</td>
<td>Sometimes</td>
<td>Consider, based on individual patient history and examination</td>
</tr>
</tbody>
</table>

HIV: human immunodeficiency virus; NSAIDs: non-steroid anti-inflammatory drugs; PPIs: proton-pump inhibitors; IBS: irritable bowel syndrome; SIBO: small-intestinal bacterial overgrowth.

* More detailed information and references can be found in Chapters 7.3.1–7.3.4.
Table 5. Identified aetiological factors and their prevalence in patients being investigated due to clinically non-responsive coeliac disease. The variables are described as a percentage of the coeliac patients investigated.

<table>
<thead>
<tr>
<th></th>
<th>Gluten (slow response)</th>
<th>RCD/EATL</th>
<th>IBD/colitis (^{a})</th>
<th>Cancer (^{b})</th>
<th>GER</th>
<th>Pancreatic insufficiency</th>
<th>Lactose intolerance</th>
<th>SIBO</th>
<th>IBS</th>
<th>Other reason (^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdulkarim et al. 2002 (n=49)</td>
<td>51</td>
<td>18</td>
<td>18</td>
<td>2</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>14</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Leffler et al. 2007 (n=113)</td>
<td>36</td>
<td>10</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>6</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>Dewar et al. 2012 (n=100)</td>
<td>45</td>
<td>9</td>
<td>18</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>9</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Stasi et al. 2016 (n=53)</td>
<td>47/ (21)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>17</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^{a}\) Includes Chrons disease, ulcerative, and microscopic colitis.
\(^{b}\) Pancreatic and colorectal cancer, duodenal adenocarcinoma.
\(^{c}\) E.g. protein-losing and medication-induced enteropathy, tropical sprue, human immunodeficiency virus, diverticulosis, anorectal dysfunction, diabetic gastroparesis, fructose intolerance, peptic ulcer, combined variable immunodeficiency, T-cell receptor gene rearrangement, anorexia nervosa.
7.3.1 Dietary transgressions

In all previous studies, ongoing advertent or inadvertent gluten consumption has been the most common finding in treated coeliac disease patients with persistent symptoms (Table 5). Globally, adherence to dietary treatment in coeliac disease varies remarkably (Table 6). The reasons for the differences are probably diverse. One reason suggested is the difficulty of recognizing the sources of gluten traces in the diet (Hollon et al. 2013, Samasca et al. 2017). On the other hand, problems of adherence probably relate to the restrictive impact of a gluten-free diet in social life, as well as to the high cost and poor availability of gluten-free products (Whitaker et al. 2009, See et al. 2015, Samasca et al. 2017). However, in a country with a good general knowledge of the disease and good availability of gluten-free products, the adherence seems to be very good, irrespective of the initial presentation at diagnosis (Viljamaa et al. 2005b, Kurppa et al. 2012b). In addition, it seems that adherence can be improved by repeated counselling, also in countries with a limited availability of gluten-free products (Rajpoot et al. 2015). Notably, current antibody tests, including the TG2-ab and EmA tests, are insufficiently sensitive in identifying small amounts of ingested gluten (Comino et al. 2016). In the future, new testing methods such as detecting immunogenic gluten peptides from faeces or urine may bring help in differential diagnostics of persistent symptoms (Comino et al. 2016, Moreno et al. 2017).

When evaluating the impact of dietary adherence on symptoms, it must be realized that the results are contradictory regarding the relationship between mucosal damage and the presence of symptoms. In some studies, villous atrophy was more present in symptomatic patients (Carroccio et al. 2008); in other studies neither the degree nor extent of mucosal damage was related to the presence of symptoms (Murray et al. 2008, Kurppa et al. 2009, Pekki et al. 2015). Hollon et al. (2013) found that 82% of the adherent yet non-responsive coeliacs responded to a highly restricted gluten contamination elimination diet, which indicates that hidden gluten may play a key role in the persistence of symptoms. Also, in all other studies (Table 5), gluten contamination seemed to be the reason for symptoms in patients with detected poor adherence. In addition, all studies agreed that strictness of the gluten-free diet is the first thing to ensure in patients with a confirmed coeliac disease diagnosis and suffering from persistent symptoms.
Table 6. Adherence to a gluten-free diet in adults suffering from coeliac disease in Western countries.

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Number of patients</th>
<th>Evaluation method</th>
<th>Strict diet, %</th>
<th>Partial diet, %</th>
<th>Poor or no diet, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Häuser et al. 2007</td>
<td>Germany</td>
<td>443</td>
<td>Self-reported</td>
<td>67</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>Usai et al. 2007</td>
<td>Italy</td>
<td>129</td>
<td>Self-reported, questionnaire</td>
<td>62</td>
<td>38</td>
<td>0</td>
</tr>
<tr>
<td>Whitaker et al. 2009</td>
<td>UK</td>
<td>147</td>
<td>Self-reported, questionnaire</td>
<td>67</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td>Rubio-Tapia et al. 2010b</td>
<td>USA</td>
<td>236</td>
<td>Interview</td>
<td>66</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>Kurppa et al. 2012b</td>
<td>Finland</td>
<td>749</td>
<td>Interview, serology</td>
<td>90</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Norström et al. 2012</td>
<td>Sweden</td>
<td>1031</td>
<td>Self-reported, questionnaire</td>
<td>96</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Sainsbury et al. 2013a</td>
<td>Australia</td>
<td>189</td>
<td>Internet-based questionnaire</td>
<td>59</td>
<td>33</td>
<td>8</td>
</tr>
<tr>
<td>Silvester et al. 2016</td>
<td>Canada</td>
<td>105</td>
<td>Self-reported, questionnaire</td>
<td>82</td>
<td>18</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

7.3.2 Concomitant diseases

Instead of or in addition to dietary transgressions, several other concomitant disorders and conditions may be found behind persistent symptoms (Tables 4 and 5). Notably, earlier studies investigating the issue were performed with relatively small study cohorts (Table 5). Primary lactose intolerance is common in the general population, its prevalence increasing with age (Usai-Satta 2012). In addition, lactose intolerance may be secondary to coeliac disease, in which case the recovery of lactase activity happens within months after beginning strict treatment (Usai-Satta et al. 2012). Microscopic colitis is a relatively common cause of chronic diarrhoea, especially in middle-aged women and the elderly.
population (Koskela et al. 2004, Green et al. 2009, Stewart et al. 2011, Yen and Bardi 2012). Some population-based studies have suggested a connection between microscopic colitis and coeliac disease (Koskela et al. 2004, Stewart et al. 2011, Thörn et al. 2013), although there are also contradictory results (Verhaegh et al. 2017). Some studies have reported an increased coexistence of inflammatory bowel disease (IBD) and coeliac disease, at least at secondary and tertiary centres (Yang et al. 2005, Leeds et al. 2007). Subsequently, a lower gastrointestinal endoscopy can be recommended in coeliac patients with diarrhoea-predominating persistent symptoms (Stewart et al. 2011).

An infectious aetiology should be taken into account, particularly in patients with a travel history to tropical areas (Rubio-Tapia et al. 2011): especially giardiasis and HIV are known to induce small-intestinal damage, from increased intraepithelial lymphocytosis to villous atrophy (Müller and von Allmen 2005, Batman et al. 2007, Koot et al. 2009). Whipple’s disease, a rare systemic bacterial infection, may also cause changes in the villous structure (Marth et al. 2016). On the other hand, post-infectious irritable bowel syndrome (IBS) may be a relevant diagnostic possibility in patients with gastroenteritis prior to the onset of symptoms and no villous atrophy (Jalanka-Tuovinen et al. 2014; Chapter 7.3.3). When steatorrhoea is present, exocrine pancreatic insufficiency or small-intestinal bacterial overgrowth (SIBO; Chapter 7.4.2) should be considered. Notably, SIBO may also induce mucosal changes and should therefore be kept in mind in treating patients with an impaired mucosal histology (Table 4).

Certain medications – namely the angiotensin 2 (AT2) receptor blocker olmesartan, non-steroid anti-inflammatory drugs (NSAIDs) and proton-pump inhibitors (PPIs) – have been shown to sometimes cause medication-induced enteropathy in the small intestine (Rubio-Tapia et al. 2012, Watanabe et al. 2013, Schiepatti et al. 2017). PPIs might also exacerbate the small-intestinal mucosal injury caused by NSAIDs, possibly by inducing dysbiosis of intestinal microbiota (Wallace et al. 2011, Watanabe et al. 2013) or transmucosal leak (Mullin et al. 2008). Further, a recent study by Mahadev and colleagues (Mahadev et al. 2017) also suggested that NSAIDs, PPIs and SSRI medications could promote persistent villous atrophy in coeliac disease, and thus anamnesis regarding these drugs belongs to differential diagnostics in patients with persistent mucosal damage.

Other possible relatively rare causes of gastrointestinal symptoms include common variable immunodeficiency (Malamut et al. 2010, Schiepatti et al. 2017) and autoimmune enteropathy (Volta et al. 2016). Both conditions respond poorly to a gluten-free diet and should be considered in patients with villous atrophy.
Besides the above-mentioned disorders, the exclusion of various malignancies becomes important, especially in elderly patients. This should be taken into account in the presence of noticeable changes in bowel habits or defecation frequency, abdominal pain, anaemia, unexplained fever, weight loss or either visible or invisible blood in the faeces (Chanq et al. 2012). However, symptoms may also be highly unspecific, and malignancies causing gastrointestinal symptoms may have different origins: either the gastrointestinal tract itself or the abdominal cavity or some other location. Diagnosis of these conditions is based on the individual examination of patients with additional investigations depending on the situation, including imaging and upper or lower gastrointestinal endoscopy and biopsies.

7.3.3 Coeliac disease and irritable bowel syndrome

IBS is a functional gastrointestinal disorder defined as recurrent abdominal pain associated with defecation and a change in bowel habits (Lacy et al. 2016), currently diagnosed according to Rome IV criteria (Mearin et al. 2016, Vork et al. 2016). Many patients can be further divided into groups based on predominant stool pattern: diarrhoea-predominant, constipation-predominant or mixed stool-pattern subtypes (Lacy et al. 2016). IBS is a common condition in the general population, with a global prevalence of about 10% (Hillilä et al. 2006, Lovell and Ford 2012). Furthermore, the prevalence of IBS-type symptoms has also been shown to be higher in patients with coeliac disease and many other gastrointestinal diseases compared with the general population (Halpin and Ford 2012, Kamp et al. 2016, Sainsbury et al. 2013b). In the meta-analysis conducted by Sainsbury et al. (2013b), the pooled prevalence of symptoms meeting the criteria for IBS was almost 30% in adherent coeliac patients. Thus, it is highly relevant to assess the possibility of IBS or other functional gastrointestinal disorders in treated coeliac patients with symptoms.

Previously, IBS was seen as a disorder with no underlying structural or biochemical basis. However, insoluble fibre was shown to exacerbate the symptoms of IBS patients as early as the 1990s (Francis and Whorwell 1994), and gastroenteritis was established as an independent risk factor for IBS several years ago (Rodríguez and Ruigómez 1999). Today, although still poorly known, IBS is thought to be associated with alterations in the so-called gut-brain axis, which is formed from the enteric and central nervous systems and the connecting autonomic nervous system, endocrine and sensory pathways (Mayer et al. 2015).
Additionally, disturbances in the brain function, dietary and genetic factors, infections, alterations in the intestinal microbiota and permeability, low-grade mucosal inflammation, immune activation, bile salt metabolism, and serotonin metabolism have been detected (Holtmann et al. 2016) and thought to interact with the gut-brain axis.

Due to the lack of knowledge of the disease mechanisms, the treatment has been targeted towards the most disturbing symptoms, often with little help during long-term follow-up (Ford et al. 2008). Symptom-based pharmacological therapies include loperamide and eluxadoline (Lacy et al. 2017). However, treatments targeted to diet, such as increasing soluble fibre (Nagarajan et al. 2015) and lowering levels of fermentable oligosaccharides, disaccharides, monosaccharides and polyols in the diet (the low FODMAP diet; Nanayakkara et al. 2016) showed some efficacy, at least in a subgroup of patients, possibly by affecting the intestinal microbiota (Chapter 7.4). The low FODMAP diet seems to be the most helpful for patients suffering from bloating, flatulence, abdominal pain and a diarrhoea-predominant stool pattern (Staudacher et al. 2017). Interestingly, this diet has shown preliminary promising results, also in treated coeliac disease patients with gastrointestinal symptoms (Imperatore et al. 2016).

7.3.4 Refractory coeliac disease

Refractory coeliac disease (RCD) is a rare entity in coeliac disease, defined as persistent villous atrophy with malabsorptive symptoms despite a strict gluten-free diet for a minimum of 6–12 months (Biagi and Corrazza 2001, Rubio-Tapia and Murray 2010a). The diagnosis requires the exclusion of other causes of villous atrophy and non-responsive coeliac disease, and overt malignancies (Abdulkarim et al. 2002, Leffler et al. 2007). RCD is divided into type I RCD, with an increased amount of IELs with normal phenotype, and type II RCD, with a clonal phenotype of IELs that has lost its normal CD3 and CD8 surface markers and instead expresses cytoplasmic CD3ε (Cellier et al. 1998, Malamut et al. 2009, Rubio-Tapia and Murray 2010a). In Finland, the prevalence of RCD is very low: 0.31% among diagnosed coeliac disease patients and 0.002% in the general population (Ilus et al. 2014). In other studies, the prevalence of RCD in coeliac patients varies between 0.4% and 10% (Arguelles-Grande et al. 2013, Sharkey et al. 2013). Symptoms in RCD I are usually comparable with those in active coeliac disease, whereas RCD II is often associated with severe ulcerative jejunitis.
(Malamut et al. 2009). Differentiating between a slow response to a gluten-free diet or dietary transgressions and RCD I may be difficult (Hollon et al. 2013).

Randomized clinical trials considering the treatment of RCD are scarce. With both RCD types, corticosteroids are used, and they often lead to a clinical response but induce histological remission only rarely (Malamut et al. 2009, Rubio-Tabia and Murray 2010a). Additional immunosuppressants are often used for RCD I (Constantino et al. 2008, Malamut et al. 2009, Rubio-Tabia and Murray 2010). For RCD II, cladribin is one drug that has been used (Al-Toma et al. 2006), but the disease is relatively resistant to all known treatments. The prognosis of RCD I is usually good, whereas RCD II has a poor prognosis with a five-year survival rate of 40–58%, mainly due to its progression to aggressive EATL (Rubio-Tabia and Murray 2010a).

7.4 Role of intestinal microbiota in coeliac disease

7.4.1 Overview of intestinal microbiota

The human intestine is colonized by a diverse community of commensal microbes described as the intestinal microbiota. The colonization starts during and after birth, the first microbes being anaerobic bacteria of the Actinobacteria and Proteobacteria phyla (Yatsunenko et al. 2012). For example, mode of delivery, antibiotic use and feeding patterns affect the colonization process (Matamoros et al. 2013). The composition of the intestinal microbiota fluctuates in small children, but stabilizes within the first two or three years (Yatsunenko et al. 2012).

In adults, the density of microbes gradually increases in the gastrointestinal tract to less than $<10^3$/ml in the stomach, $10^3$–$10^4$/ml in the duodenum, $10^7$/ml in the distal ileum and finally, up to $10^{12}$/ml in the colon (Aron-Wisnewsky et al. 2012). Most of the microbes colonizing the gastrointestinal tract are either strictly or facultatively anaerobic bacteria. The composition of the colonic microbiota has been more extensively investigated compared with that in the duodenum. The duodenal microbiota is affected by bile and pancreatic secretions, which are toxic to many micro-organisms. Thus, the composition differs from that seen in the large intestine (Angelakis et al. 2015, Li et al. 2015). The most common phyla seen in the duodenum are Firmicutes and Proteobacteria (Aron-Wisnewsky et al. 2012, Li et al. 2015), and Proteobacteria may comprise as much as 40% of the microbial composition (Li et al. 2015). The microbiota is most diverse in the large
intestine, where the most abundant phyla are Bacteroidetes and Firmicutes, while Proteobacteria, Actinobacteria, Fusobacteria and Verrucomicrobia account for less than 10% of the total population (Eckburg et al. 2005).

There is a great deal of interindividual variation in the composition of intestinal microbiota at the species level, but there are common patterns in the higher-taxa levels (Eckburg 2005, Hooper and Macpherson 2010). The composition of the microbiota varies remarkably between individuals from different geographic locations (Yatsunenko et al. 2013). Also, many other life habits and environmental factors affect the microbial composition (Figure 3). Nevertheless, the composition remains relatively stable at the individual level, at least in the short term (Jalanka-Tuovinen et al. 2011). The intestinal microbiota probably plays a considerable role in human health (Figure 3).

![Figure 3](image-url)  
*Figure 3.* Examples of the effects of host and environmental factors on microbiota and the beneficial impact of the intestinal microbiota on human health. Information obtained from Thursby and Juge 2017.

7.4.2 Intestinal microbiota and coeliac disease

Due to the impact of commensal microbes on human health, their consequences in the case of pathologic conditions have also been investigated. Indeed, alterations in the intestinal microbiota has been associated with several gastrointestinal disorders and autoimmune diseases (Table 7). Additionally, changes associated with coeliac disease have been found in many studies when compared with healthy
The intestinal microbiota has also been shown to vary in untreated coeliac patients with gastrointestinal versus extraintestinal manifestations (Wacklin et al. 2013). Most of the studies investigating the intestinal microbiota in coeliac patients are conducted with paediatric patients; studies with treated adult patients are scarce (Table 8).

In general, it is unclear whether the detected alterations in microbiota play a role in coeliac disease pathogenesis or are a consequence of active disease (Galipeau and Verdu 2014). Interestingly, in treated coeliac disease patients, the gluten-free diet itself may also be a factor affecting the microbiota composition. This is supported by the finding that treated coeliac patients have a reduced diversity of Lactobacillus and Bifidobacterium genera compared with untreated

<table>
<thead>
<tr>
<th>Research method</th>
<th>Findings in microbiota</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 diabetes 16S rRNA gene pyrosequencing</td>
<td>Firmicutes↑; Proteobacteria↓; Firmicutes/Bacteroidetes↑</td>
<td>Pellegrini et al. 2017</td>
</tr>
<tr>
<td>IBS DGGE FISH</td>
<td>Pseudomonas sp↑; Bifidobacteria↓</td>
<td>Kerckhoffs et al. 2011; Kerckhoffs et al. 2009</td>
</tr>
<tr>
<td>Liver cirrhosis 16S rRNA gene pyrosequencing</td>
<td>Firmicutes↑; Proteobacteria↓</td>
<td>Chen et al. 2016</td>
</tr>
<tr>
<td>IBD qPCR</td>
<td>Firmicutes↓; Proteobacteria↑</td>
<td>Frank et al. 2011</td>
</tr>
<tr>
<td>IBS qPCR + phylogenetic microarray</td>
<td>Firmicutes↑; Bacteroidetes↓; Firmicutes/Bacteroidetes↓</td>
<td>Rajilić-Stojanović et al. 2011</td>
</tr>
<tr>
<td>Colorectal cancer 16S rRNA gene pyrosequencing</td>
<td>Firmicutes↑; Fusobacteria↑; Proteobacteria↓</td>
<td>Gao et al. 2015</td>
</tr>
</tbody>
</table>

IBS: irritable bowel syndrome; DGGE: denaturing gradient gel electrophoresis; FISH: fluorescent in situ hybridization; qPCR: quantitative Polymerase Chain Reaction; IBD: inflammatory bowel disease.
coeliacs and healthy controls (Nistal et al. 2012). Further, the gluten-free diet has been shown to lead to a reduction in beneficial bacteria and impairments in the ability of fecal bacteria to stimulate the host’s immune response even in healthy individuals (De Palma et al. 2009).

Table 8. Changes in the intestinal microbiota detected in untreated and treated coeliac disease compared with healthy individuals.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Research method</th>
<th>Findings in microbiota</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Untreated coeliac disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nadal et al. 2007 (n=20)</td>
<td>Duodenal biopsy, FISH</td>
<td>Lactobacillus ↓; Bifidobacteria↓; Bacteroides-Prevotella ↑ E. Coli ↑</td>
<td>Children, partially normalized on a GFD</td>
</tr>
<tr>
<td>Collado et al. 2009 (n=30)</td>
<td>Duodenal biopsy + fecal sample, qPCR</td>
<td>Bacteroides, C leptum, Streptococci, E. Coli↑; Bifidobacterium↓</td>
<td>Children, partially normalized on a GFD</td>
</tr>
<tr>
<td>De Palma et al. 2010b (n=24)</td>
<td>Fecal sample, FISH</td>
<td>Bifidobacteria↓; certain Firmicute species ↓; Bacteroides-Prevotella↑; IgA-coated faecal bacteria ↓</td>
<td>Children, partially normalized on a GFD</td>
</tr>
<tr>
<td>Nistal et al. 2012 (n=10)</td>
<td>Fecal sample, DGGE</td>
<td>Bifidobacterium bifidum↑</td>
<td>Adults, partially normalized on a GFD</td>
</tr>
<tr>
<td><strong>Treated coeliac disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nadal et al. 2007 (n=10)</td>
<td>Duodenal biopsy, FISH</td>
<td>Ratio of Lactobacillus--Bifidobacterium to Bacteroides--E. coli ↓</td>
<td>Children, GFD 1-2 yr</td>
</tr>
<tr>
<td>Collado et al. 2009 (n=18)</td>
<td>Duodenal biopsy + fecal sample, qPCR</td>
<td>Bacteroides, C leptum↑; Bifidobacterium↓</td>
<td>Children, GFD &gt;2 yr</td>
</tr>
<tr>
<td>De Palma et al. 2010b (n=18)</td>
<td>Fecal sample, FISH</td>
<td>IgA, IgG, and IgM coated faecal bacteria↓</td>
<td>Children, GFD &lt;2 yr</td>
</tr>
<tr>
<td>Nistal et al. 2012 (n=11)</td>
<td>Fecal sample, DGGE</td>
<td>Diversity of Lactobacillus and Bifidobacterium ↓</td>
<td>Adults, GFD &gt; 2 yr</td>
</tr>
</tbody>
</table>

FISH: fluorescent in situ hybridization; GFD: gluten-free diet; qPCR: quantitative Polymerase Chain Reaction; DGGE: denaturing gradient gel electrophoresis; Ig: immunoglobulin.
Another microbiota-related condition relevant to coeliac disease is SIBO, a heterogeneous condition characterized as an increased growth of bacteria in the small intestine (Bures et al. 2010). Usually dysbiosis, especially colonization by microbes typical in the colon, is present, in addition to quantitative changes (Bouhnik et al. 1999). SIBO is suggested to especially relate to diseases affecting gastrointestinal motility (Bures et al. 2010). Some studies suggest that the prevalence of SIBO is high in treated coeliac patients with persistent symptoms (Abdulkarim et al. 2003, Tursi et al. 2003b, Rubio-Tapia et al. 2009b), yet the prevalence seems to vary considerably between the studies, i.e. between 5% and 67% (Losurdo et al. 2017). However, there are several challenges in the diagnostics of SIBO, as cultivation of jejunal aspiration is difficult (Bures et al. 2010), and lactulose and glucose breath tests have insufficient sensitivity and specificity (Rana et al. 2012, Erdogan et al. 2015). This may complicate the identification of patients that would truly benefit from the treatment of SIBO. Accordingly, rifaximin failed to alleviate persistent symptoms of treated coeliac disease in a randomized, placebo-controlled trial (Chang et al. 2011), even though it is considered the most effective treatment for SIBO (Bures et al. 2010).

7.4.3 Intestinal microbiota and persistent gastrointestinal symptoms

There is little evidence of possible causal connections between intestinal microbiota and gastrointestinal symptoms in any disorder, including coeliac disease. Jalanka-Tuovinen and colleagues (2011) found out that healthy individuals expressing abdominal pain had over five-fold less Bifidobacteria than asymptomatic subjects. Findings pointing towards SIBO are also more common in patients with gastrointestinal symptoms (Martins et al. 2017). Furthermore, microbiota-modulating treatments alleviate the symptoms in a subgroup of patients with functional gastrointestinal disorders, which indirectly supports the idea of dysbiosis affecting the development of symptoms (Kajander et al. 2008, Nagarajan et al. 2015, Nanayakkara et al. 2016). However, the issue is probably more complex, as there are also contradictory results, especially in the case of treatment with probiotics (Simrén et al. 2010, Hod et al. 2017).

When talking about coeliac disease, Smecuol et al. (2013) showed that treatment with Bifidobacterium alleviated gastrointestinal symptoms and lowered TG2-ab levels in untreated coeliac patients on a gluten-containing diet. However, the studies of treated coeliac patients are either conducted with asymptomatic coeliac patients or without any reporting of the presence or absence of persistent
symptoms (Table 8). Thus, causality between gastrointestinal symptoms and microbial changes in general should be further investigated: there is no current evidence regarding the role of microbial changes in persistent gastrointestinal symptoms in treated coeliac disease patients.

7.5 Managing persistent symptoms in treated coeliac disease

As there are several causes of persistent symptoms in coeliac disease, management of and prognosis for symptoms is highly dependent on the underlying condition. In addition, the long-term prognosis of symptoms is poorly investigated. In the study by Dewar and colleagues (2012), the situation after two years was good in patients whose symptom causes had been identified, as majority of patients felt that their symptoms had been alleviated. However, the situation might be different in patients without any identifiable reason for their symptoms. This hypothesis is supported by the knowledge that treatment of functional gastrointestinal disorders is often challenging (Ford et al. 2008): localizing somatic symptoms outside the gastrointestinal tract is also common (Hillilä et al. 2007). Moreover, a considerable psychiatric comorbidity, burden and reduced quality of life may be associated with these disorders (Hillilä et al. 2007, DiBonaventura et al. 2011, Buono et al. 2017).
THE PRESENT STUDY

8 Aims

The aims of this dissertation were to investigate the prevalence and severity of gastrointestinal symptoms in treated coeliac disease patients and elucidate the mechanisms for the persistence of symptoms.

The specific aims were

1. To assess the prevalence and severity of gastrointestinal symptoms in untreated, short-term treated and long-term treated coeliac disease and to compare the symptom severity in treated coeliac disease patients with that of other common gastrointestinal diseases (I).

2. To investigate the factors associated with persistent gastrointestinal symptoms and a reduced health-related quality of life in coeliac disease patients in long-term dietary treatment (II).

3. To compare the duodenal microbial composition of long-term treated coeliac disease patients with and without persistent gastrointestinal symptoms (III).

4. To compare dietary factors and markers of small-bowel mucosal innate immunity and epithelial stress and integrity in treated coeliac disease patients with and without persistent symptoms (IV).
9 Patients

9.1 Patients in Study I

The study cohort was obtained from the prospectively collected research database maintained by the Celiac Disease Research Centre. It comprised 856 consecutive patients with confirmed coeliac disease diagnoses. Exclusion criteria were age <15 years and an uncertain initial diagnosis. All patients received dietary counselling by professional dietitians and started a strict gluten-free diet soon after their diagnosis was confirmed. The demographic characteristics of the study cohort are described in Table 9.

For further statistical analysis, the study subjects were divided into three groups based on the duration of their gluten-free diet: newly diagnosed patients (no diet), short-term treated patients (diet of one to two years) and long-term treatment patients (diet ≥3 years). In addition, the long-term treatment group was further divided into subjects who had been on a gluten-free diet either 3–5 years, 6–10 years or more than 10 years.

9.2 Patients in Study II

Participants were volunteering coeliac disease patients recruited by a nationwide search using newspaper advertisements and with the help of national and local coeliac disease societies. The final study cohort comprised 596 adults (age ≥18 years) with biopsy-proven coeliac disease confirmed from the medical records. Patients who had been on a gluten-free diet less than one year or had refractory coeliac disease, or whose coeliac disease diagnosis could not be verified were excluded. Patient characteristics are described in Table 9.
9.3 Patients in Study III

The patients were recruited at the University of Tampere and sampled at the Tampere University Hospital. The recruitment was done by inviting volunteer long-term treated coeliac disease patients to take a health survey. None of them had consulted a doctor due to ongoing symptoms. The inclusion criteria were age \(\geq 18\) years, maintenance of a gluten-free diet for \(\geq 3\) years and strict adherence to dietary treatment as defined by a minor inadvertent gluten intake of less than once a month combined with negative serology. Furthermore, the diagnosis of coeliac disease had to be based on the combination of positive celiac disease autoantibodies and subtotal or total small bowel mucosal villous atrophy. To minimize possible confounding factors and increase the probability of detecting differences in microbiota, only patients with gastrointestinal symptoms as an initial presentation of coeliac disease were included. Also, subjects with a recent or current use of medications that could noticeably affect bowel function (antibiotics, opioids, laxatives or anti-diarrhoeal drugs) were excluded.

A total of 177 participants underwent a combination of clinical examination, dietary assessment, upper gastrointestinal endoscopy with duodenal biopsies, and measurement of celiac disease serology, genetics and basic laboratory parameters. Subjects with both negative coeliac disease antibodies and normal small bowel mucosa (n=164) were selected for further assessment of current gastrointestinal symptoms on the Gastrointestinal Symptom Rating Scale (GSRS; Chapter 10.2). Based on the symptom scoring, a total of 18 subjects with the most severe symptoms and 18 subjects with least amount of symptoms were selected for analysis of their intestinal microbiota (Table 10).

9.4 Patients in Study IV

The initial patient cohort comprised of 167 coeliac disease patients who had voluntarily participated in a health survey at Tampere University and had not sought medical help for gastrointestinal symptoms. The inclusion criteria were biopsy-proven celiac disease, age \(\geq 18\) years, a duration of gluten-free diet of \(\geq 2\) years and a strict self-reported dietary adherence combined with a negative serum TG2-ab level at the time of the study. All subjects underwent systematic clinical and dietary evaluation, assessment of gastrointestinal symptoms, upper gastrointestinal endoscopy and evaluation of laboratory parameters related to
malabsorption and coeliac disease serology. Based on the results of a validated GSRS questionnaire (Chapter 10.2), 25 subjects with most severe symptoms and 25 of the most asymptomatic subjects were chosen for further comparison (Table 10).

9.5 Healthy controls in Studies I–II

In Study I, a total of 160 individuals and, in Study II, a total of 110 subjects were used as non-coeliac control groups (Table 9). They were recruited from among the friends and close neighbourhood of the study patients and had no first-degree relatives with coeliac disease.

9.6 Ethical considerations

In all studies, the protocol was approved by the Regional Ethics Committee of Tampere University Hospital District (former Ethical Committee of Tampere University Hospital). All participants gave written informed consent.
Table 9. Demographic and clinical data for coeliac disease patients with and without a gluten-free diet (GFD) in Studies I and II.

<table>
<thead>
<tr>
<th></th>
<th>Study I</th>
<th>Study II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated patients, n=128</td>
<td>Treated patients, n=596</td>
</tr>
<tr>
<td></td>
<td>Short treatment, n=93</td>
<td>Non-coeliac controls, n=110</td>
</tr>
<tr>
<td></td>
<td>Long treatment, n=635</td>
<td></td>
</tr>
<tr>
<td>Females, %</td>
<td>76</td>
<td>76</td>
</tr>
<tr>
<td>Current age, median (range), years</td>
<td>47 (15–72)</td>
<td>55 (19-92)</td>
</tr>
<tr>
<td>Duration of GFD, median (range), years</td>
<td>0</td>
<td>55 (23–87)</td>
</tr>
<tr>
<td></td>
<td>1 (1–2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 (3–48)</td>
<td></td>
</tr>
<tr>
<td>Mode of presentation at diagnosis, %</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>66</td>
<td>69</td>
</tr>
<tr>
<td>Extraintestinal a</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Screen-detected b</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>Coeliac disease in family, %</td>
<td>47</td>
<td>62</td>
</tr>
<tr>
<td>Strictness of GFD, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strict diet</td>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td>Occasional gluten</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>No lapses</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

a Dermatitis herpetiformis, aphthae, enamel defects, elevated liver enzymes, neurological symptoms, reproductive problems.
b Family history of coeliac disease, type I diabetes, thyroidal disease, Sjögren's syndrome, Addison's disease or IgA nephropathy.
Table 10. Demographic and clinical characteristics of coeliac patients with and without persistent symptoms on a gluten-free diet (GFD) in Studies III and IV.

<table>
<thead>
<tr>
<th></th>
<th>Study III</th>
<th></th>
<th>Study IV</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic</td>
<td>No symptoms</td>
<td>Symptomatic</td>
<td>No symptoms</td>
</tr>
<tr>
<td>Age, median (range), yrs.</td>
<td>n=17</td>
<td>n=17</td>
<td>n=25</td>
<td>n=22</td>
</tr>
<tr>
<td></td>
<td>54 (27–72)</td>
<td>63 (42–75)</td>
<td>53 (27–72)</td>
<td>61 (30–75)</td>
</tr>
<tr>
<td>Females, %</td>
<td>94</td>
<td>71</td>
<td>96</td>
<td>82</td>
</tr>
<tr>
<td>GFD, median (range), yrs.</td>
<td>10 (3–23)</td>
<td>8 (4–35)</td>
<td>9 (2–23)</td>
<td>10 (4–35)</td>
</tr>
<tr>
<td>Celiac disease in family, %</td>
<td>29</td>
<td>53</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td>Use of NSAIDs, %</td>
<td>11</td>
<td>14</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Use of PPIs, %</td>
<td>19</td>
<td>1</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Use of oats, %</td>
<td>87</td>
<td>76</td>
<td>70</td>
<td>68</td>
</tr>
<tr>
<td>Use of fibre, g/day(^a)</td>
<td>15.7 (13.4–18)</td>
<td>19.6 (14.6–24.5)</td>
<td>15.2 (13.2–17.1)</td>
<td>20.2 (16.1–24.4)</td>
</tr>
<tr>
<td>Blood haemoglobin, g/l</td>
<td>138 (133–143)</td>
<td>141 (133–149)</td>
<td>137 (131–144)</td>
<td>137 (131–144)</td>
</tr>
<tr>
<td>Serum total iron, µmol/l</td>
<td>16.5 (13.8–19.2)</td>
<td>17.8 (15.9–19.8)</td>
<td>16.9 (15.0–18.9)</td>
<td>19.2 (16.6–21.7)</td>
</tr>
<tr>
<td>Erythrocyte folate, nmol/l</td>
<td>559 (448–670)</td>
<td>600 (470–729)</td>
<td>670 (486–854)</td>
<td>616 (445–788)</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>25.3 (23.5–27.2)</td>
<td>26.2 (24.8–27.5)</td>
<td>25.4 (23.7–27.1)</td>
<td>24.5 (23.2–25.8)</td>
</tr>
<tr>
<td>TG2ab, median (range), units/ml</td>
<td>0.3 (0.0–0.6)(^b)</td>
<td>0.8 (0.1–1.4)</td>
<td>0</td>
<td>0 (0–0.9)</td>
</tr>
<tr>
<td>VH/CrD</td>
<td>3.2 (2.9–3.4)</td>
<td>3.0 (2.8–3.2)</td>
<td>3.1 (2.9–3.4)</td>
<td>2.9 (2.7–3.0)</td>
</tr>
<tr>
<td>GRSRS total score</td>
<td>2.8 (2.6–3.0)</td>
<td>1.3 (1.2–1.4)</td>
<td>2.8 (2.6–2.9)</td>
<td>1.2 (1.2–1.2)</td>
</tr>
</tbody>
</table>

NSAIDs: non-steroid anti-inflammatory drugs; PPIs: proton-pump inhibitors; TG2ab: transglutaminase 2 antibodies; VH/CrD: Villous Height Crypt Depth Ratio; GRSRS: Gastrointestinal Symptom Rating Scale.
\(^a\)Quantitative data, excluding age and duration of GFD, are expressed as means with 95% confidence intervals.
10 Methods

10.1 Demographic and clinical data (Studies I–IV)

In all studies, the participants were systematically interviewed by a study nurse or physician with expertise in coeliac disease. They were asked about demographic information and family history of coeliac disease, clinical presentation of the disease at the time of diagnosis, adherence to a gluten-free diet and possible consumption of oats or purified wheat starch. Furthermore, participants were asked about the presence of coeliac disease-associated disorders (such as type one diabetes, thyroidal disease and Sjögren’s syndrome) or other significant conditions. In Studies I and II, participants were also asked about time and site of diagnosis (primary, secondary or tertiary healthcare), presence of dietary counselling after diagnosis and regular follow-up. In Studies III and IV, participants were asked about any current use of certain medications, including NSAIDs, PPIs and AT2 receptor blockers, namely olmesartan.

Based on interviews, the main mode of presentation of coeliac disease at the time of diagnosis was classified by gastrointestinal symptoms (e.g. indigestion, diarrhoea, abdominal pain and signs of malabsorption), extraintestinal symptoms (e.g. DH, dental enamel defects and neurological symptoms) and patients detected by screening at-risk groups (coeliac disease in family, type I diabetes, thyroid disease, Sjögren’s syndrome, Addison’s disease and IgA nephropathy).

10.2 Clinical symptoms (Studies I–IV)

In Studies I–IV, participants self-assessed their gastrointestinal symptoms using the GSRS, which is a structured and validated questionnaire used widely for evaluating symptoms in gastrointestinal disorders (Svedlund et al. 1988, Revicki et al. 1998). The questionnaire had been translated into Finnish (Appendix 1) but not validated in a Finnish population. It consists 15 separate items that cover five sub-dimensions of gastrointestinal symptoms: indigestion, diarrhoea, constipation, abdominal pain and reflux. Separate values for each sub-dimension
score are calculated as a mean value of the respective items, and the GSRS total score is calculated as a mean of all 15 items. Scoring is based on a seven-grade Likert Scale, in which one point is minimal symptoms and seven points the most severe symptoms. Patients are asked to fill in the questionnaire based on the previous week.

To define patients with substantial gastrointestinal symptoms, a cut-off value for persistent symptoms was stated in Studies I and II. A patient was considered to have persistent gastrointestinal symptoms if they had a GSRS total score (in Studies I–II) or sub-dimension score (in Study I) higher than one standard deviation (SD) compared with the mean value of healthy controls. In Studies III and IV, the necessary number of most symptomatic and asymptomatic subjects were selected based on GSRS scores.

In Study I, the mean GSRS sub-dimension scores of untreated and long-term treated coeliac patients were compared with the corresponding GSRS scores of other common gastrointestinal disorders (peptic ulcer, gastro-oesophageal reflux disease, IBD and IBS) found in the literature.

In addition to the use of the validated questionnaire, patients in Study II were interviewed about the duration and severity of their self-perceived symptoms before the diagnosis. The duration of symptoms was defined as first experienced symptom until diagnosis, and three subgroups were formed as follows: no symptoms, symptoms for ten years or less, and symptoms for more than ten years. Likewise, based on symptom severity, three subgroups were formed: no symptoms, moderate symptoms and severe symptoms (Kivelä et al. 2015).

10.3 Health-related quality of life (Study II)

Health-related quality of life was assessed using the structured and validated Psychological General Well-Being questionnaire (Dupyi 1984). The questionnaire has been translated into Finnish (Appendix 2) but not validated in a Finnish population. The survey consists of 22 separate items with six sub-dimensions: anxiety, depression, well-being, self-control, general health, and vitality. In the scoring, based on a six-grade Likert scale, higher scores indicate better quality of life. Values are calculated separately for the PGWB total score and six sub-dimension scores are calculated as a sum of related items. The PGWB total score ranges from 22 to 132 points. Patients are asked to fill in the questionnaire based on the previous week. Patients with a PGWB mean score
lower than 1 SD compared with the mean value of healthy controls were considered to have a reduced quality of life.

10.4 Dietary assessment (Studies I–IV)

In all studies, adherence to a gluten-free diet was evaluated by a combination of self-assessment, interview by an experienced study nurse or physician, and serological tests (Chapter 10.5). Patients having a minor and inadvertent gluten intake less than once a month combined with negative coeliac disease serology were considered strictly adherent to the dietary treatment. In Studies III and IV, patients also kept a four-day food diary, which was used by an experienced dietitian to evaluate adherence to a gluten-free diet, use of oats and gluten-free wheat starch, and daily fibre intake (g/day).

10.5 Serological tests (Studies I–IV)

Serum IgA class EmA levels (Studies I–III) were investigated by an indirect immunofluorescence method employing human umbilical cord as the substrate. A dilution of 1: ≥5 was considered positive. Positive samples were further diluted to 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000 until negative. Serum IgA class TG2-ab levels (Studies I, III and IV) were measured by enzyme-linked immunosorbent assay (QUANTA Lite h-tTG IgA, INOVA Diagnostics, San Diego, CA, USA in Study I and Celik and Phadia, Freiburg, Germany in Studies III–IV). As instructed by the manufacturer, TG2-ab units either ≥30 U/l (INOVA Diagnostics) or ≥5 U/l (Phadia) were considered positive. For both EmA and TG2-ab testing, corresponding IgG class antibodies were used in cases of known IgA deficiency.

10.6 Laboratory parameters and genetics (Studies III–IV)

Blood haemoglobin (reference values: men 134–167 g/l and women 117–155 g/l), serum total iron (reference values: 9.0–34.0 µmol/l), and erythrocyte folic acid (reference values: 200–700 nmol/l) were measured using standard laboratory methods. Coeliac disease-associated HLA haplotypes (HLA)-DQB1*02 and DQB1*0302 alleles (DQ2 and DQ8) were detected in Study III by using either
the DELFIA Celiac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or the SSP DQB1 low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden/Qiagen Vertriebs GmbH, Vienna, Austria), as instructed by the manufacturer.

10.7 Small-bowel mucosal biopsy

10.7.1 Upper gastrointestinal endoscopy (Studies III–IV)

All patients had undergone upper gastrointestinal biopsies at the time of diagnosis according to national guidelines (Coeliac Disease, Current Care Guidelines 2010). Besides, an upper gastrointestinal endoscopy was performed at the time of the study on all participants in Studies III–IV. During the endoscopy, a minimum of seven forceps biopsy samples were taken from the duodenal mucosa. Depending on the later use, the biopsy specimens were either freshly embedded in an optimal cutting temperature compound (Tissue-Tec, Miles Elkhart, Indiana), snap-frozen in liquid nitrogen and then stored at -70°C, or fixed in formalin and embedded in paraffin. Furthermore, upon the endoscopy, biopsy samples were taken from the corpus and antrum of the stomach for routine histological assessment and Helicobacter pylori staining.

10.7.2 Mucosal morphology and inflammation (Studies III–IV)

For morphological analysis, a minimum of three formalin-fixed biopsy samples were stained with haematoxylin and eosin (H&E). Measurements of VH/CrD were performed according to the validated standard operating procedure (SOP) of the study group (Taavela et al. 2013). Following the SOP, an experienced evaluator assessed the cutting of a biopsy sample based on the orientation of crypts of Lieberkühn. Only specimens with a cutting perpendicular to the luminal surface were evaluated, and tangential cuttings were recut until reliable morphological readouts were reached. A ratio below 2.0 was considered to indicate active celiac disease (Kuitunen et al. 1982, Taavela et al. 2013).

The density of IELs was measured from the frozen sections by staining CD3+ IELs with monoclonal antibody Leu4 (Becton Dickinson, San Jose, CA, USA) and γδ+ IELs with T cell receptor γ-antibody (Endogen, Woburn, MA, USA). A
blind observer calculated the density as cells/mm by counting the number of positively stained cells with a 100 x flat-field microscope objective, from a minimum of 30 microscopic fields measuring 1.6 mm in epithelial length. The reference values for normal cell counts were <37 cells/mm for CD3+ IELs and <4.3 cells/mm for γδ+ IELs (Järvinen et al. 2003).

10.7.3 Markers of epithelial stress, innate immunity and epithelial integrity (Study IV)

The densities of CD25+ and FOXP3+ T-cells and CD117+ mast cells and the expression of the tight junction proteins claudin-3 and occludin, HSP60, IL15 and Toll-like receptor 2 and 4 (TLR2 and 4) were evaluated using immunohistochemical methods from the paraffin-embedded duodenal biopsy blocks. The blocks were cut into 5 µm sections and deparaffinized in xylene and rehydrated through graded ethanol series. After epitope retrieval, endogenous peroxidase activity was quenched, and nonspecific antibody binding sites were blocked using normal goat or horse serum (1:20) in 5% bovine serum albumin for 30 minutes. Subsequently, the samples were incubated with primary antibody (Supplementary Table 1 in original publication IV). The sections were washed with phosphate buffered saline and then incubated with biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) for 30 minutes and immunodetection was performed using an avidin-biotin complex method. Thereafter, the sections were counterstained in Harris haematoxylin. An Olympus BX60F5 bright field/fluorescence microscope, a ColorView III digital camera and Cell^D software version 2.7 (Olympus Co Ltd, Tokyo, Japan) were used for visualizing and imaging the stained samples.

When analysing the immunohistochemical stainings, the amount of CD25+, CD117+ and FOXP3+ was defined by calculating the number of positively stained cells per mm² lamina propria using ImageJ software. In addition, IL-15, HSP60, Claudin-3, Occludin and TLR2 and TLR4 stainings were scored blindly as instructed beforehand by at least two independent observers. The final scores were calculated as the median result of the observers. In IL-15 and HSP60 analysis, the samples were scored as either negative (0) or positive (1). IL-15 was scored separately for epithelium and lamina propria and HSP60 for crypt epithelium, villus epithelium and brush border. In claudin-3 staining, the scoring was based on overall intensity and the extent of honeycomb-like structure in the epithelium, while the scoring of occludin was based on the combination of the
intensity and area of the staining. Both claudin-3 and occluding staining were defined as either negative/weak or strong. The expression of TLR2 was assessed by counting the number of positively stained cells in the epithelium, and the result was expressed as cells/mm. TLR4 was scored as either negative/weak, medium or strong based on the overall intensity and distribution of the staining in the epithelial cells.

10.8 Assessment of intestinal microbiota

10.8.1 DNA extraction (Study III)

Total DNA was extracted from the frozen biopsy samples using the mechanical lysis of bacterial cells combined with the QIAamp® Mini Kit (Qiagen, Valencia, CA). Thereafter, the biopsy samples were lysed by incubating the sample in ATL lysis buffer with proteinase K overnight at 56°C, followed by mechanical lysis with Fastprep® instrument (MP Biomedical, Carlsbad, CA) for one minute at the level of 6.0 m/s, purified with spin columns, and eluted with 400 µl of buffer AE. The DNA concentrations were evaluated using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE). The extracted DNA was stored at -20°C.

10.8.2 The 16S rRNA gene pyrosequencing (Study III)

To profile duodenal microbiota, the bar-coded pyrosequencing method was used. The V4-V6 region of the 16S rRNA gene was amplified by polymeric chain reaction (PCR) in three replicates using a bacterial primer pair (F515 5’-TGYCAGCMGCCGCGGTAG-3’; 1061R 5’-TCACGRCACGAGCTGACG-3’). The V4-V6 variable region was chosen based on the results of the pretesting of PCR amplification. The PCR products were purified, quantified, and pooled in equal amounts and sequenced using an FLX Titanium Genome Sequencer (Roche) in the Eurofins MWG (Ebersberg, Germany). The raw sequences were trimmed using Mothur v.1.31.2 (Schloss et al. 2009). The sequences were included the analysis if they had an averaged quality score of over 25 within a 50 bp window, a length of over 250 bases, a maximum of two mismatches to barcode tags and the forward primer, no ambiguous bases, and no homopolymers longer than 8 bp, and were non-chimeric according to the Uchime (Robert C.
Edgar, http://drive5.com/uchime). The high-quality sequences were aligned using Greengenes as the reference database (May 2013 release, http://greengenes.secondgenome.com). A total of 960 sequences were randomly subsampled from each sample using Mothur to avoid the bias of varying sequencing coverage in samples. This subsampled dataset (32,640 sequences) was used in the later analysis. The sequences were binned into operational taxonomic units (OTUs) applying a sequence similarity of over 97%. Furthermore, the sequences were classified into bacterial taxa using the Wang approach in Mothur and SILVA, release 111, as a reference database (July 2012; Pruesse et al. 2007), and a threshold of certainty of over 60%.

10.9 Statistical analysis

In all studies, categorical data were described using percentages. Quantitative data were described using means with 95% confidence intervals (CIs) or medians with ranges or quartiles, as appropriate. Normality of the variables was analysed by using either a Kolmogorov-Smirnov or a Shapiro-Wilk test with box-plot pictures. In all statistical analysis, a p-value of <0.05 was considered statistically significant.

To compare categorical variables, cross-tabulation with Pearson’s χ2 test or Fisher’s exact test was used. To compare means between two variables (Studies I–IV), an independent-sample t-test with two-tailed significance was used in normally distributed variables, and the Mann-Whitney U test was used for non-parametric variables. To compare means between more than two independent samples, one-way ANOVA with Bonferroni post hoc analysis was used in normally distributed variables and the Kruskal-Wallis test in non-parametric variables (Study I). In Study II, binary logistic regression analysis was used to identify factors associated with persistent gastrointestinal symptoms and reduced quality of life, and the results were presented as odds ratios (ORs) with a 95% CI. To test correlation, Spearman’s Rank-Order Correlation with scatter-dot plots was used (Study IV). In all the above-mentioned analysis, the Statistical Package for the Social Sciences (SPSS) Statistics Version 19 or newer (IBM Corporation, Armonk, NY, USA) was used.

In Study III, the analysis of intestinal microbiota was performed using either R or Mothur statistical programs. The inverse Simpson and Shannon microbial diversity indices reflecting the number, abundance, evenness and richness of
bacterial species were calculated using R version 3.0.2 (R Development Core Team 2012) and its extension package Vegan (Oksanen et al. 2012). The comparisons between average microbial richness were computed using a c2m randomization test with 9,999 permutations in R package rich (Rossi 2011). The difference in mean diversity and in the relative abundance of taxa between the study groups was assessed by ANOVA in R. The sequence coverage was assessed using rarefaction analysis and Good’s coverage calculation in Mothur. Redundancy analysis (RDA) based on Hellinger transformed data and multidimensional scaling (MDS) based on Bray-Curtis distances were evaluated in R and its extension package Vegan. ANOVA for RDA clustering was completed with a full model using 999 permutations in R. Weighted and Unweighted Unifrac (Lozupone et al. 2005) and hierarchical clustering applying a parsimony method with 1,000 replications was computed as implemented in Mothur.

In Study I, covariance analysis was performed to take account of the effect of age. Furthermore, first the whole study cohort and then separately males and females were analysed. In Study III, all analysis was repeated using a dataset containing only the patients without gastrointestinal disease other than coeliac disease in order to exclude the possible confounding effect of other gastrointestinal diseases.
11 Results

11.1 Prevalence and severity of persistent gastrointestinal symptoms (Studies I–II)

Untreated coeliac disease patients had higher GSRS mean scores on all sub-dimensions except constipation and reflux compared with treated coeliac patients and healthy controls (Figure 1 in original publication I). The short-term treated patients only had more diarrhoea than healthy controls (Figure 1 in original publication I). Long-term treated patients had significantly higher mean GSRS reflux scores than the healthy controls (Figure 1 in original publication I). In more detailed analysis, reflux was seen particularly in patients treated for >10 years. There were no differences between short-term and long-term treated patients in GSRS mean scores.

When comparing the considerably increased symptoms (>1 SD compared with mean values of healthy controls), all coeliac disease patients expressed more symptoms than healthy controls on the basis of GSRS total score (Figure 3, Table 2 in original publication I). The untreated patients also showed significant overrepresentation in all GSRS sub-dimensions except constipation compared with all the other study groups (Table 2 in the original publication I). Moreover, there was an overrepresentation of short-term treated patients with diarrhoea compared with long-term treated patients and healthy controls (Table 2 in original publication I). In long-term treated patients, excessive reflux symptoms were seen compared with healthy controls (Table 2 in original publication I).

When performing the analysis in both genders separately, the detected differences in diarrhoea were mainly seen in short-term treated men compared with healthy controls (mean values 2.4 vs. 1.5, p < 0.001) and short-term treated women (mean values 2.4 vs. 1.7, p=0.003). The long-term treated women had higher total scores (2.0 vs. 1.8, p=0.001) and indigestion scores (2.5 vs. 2.3, p=0.034) compared with long-term treated men. The differences seen in GSRS reflux and total scores of long-term treated patients remained significant in women, but not in men (p=0.002 and p=0.015 respectively).
Figure 4. Percentage of coeliac disease patients (bars: Studies I–II) and healthy controls (dotted line: Study I) with increased gastrointestinal symptoms (defined as GSRS total score >1 SD compared with the mean value of healthy controls in the study in question). GSRS: Gastrointestinal Symptom Rating Scale; SD: standard deviation.

11.2 Comparison of gastrointestinal symptoms between coeliac disease and other intestinal diseases (Study I)

The GSRS scores of untreated and long-term treated coeliac disease patients were compared with GSRS scores seen in other common gastrointestinal disorders retrieved from the literature (Figure 2 in original publication I). The patients suffering from other gastrointestinal diseases were otherwise untreated, except for IBD patients, who had a clinical response to treatment. In general, the untreated coeliac disease patients were seen to suffer from a wider spectrum of symptoms than other gastrointestinal disease groups. The most severe symptoms in untreated coeliac disease were indigestion, diarrhoea and abdominal pain. In long-term treated patients, the gastrointestinal symptoms were clearly milder (Figure 2 in original publication I).
11.3 Epidemiological factors associated with persistent symptoms (Study II)

The epidemiological factors associated with the presence of persistent gastrointestinal symptoms at the time of the study are summarized in Table 11. Notably, patients diagnosed with extraintestinal symptoms had fewer current symptoms than those with initial gastrointestinal presentation (Table 2 in original publication II). None of the other investigated factors were associated with persistent symptoms, which included, for example, current age and gender and dietary non-adherence (Table 2 in original publication II).

Table 11. The factors associated with persistent symptoms or reduced quality of life in treated coeliac disease patients in original publication II.

<table>
<thead>
<tr>
<th>Persistent gastrointestinal symptoms&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis between the age of 25 and 60</td>
<td>0.05</td>
</tr>
<tr>
<td>Duration of symptom &gt;10 years before diagnosis</td>
<td>0.01</td>
</tr>
<tr>
<td>Moderate/severe symptoms before diagnosis</td>
<td>0.037/ 0.013</td>
</tr>
<tr>
<td>Thyroid disease</td>
<td>0.002</td>
</tr>
<tr>
<td>Non-coeliac food intolerance</td>
<td>0.012</td>
</tr>
<tr>
<td>Other gastrointestinal disease</td>
<td>0.009</td>
</tr>
<tr>
<td>Any coeliac disease-related comorbidity</td>
<td>0.027</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reduced quality of life&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms (more or less than 10 years) before diagnosis</td>
<td>0.038/ 0.017</td>
</tr>
<tr>
<td>Psychiatric comorbidity</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Presence of current gastrointestinal symptoms</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Defined as Gastrointestinal Symptom Rating Scale total score >1SD compared with mean value of healthy controls.

<sup>b</sup> Defined as Psychological General Well-Being score <1 SD compared with mean value of healthy controls.
11.4 Dietary factors in persistent symptoms (Studies II–IV)

In Study IV, the treated coeliac disease patients without symptoms had a significantly higher daily fibre intake than did patients with persistent symptoms (Figure 1 in original publication IV). The use of fibre did not have a clear correlation with GSRS total or sub-scores (-0.3< r <0.3 and p >0.05 in each), but all patients using the recommended amount of fibre (≥25 g/day, Nordic Council of Ministers 2014) belonged to the asymptomatic group. There was a similar but non-significant trend in fibre intake in Study III. In Studies III and IV, 68–87% used oats as a part of a gluten-free diet, and there were no differences between patient with and patients without persistent symptoms. Furthermore, the consumption of oats was not associated with persistent symptoms in the logistic regression analysis performed in Study II.

11.5 Differences in duodenal microbiota composition (Study III)

The profiling of microbiota was successful for 17 subjects in each group. In general, the microbiota analysis indicated that the treated coeliac patients with persistent symptoms had an altered duodenal microbiota composition compared with treated patients without symptoms. Differences in the microbiota composition were evaluated using several clustering methods, all of which provided congruent results at the genera level and mostly similar results at the OTU level (Table 12, Figures 1–2, and Supplementary Figures 1A–B in original publication III). In the more detailed analysis of duodenal microbiota, the coeliac patients with persistent symptoms had a lower relative abundance of Bacteroidetes and Firmicutes and a higher relative abundance of Proteobacteria than did treated patients with no symptoms (Figure 3 in original publication III). The microbiota composition also differed between the groups at the genus level in a total of seven taxa, including the highly abundant genus Prevotella (Table 2 in original publication III).
Table 12. Differences in duodenal microbial composition in patients with and without persistent gastrointestinal symptoms on a gluten-free diet (Study III).

<table>
<thead>
<tr>
<th>Clustering method</th>
<th>Genera level</th>
<th>OTU level</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDA&lt;sup&gt;a, b&lt;/sup&gt;</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Weighted Unifrac</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Unweighted Unifrac</td>
<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>Hierarchical clustering&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.07</td>
<td>0.71</td>
</tr>
</tbody>
</table>

<sup>a</sup>The results were also supported by the MDS analysis.
<sup>b</sup><em>p</em>=0.06 in the analysis without patients with other gastrointestinal diseases.
<sup>c</sup>Applying a parsimony method.

OTU: operational taxonomic units; RDA: redundancy analysis; MDS: multifactorial scaling.

The richness of duodenal microbiota was reduced in the treated patients with persistent symptoms when measured as a number of detected genera or OTUs (Table 13, Figure 4 and Supplementary Figure 1C in original publication <strong>III</strong>). Likewise, rarefaction curves based on OTUs and genera indicated a lower level of richness in the symptomatic patients that was significant for OTUs (Supplementary Figures 1D and 2 in original publication <strong>III</strong>). On inverse Simpson or Shannon diversity indices taking into account both the richness (number of species) and the abundances and evenness of species, no differences were observed between study groups. The richness is part of diversity estimates, so they usually correlate well. Thus, the absence of significance in diversity between the groups indicates that the abundance of the dominating species in subjects with persistent symptoms was becoming more even (their evenness was increasing) along with the decrease of richness. This indicates the possibility that the abundance of different species was affected differently in patients with persistent symptoms. Based on the rarefaction curves (Supplementary Figures 1D and 2 in original publication <strong>III</strong>) and Good’s coverage for genera, the sequencing effort was adequate to capture most of the bacterial diversity.
Table 13. Differences in duodenal microbial richness in patients with and without persistent gastrointestinal symptoms on a gluten-free diet (Study III).

<table>
<thead>
<tr>
<th></th>
<th>Persistent symptoms</th>
<th>No symptoms</th>
<th>p-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genera</td>
<td>32 (117)</td>
<td>37 (721)</td>
<td>0.05</td>
</tr>
<tr>
<td>OTU</td>
<td>72 /721</td>
<td>106 (1016)</td>
<td>0.007$^b$</td>
</tr>
</tbody>
</table>

$^a$ Assessed by c2m randomization test.
$^b$ Statistically significant also without patients with other gastrointestinal diseases, p=0.03.
OTU: operational taxonomic units.

To rule out the possible contribution of concomitant gastrointestinal diseases, the analysis was repeated after excluding the patients with these disorders (eight patients in the persistent symptoms group and one patient in the no-symptoms group). The results were mostly congruent with those seen in the entire study cohort (Tables 12 and 13, Supplementary Figure 3 in original publication III), although only the abundance of Bacteroidetes (p=0.03) was statistically significant.

11.6 Intestinal morphology, inflammatory cells and markers of innate immunity and epithelial integrity with persistent symptoms (Study IV)

The small-bowel mucosal VH/CrD ratio at the time of the study was similar in symptomatic and asymptomatic coeliac disease patients on a strict gluten-free diet (mean ratio 3.1 and 2.9 respectively; p=0.104), but the asymptomatic patients had a higher density of mucosal CD3+ IELs compared with the symptomatic patients. There was a similar trend in the γδ+ IELs, but this was non-significant (Figure 1 in original publication IV). Nevertheless, there were no differences in the expression of FOXP3+, CD117+ (Figure 2 in original publication IV) or CD25+ (median cell density symptomatic 30 cells/mm$^2$ vs. asymptomatic 26 cells/mm$^2$, p=0.835) in the lamina propria of symptomatic and asymptomatic subjects.

The staining of HSP60 was assessed separately for crypts, villi and brush borders, but no differences were observed in the percentage of patients with positive HSP60 (Figure 3 in original publication IV). The expression of IL15 in
the epithelium and in the lamina propria was observed to be rather low in both groups, and no significant differences were detected (Figure 3 in original publication IV). There were also no significant differences between the groups in the number of subjects expressing strong staining for occludin and claudin-3 in the epithelium (Figure 4 in original publication IV). The expression of TLR2 and TLR4 in the intestinal epithelium was also similar in patients with and without persistent symptoms.

11.7 Health-related quality of life in long-term treated coeliac disease
(Study II)

The coeliac disease patients on a gluten-free diet had lower mean PGWB scores than healthy subjects, with a mean PGWB total score of 102.8 (range 29.0–132.0) in treated coeliac patients and 105.3 (range 65.0–126.0) in the healthy controls (p=0.05). A total of 25% of the long-term treated coeliac disease patients had reduced quality of life (a PGWB score lower than 1 SD compared with the control mean), compared with 12% in the healthy controls. Factors associated with reduced health-related quality of life at the time of the study are summarized in Table 11.
12 Discussion

12.1 Prevalence and severity of persistent gastrointestinal symptoms

The present results indicate that long-term treated coeliac disease patients have more symptoms than healthy individuals. In fact, persistent gastrointestinal symptoms were seen in almost a quarter of the long-term treated coeliac patients following a strict dietary treatment. This is in line with earlier studies reporting a considerable proportion of treated patients with insufficient clinical response (Pink and Creamer 1967, Midhagen et al. 2003, Cranney et al. 2007, Stasi et al. 2016). Instead, the symptoms seen here were mild to moderate, whereas in previous studies the persistent symptoms tended to be rather severe, with excessive diarrhoea and even malabsorptive symptoms (Abdulkarim et al. 2003, Dewar et al. 2012). These differences are probably due to various reasons, perhaps most importantly differences in study design. The majority of the previous studies investigating this issue were conducted at referral centres (Abdulkarim et al. 2003, Leffler et al. 2007, Dewar et al. 2012, Stasi et al. 2016), where only the most symptomatic patients are sent. This increases the risk of overrepresentation of patients with severe symptoms and concomitant disorders.

In Finland, coeliac disease is commonly managed in the primary care system (Collin et al. 2007, Fuchs et al. 2014). The shift of diagnostics and treatment from tertiary to primary care has neither lengthened the diagnostic delay nor lowered the clinical prevalence of the disease (Fuchs et al. 2014). This means that, in Finland, the training of primary care physicians has been effective and that coeliac disease patients with milder symptoms are found at a relatively early stage (Fuchs et al. 2014). Against this backdrop, the prevalence of persistent symptoms in over 20% of treated patients feels highly relevant: it means that many coeliac disease patients accept the continuous presence of symptoms as an inevitable part of their daily life.

In the present study, diarrhoea seemed to be alleviated later than other gastrointestinal symptoms. Earlier, Pulido and colleagues (2013) also observed a slow alleviation of diarrhoea, but there are also inconsistent results (Murray et al. 2004). Nevertheless, diarrhoea was shown to be alleviated in the long-term treated
patients both in here and in the study by Pulido et al. (2013). It seems that the response to a gluten-free diet takes a variable amount of time, and patients should be encouraged to maintain a strict diet even if some symptoms were slow to be alleviated.

The reason for the presence of excessive reflux symptoms in the long-term treated patients remains unclear. In earlier studies, reflux was approximately as common in coeliac disease patients as in the general population (Mooney et al. 2015), and was alleviated by a gluten-free diet in both the short and long term (Cuomo et al. 2003, Nachman et al. 2011). However, a gluten-free diet often contains more fat than an unrestricted diet (Wild et al. 2010), which may increase reflux symptoms (Kubo et al. 2014). A high BMI may also cause reflux symptoms (Vaishnav et al. 2017), but unfortunately there was no available information regarding the differences in BMI between coeliac patients and controls.

12.2 Predictors of persistent symptoms

12.2.1 Dietary adherence

Adherence to the gluten-free diet was very good, as 88–94% of coeliac patients in Studies I–II followed a strict diet. This is in line with previous Finnish studies (Viljamaa et al. 2005b, Paavola et al. 2012). The excellent adherence is probably at least partly due to the high level of general knowledge and availability of gluten-free products in Finland. Globally, dietary lapses are the most frequent reason for poor symptom response (Abdulkarim et al. 2003, Leffler et al. 2007, Dewar et al. 2012, Stasi et al. 2016). In addition, inadvertent gluten intake due to gluten traces in food is common also in patients aiming at a strict diet (Hollon et al. 2013). Thus, gluten intake should be the first thing to investigate as a plausible reason for persistent symptoms. Unfortunately, there are no good methods to detect minor dietary lapses (Comino et al. 2016). In the future, detecting immunogenic gluten peptides from faeces or urine samples, for example, may prove useful in these situations (Comino et al. 2016, Moreno et al. 2017).

When adherence to dietary treatment seems truly strict, as it was in the patients in these studies, other reasons for symptoms must be considered. This means the exclusion of possible coexisting diseases. Most notably, the possibility of refractory coeliac disease should be evaluated, although this condition is rare in Finland (Ilus et al. 2014). In this case, although intestinal biopsies were not
obtained in Studies I–II, the patients had normal serological tests. Furthermore, in Studies III–IV, upper gastrointestinal endoscopies were performed to exclude persistent villous atrophy. Thus, refractory coeliac disease is considered highly unlikely in any of the patients.

12.2.2 The associations of diagnostic delay, microbiota and diet with persistent symptoms

A diagnostic delay and severe symptoms before diagnosis were associated with persistent symptoms. Although the delay has been reduced over the past few decades (Norström et al. 2011, Fuchs et al. 2014), it remains unacceptably long in some patients (Green et al. 2001, Norström et al. 2011, Fuchs et al. 2014). In line with our findings, our Sansotta colleagues (Sansotta et al. 2017) very recently showed diagnostic delay to be associated with poor symptom response.

Chronic pain has been investigated, in particular in patients with IBS, in which the abdominal pain is by definition long-lasting. In IBS, excessive sensitivity to visceral pain is suggested to contribute to the symptoms (Kanazawa et al. 2008). This is thought to be associated with alterations in the so-called gut-brain axis (Mayer et al. 2015). In addition, IBS is probably associated with changes in intestinal permeability, immune function, and intestinal microbiota (Holtmann et al. 2016). All these components are affected by brain signalling and correspondingly provide feedback to the central nervous system, forming several two-way loops (Mayer et al. 2015). Even though most coeliac disease patients with ongoing symptoms were not diagnosed with IBS, it is possible that long-lasting pain might contribute similar disturbances in the gut-brain axis.

Treated coeliac patients suffering from persistent symptoms had different compositions of duodenal microbiota and a reduced microbial richness compared with those of asymptomatic patients. Alterations in intestinal microbiota have previously been detected in untreated coeliac disease, with some of the changes present even in patients on a gluten-free diet (Nadal et al. 2007, Collado et al. 2009, DePalma 2010b, Nistal et al. 2012). Interestingly, an increase in Proteobacteria comparable with that found in the present study has also been detected with IBD (Frank et al. 2011). Also other disturbances, such as antibiotic treatment, have been shown to induce long-term dysbiosis (Jernberg et al. 2007, Dethlefsen and Relman 2011). It is therefore possible that active coeliac disease contributes to long-term or even irreversible dysbiosis of the intestinal
microbiota. However, the possible causality between dysbiosis and ongoing symptoms requires further investigation before any conclusions can be drawn.

Changes in the expression of TLRs could be hypothesized as connected with dysbiosis. TLRs are a group of receptors that recognize ligands from commensal microbiota or pathogens. Subsequently, they activate specific signalling pathways to maintain intestinal homeostasis (Valentini et al. 2014). Notably, alterations in the expression of distinct TLR genes have previously been detected in untreated coeliac disease compared with healthy controls (Szebeni et al. 2007, Kalliomäki et al. 2012). Differences between treated and untreated coeliac patients have been observed in children as well (Szebeni et al. 2007). In general, Gram-negative bacteria such as Bacteroidetes and Proteobacteria are recognized by TLR4 and Gram-positive bacteria, including Firmicutes, by TLR2 (Frosali et al. 2015). As changes in Proteobacteria and Firmicutes were detected in Study III, investigating TLR 2 and 4 seemed relevant here. However, no differences were detected in either of the receptors between symptomatic and asymptomatic treated coeliac patients. However, as only two TLR types were investigated, further investigation of the other types is needed.

In Study III, concomitant gastrointestinal disorders were overrepresented in treated patients with persistent symptoms. Many gastrointestinal disorders have been shown to promote alterations in the intestinal microbiota (Frank et al. 2011 Rajilić-Stojanović et al. 2011, Chen et al. 2016), which could affect the present results. However, a majority of the observed differences were preserved after the exclusion of patients with concomitant gastrointestinal diseases, supporting the idea that detected alterations in the microbiota are caused by some coeliac disease-related factor. Symptomatic patients were also somewhat older than asymptomatic patients. However, this is not likely to affect the results, as the intestinal microbiota remains relatively stable during adulthood (Biagi et al. 2010).

It could be argued that undiagnosed IBS might be the main cause of symptoms in treated coeliacs, even without the presence of microbial changes, as it is a common disorder in the general population (Hilliliä et al. 2008, Lovell and Ford 2012). Notably, even though there are diagnostic criteria defining IBS, differentiating between symptoms caused by IBS and symptoms of other aetiologies may be challenging, as there are no diagnostic tests or symptoms pathognomonic to the disorder. However, the fact that as many as 25% of the patients were suffering from symptoms in the present study is clearly a higher percentage than was found in another study investigating gastrointestinal
symptoms in the Finnish population (Hillilä et al. 2008). It thus seems unlikely that IBS could explain all the symptoms here. Nevertheless, it is possible that persistent symptoms in treated coeliac disease share common mechanisms with IBS, as discussed earlier.

The asymptomatic patients in the Study IV had a significantly higher intake of fibre than patients with symptoms did. Notably, all subjects using recommended amounts of fibre (Nordic Council of Ministers 2014) were asymptomatic. Diet has been shown to be an important modulator of the intestinal microbiota (Simpson and Campbell 2015). A Western diet with a low fibre, but high fat and sugar content has been shown to promote pro-inflammatory changes in the microbiota, including elevated levels of Proteobacteria comparable with those in present study (De Filippo et al. 2010, Wu et al. 2011, Devkota et al. 2012, Martinez-Medina et al. 2014). The amount of dietary fibre has also been shown to change the composition of microbiota-produced metabolites, emphasizing that fibre intake likely actually affects the functioning of intestinal microbiota (de Mello et al. 2017). A gluten-free diet is known to predispose to a low fibre intake (Thompson et al. 2005, Wild et al. 2010). Subsequently, abdominal complaints of treated coeliac patients are often presumed to be caused by constipation related to this circumstance. Based on the present results, also the microbiota-modulating effects of an insufficient fibre intake should be considered a cause of ongoing symptoms.

Taken together, aiming at an early coeliac disease diagnosis seems justifiable in order to shorten the diagnostic delay and thus achieve a better symptom response. Even though the relationship between fibre, dysbiosis and persistent gastrointestinal symptoms requires further study, paying attention to a sufficient fibre intake in coeliac disease patients can be recommended. In the future, interventions with a low FODMAP diet and microbiota-modulating treatments such as prebiotics and soluble fibre may further elucidate the mechanisms of symptom development in treated coeliac disease.

12.2.3 Inflammatory cells

Intraepithelial lymphocytosis is characteristic of active coeliac disease (Leon 2011) and is generally presumed to be associated with ongoing symptoms. However, the issue may be more complex, as the degree and extent of mucosal lesions correlates relatively poorly with the severity of symptoms (Murray et al. 2008, Kurppa et al. 2009, Pekki et al. 2015). In the present study, treated
asymptomatic coeliac disease patients had significantly more CD3+ IELs in their intestinal mucosa than did symptomatic patients. A similar trend was detected in γδ+ IELs.

Earlier, the αβ+ IELs have been shown to correlate with the activity of coeliac disease and to diminish with a gluten-free diet (Kutlu et al. 1993). On the other hand, although the γδ+ IELs are more specific to coeliac disease, they tend to persist despite a strict dietary treatment (Camarero et al. 2000, Koskinen et al. 2010, Calleja et al. 2011). Interestingly, intraepithelial lymphocytosis does not increase symptoms in strictly adherent patients having otherwise normal mucosa (Tuire et al. 2012). It has been suggested that γδ+ IELs may even have protective effects in coeliac disease by limiting the cytotoxic activity of αβ+ IELs (Bhagat et al. 2008), and the present results support this hypothesis to some degree.

No differences in the number of mast cells were detected. Just recently, Frossi and colleagues (2017) showed mast cells to be associated with the onset and progression of active coeliac disease, the number of activated mast cells correlating directly with Marsh classification. In addition, an increased number of mast cells has been detected in patients with IBS (DeSilva et al. 2012, Martinez et al. 2013). In these patients, the number of activated mast cells has been suggested to be more important than the total cell count (Martinez et al. 2013). The present results do not support the role of mast cells in symptoms of treated coeliac disease patients, but further studies measuring also mast cell activation are needed.

12.2.4 Epithelial stress and alterations of epithelial integrity

The overexpression of HSP60 was not seen in treated coeliac disease patients and parallel negative results were obtained with IL-15. Both are thought to play a role in the pathogenesis of coeliac disease (Iltanen et al. 1999a, Abadie and Jabri 2014), but studies investigating their impact on treated coeliac disease are scarce. Setty et al. (2015) recently studied HSP27 and IL-15 in different phases of coeliac disease. They detected no differences in the expression of HSP27 in treated patients and in controls. This supports the present results, although the HSP proteins were different in the two studies. Contradictory to the present study, Setty and colleagues (2015) reported increased epithelial expression on IL-15 in treated coeliac patients. However, the presence of gastrointestinal symptoms in those patients was not reported, making a comparison of the results difficult.
Based on the present results, epithelial stress does not seem to play a marked role in the persistence of symptoms in coeliac disease. The expression of junctional proteins occluding and claudin-3 were also investigated, yet no differences were detected between the treated coeliac patients with and without symptoms. Gastrointestinal symptoms in untreated coeliac disease have been associated with the disrupted intestinal barrier (Montalto et al. 2002, Ciccocioppo et al. 2006, Rauhavirta et al. 2014). In addition, a tight-junction-modulator larazotide acetate has been investigated in treated coeliac patients with symptoms, with contradictory results (Leffler et al. 2012, Kelly et al. 2013, Leffler et al. 2015b). Some symptom relief was seen, indirectly supporting the role of altered epithelial integrity in the symptoms. As in coeliac disease, alterations in the intestinal permeability have been detected in IBS as well (Martinez et al. 2012, Martinez et al. 2013). Although the role of epithelial integrity in the persistent symptoms cannot be established based on the present results, it must be remembered that there are several intestinal barrier markers that were not investigated.

12.2.5 Comorbidities and age at diagnosis

Autoimmune thyroid disease, non-coeliac food intolerance, other gastrointestinal disease and the presence of any coeliac disease-related co-morbidity increased the risk of persistent gastrointestinal symptoms. The results are contradictory to a recent study by Sansotta and colleagues (2017) in which none of these associations were detected. The reason for the differences remains unclear. In general, untreated thyroid disease may cause abdominal complaints that are usually alleviated during appropriate treatment of the disease (Ebert 2010). Considering that the thyroid disease in the patients in the present study was being treated, this finding does not fit in with the idea of symptoms being thyroid-related. Instead, an increased risk of excessive gastrointestinal symptoms in coeliac disease patients with other concomitant gastrointestinal condition seems logical. As comorbidity is also shown to affect the general experience of health (Lorem et al. 2017), coeliac disease patients with concomitant diseases might need additional support from the healthcare system.

A coeliac disease diagnosis at working age (25–60 years) was also associated with persistent symptoms. In line with this result, Sansotta and colleagues (2017) recently found that patients diagnosed in adulthood had poorer symptom relief. It is possible that patients at working age have already other challenges in their
working and family life. In such a situation, adapting to a totally new dietary regime may be difficult. In addition, concomitant disorders start to emerge during adulthood (Stewart et al. 2011).

12.3 Reduced health-related quality of life in treated coeliac disease

A total of 25% of the long-term treated patients suffered from reduced health-related quality of life, which is in line with earlier studies (Häuser et al. 2007, Nachman et al. 2010, Paavola et al. 2012, Shah et al. 2014). In general, many factors, including social support, self-care and adherence to treatment, have been shown to affect the ability to cope with a chronic disease (Stewart and Yen 2011). In the present study, non-adherence was not a predictor for a reduced quality of life. However, the number of patients with dietary lapses was very small. It is possible that the impact of adherence is greater in countries in which dietary transgressions are more frequent.

Interestingly, both having gastrointestinal symptoms before coeliac disease diagnosis or having them at the time of the study predisposed patients to a reduced quality of life. As stated earlier, patients with a long diagnostic delay and severe symptoms before diagnosis had more current gastrointestinal symptoms. It is possible that these patients are more vulnerable to impairments in quality of life and may require additional support from the healthcare system.

The presence of psychiatric comorbidity was also a risk factor in reduced quality of life. This seems reasonable, as the burden of ill health and a reduced quality of life comparable to that of physical disorders is often associated with psychiatric conditions (Busija et al. 2017, Vrbova et al. 2017). The absence of regular follow-up on coeliac disease did not affect quality of life. However, it must be recognized that the presence of follow-up does not necessarily mean that the patient would receive comprehensive support, as follow-up may only consist of serological testing. Thus, support from the healthcare system may still be effective in patients having difficulties in coping with coeliac disease. Indeed, especially coeliac patients with affective disorders have been shown to benefit from psychological support (Addolorato et al. 2004).
12.4 Strengths and limitations of the study

The major strengths of all the studies were the representative cohorts of coeliac disease patients. In Studies I–II, the study populations were very large and included considerable numbers of screen-detected patients and those with extraintestinal presentations. In Studies III–IV, the final study groups were relatively small, but they were chosen from representative cohorts of patients. It must be noted that patients were not recruited while consulting a physician for persistent symptoms in any of the studies. The recruitment method applied in the present study decreases the risk of selection bias and provides a good general picture of the Finnish coeliac population. Furthermore, in all studies, coeliac disease diagnoses and relevant medical data were obtained from medical records. Adherence to a gluten-free diet was usually confirmed by a combination of self-reporting, expert interview and serological testing.

Another strength was the use of validated and widely used questionnaires for the assessment of symptoms and quality of life. Moreover, in Study III, the duodenal microbiota was investigated instead of using fecal samples. This can be considered a strength, since coeliac disease especially affects the duodenum, and microbiota from this site is poorly represented in fecal samples. In general, the duodenal microbiota has scarcely been investigated, so this study brings novel information to the field.

In Studies I–II, recruitment of patients was carried out mainly through coeliac disease societies. This enhances the enrollment, but simultaneously predisposes to selection bias. It is also possible that some of the patients that cope poorly did not participate in the present study. Another problem is that the excellent adherence of patients to a gluten-free diet may have concealed the association between dietary lapses and a reduced quality of life or persistent symptoms. Thus, the results cannot be directly generalized to countries with poorer adherence.

The lack of serological testing of the control population may also be considered a limit. There is a possibility that few suffered from asymptomatic coeliac disease. However, this would be a very small proportion of the controls. Furthermore, it is possible that recruiting controls among the patients’ friends and neighbours could have caused some selection bias, as the healthiest individuals might be more likely to take part in the study. On the other hand, the controls were not required to be entirely healthy, so some of them may have had a non-coeliac illness causing gastrointestinal symptoms, such as IBS.
It should be remembered that the patient groups in Studies III–IV were relatively small, which increases the risk of false negative results. The food diaries in these studies were only four days long, and it can be argued that a diary covering the entire week would have given a more truthful picture. It is also possible that changes in the intestinal mucosa may be patchy and missed, even when taking several biopsies. Finally, even though the use of duodenal biopsies is a strength of the present study, it makes comparison with previous studies using fecal samples more difficult.
13 Summary and conclusions

This dissertation demonstrates that, although the short-term response to a gluten-free diet is generally good, persistent symptoms are common in long-term treated coeliac disease patients. Consistent with previous Finnish studies (Viljamaa et al. 2005b, Paavola et al. 2012), the dietary adherence in these studies was excellent. Even though dietary transgressions are the most frequent cause of poor symptom response in coeliac disease globally (Abdulkarim et al. 2003, Leffler et al. 2007, Dewar et al. 2012, Stasi et al. 2016), other reasons for symptoms must be sought in patients with proven strict adherence.

A majority of the patients did not have a diagnosis of IBS. Differential diagnosis and treatment of IBS is a challenge. This disorder was not systematically excluded in this study, yet the existing Rome criteria would probably not have been fulfilled in patients with mild symptoms. However, it could be hypothesized that persistent gastrointestinal symptoms in coeliac disease and IBS might nevertheless share common mechanisms. Interestingly, diagnostic delay was shown to predispose to ongoing symptoms later in life. This might be due to the alterations in the neural signalling and gut-brain axis comparable to those suggested for IBS (Mayer 2015). Moreover, altered intestinal microbiota and a low fibre intake might contribute to the persistence of symptoms similar to those of IBS. The results with respect to innate immunity, epithelial stress and epithelial integrity do not support the idea of them playing a marked role in persistent symptoms in treated coeliac disease.

This dissertation opens a new research field to be elucidated in the development and treatment of persistent symptoms in treated coeliac disease. Firstly, aiming at early coeliac disease diagnosis is recommended in order to achieve an optimal symptom response. Secondly, the low FODMAP diet and microbiota-modulating treatments such as pre- or probiotics and soluble fibre should be investigated in treated coeliacs. In any case, paying attention to sufficient fibre intake in a gluten-free diet is recommended. It should also be realized that coeliac patients with a long diagnostic delay or concomitant disorders are vulnerable to a poor symptom response. These patients may benefit from additional support from healthcare professionals. Despite a persistence of
symptoms, coeliac disease patients should always be encouraged to maintain a strict dietary treatment.
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APPENDIX 1: GSRS QUESTIONNAIRE
THE GASTROINTESTINAL SYMPTOM RATING SCALE
(GSRS)

Nimi______________________________________________

Lue tämä ensin:
Tutkimus sisältää kysymyksiä voinnistasi ja tilastasi kuluneen viikon aikana. Merkitse rastilla (X) se vaihtoehto, joka sopii parhaiten sinuun ja tilaasi.

1. Onko Sinulla ollut VÄTSAKIPUJA kuluneen viikon aikana? (Vatsakivuilla tarkoitetaan kaikenlaista kipua tai särkyä vatsassa.)
   □ Ei minkäänlaisia vaivoja
   □ Vähäpäätöisiä vaivoja
   □ Lieviä vaivoja
   □ Kohtalaisia vaivoja
   □ Melko pahoja vaivoja
   □ Pahoja vaivoja
   □ Erittäin pahoja vaivoja

2. Onko Sinulla ollut NÄRÄSTYSTÄ kuluneen viikon aikana? (Närästyksellä tarkoitetaan kirvelevää tai polttavaa pahanolontunnetta rintalastan takana.)
   □ Ei minkäänlaisia vaivoja
   □ Vähäpäätöisiä vaivoja
   □ Lieviä vaivoja
   □ Kohtalaisia vaivoja
   □ Melko pahoja vaivoja
   □ Pahoja vaivoja
   □ Erittäin pahoja vaivoja
3. Onko Sinulla ollut HAPPAMIA RÖYHTÄISYJÄ kuluneen viikon aikana? (Happamilla röyhtäisyillä tarkoitetaan äkillisiä, hapanta vatsanestettä sisältäviä röyhtäisyjä.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja

4. Onko Sinua HIUKAISSUT kuluneen viikon aikana? (Hiukaisulla tarkoitetaan vatsassa olevaa hiukovaa tunnetta, johon liittyy tarve syödä aterioiden välillä.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja

5. Onko Sinulla ollut PAHOINVOINTIA kuluneen viikon aikana? (Pahoinvoinnilla tarkoitetaan pahanolontunnetta, joka saattaa muuttua kuvotukseksi tai oksentamiseksi.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja
6. Onko vatsasi KURISSUT kuluneen viikon aikana? (Kurinalla tarkoitetaan vatsassa tuntuvaan värinä tai ”murinaa”.)
- Ei minkäänlaisia vaivoja
- Vähäpätöisiä vaivoja
- Lieviä vaivoja
- Kohtalaisia vaivoja
- Melko pahoja vaivoja
- Pahoja vaivoja
- Erittäin pahoja vaivoja

7. Onko vatsaasi TURVOTTANUT kuluneen viikon aikana? (Turvotuksella tarkoitetaan vatsassa tuntuvaa pingotusta, johon usein liittyy tuntemuksia ilmavaivoista.)
- Ei minkäänlaisia vaivoja
- Vähäpätöisiä vaivoja
- Lieviä vaivoja
- Kohtalaisia vaivoja
- Melko pahoja vaivoja
- Pahoja vaivoja
- Erittäin pahoja vaivoja

8. Onko Sinua vaivannut RÖYHTÄILY kuluneen viikon aikana? (Röyhtäilyllä tarkoitetaan tarvetta päästää ilmaa suun kautta, minkä yhteydessä vatsassa tuntuva pingotus usein helpottuu.)
- Ei minkäänlaisia vaivoja
- Vähäpätöisiä vaivoja
- Lieviä vaivoja
- Kohtalaisia vaivoja
- Melko pahoja vaivoja
- Pahoja vaivoja
- Erittäin pahoja vaivoja
9. Onko Sinulla ollut ILMAVAIVOJA kuluneen viikon aikana? (Ilmavaivoilla tarkoitetaan tässä tarvetta päästää ilmaa, jonka yhteydessä vatsassa tuntuva pingotus usein helpottuu.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja

10. Onko Sinua vaivannut UMMETUS kuluneen viikon aikana? (Ummetuksella tarkoitetaan ulostuskertojen harventumista.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja

11. Onko Sinua vaivannut RIPULI kuluneen viikon aikana? (Ripulilla tarkoitetaan ulostuskertojen lisääntymistä.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja
12. Onko Sinua vaivannut LÖYSÄ VATSA kuluneen viikon aikana? (Jos ulosteesi on välillä ollut kovaa ja välillä löysää, ilmoita vain, missä määrin ulosteesi löysyys on Sinua vaivannut.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja

13. Onko Sinua vaivannut KOVA VATSA kuluneen viikon aikana? (Jos ulosteesi on välillä ollut kovaa ja välillä löysää, ilmoita vain, missä määrin ulosteesi kovuus on Sinua vaivannut.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja


☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja
15. Onko Sinulla kuluneen viikon aikana ollut ULOSTAMISEN YHTEYDESSÄ TUNNE, ETTÄ SUOLI EI OLE TYHJENTYNYT KOKONAAN? (Tällä tarkoitetaan, että suoli ei ponnistuksista huolimatta tunnu tyhjentyneen kunnolla.)

☐ Ei minkäänlaisia vaivoja
☐ Vähäpätöisiä vaivoja
☐ Lieviä vaivoja
☐ Kohtalaisia vaivoja
☐ Melko pahoja vaivoja
☐ Pahoja vaivoja
☐ Erittäin pahoja vaivoja

16. ONKO SINULLA VIIMEISEN KUUKAUDEN AIKANA ESIINTYNYT SEURAAVIA OIREITA (rengasta sopivat vaihtoehdot)

a. kielikipuja
b. haavaumia suussa
c. luustokipuja
d. puutumista
e. muuta, mitä ______________________________________________________________

TARKISTA, ETTÄ OLET VASTANNUT KAIKKIIN KYSYMYKSIIN, ENNEN KUIN PALAUTAT LOMAKKEEN.

KIITOS AVUSTASI!
APPENDIX 2: PGWB QUESTIONNAIRE
Tutkimuksen tämä osa sisältää kysymyksiä siitä, miltä Teistä tuntuu ja kuinka Teillä on mennyt VIIMEKSI KULUNEEN VIIKON AIKANA. Jokaisen kysymyksen osalta rastittakaa (X) se vaihtoehto, joka parhainten sopii Teidän kohdallenne.

1. Miltä Teistä on YLEISESTI ottaen TUNTUNUT viimeksi kuluneen viikon aikana?
   - Mielialani on ollut erinomainen
   - Mielialani on ollut oikein hyvä
   - Mielialani on ollut enimmäkseen hyvä
   - Mielialani on vaihdellut paljon
   - Mielialani on ollut enimmäkseen huono
   - Mielialani on ollut hyvin huono

2. Kuinka usein Teitä on VAIVANNUT JOKIN SAIRAUS, RUUMIILLINEN VAIVA, SÄRYT tai KIVUT viimeksi kuluneen viikon aikana?
   - Joka päivä
   - Melkein joka päivä
   - Noin puolet ajasta
   - Silloin tällöin, mutta vähemmän kuin puolet ajasta
   - Harvoin
   - Ei koskaan
3. Tunsitko itsenne MASENTUNEEKSI viimeksi kuluneen viikon aikana?
   □ Kyllä – niin paljon, että minusta tuntui siltä, että ottaisin itseni hengiltä
   □ Kyllä – niin paljon, etten välittänyt mistään
   □ Kyllä – hyvin masentuneeksi melkein joka päivä
   □ Kyllä – melko masentuneeksi useita kertoja
   □ Kyllä – lievästi masentuneeksi silloin tällöin
   □ Ei – en ole kertaakaan tuntenut itseäni lainkaan masentuneeksi

4. Oletteko pystynyt HALLITSEMAAN KÄYTTÄYTMISTÄNNE, AJATUKSIANNE, MIELIALOJANNE tai TUNTEITANNE viimeksi kuluneen viikon aikana?
   □ Kyllä, ehdottomasti
   □ Kyllä – useimmiten
   □ Yleensä
   □ En kovin hyvin
   □ En, ja se häiritsee minua jonkin verran
   □ En, ja se häiritsee minua kovasti

5. Onko Teitä vaivannut HERMOSTUNEISUUS tai LEVOTTOMUUS viimeksi kuluneen viikon aikana?
   □ Erittäin paljon, jopa niin, että en ole voinut tehdä työtä tai huolehtia asioista
   □ Hyvin paljon
   □ Melko paljon
   □ Jonkin verran, niin että se on vaivannut minua
   □ Vähän
   □ Ei lainkaan
6. Kuinka paljon TARMOA, PIRTEYTTÄ tai ELINVOIMAA Teillä on ollut viimeksi kuluneen viikon aikana?

☐ Hyvin täynnä tarmoa – erittäin pirteä
☐ Melko tarmokas suurimman osan ajasta
☐ Tarmokkuuteni on vaihdellut melkoisesti
☐ Yleensä vähän tarmoa tai pirteyttä
☐ Hyvin vähän elinvoimaa tai tarmoa suurimman osan ajasta
☐ Ei lainkaan tarmoa tai elinvoimaa – olen tuntenut itseni loppuun ajetuksi tai loppuun kuluneeksi

7. Olen tuntenut itseni ALAKULOISEKSI JA SYNKKÄMIELISEKSI viimeksi kuluneen viikon aikana?

☐ En kertaakaan
☐ Vähän tänä aikana
☐ Jonkin verran tänä aikana
☐ Melkoisen osan tästä ajasta
☐ Suurimman osan tästä ajasta
☐ Koko ajan

8. Oletteko yleisesti ollut KIREÄ tai tuntenut itsenne JÄNNITTYNEEKSI viimeksi kuluneen viikon aikana?

☐ Kyllä, erittäin jännittyneeksi suurimman osan ajasta tai koko ajan
☐ Kyllä, hyvin jännittyneeksi suurimman osan ajasta
☐ En ole ollut koko ajan kireä, mutta olen tuntenut itseni melko jännittyneeksi useita kertoja
☐ Olen tuntenut itseni vähän jännittyneeksi muttamia kertoja
☐ En ole yleensä tuntenut itseäni jännittyneeksi
☐ En ole lainkaan tuntenut itseäni jännittyneeksi
9. Kuinka ONNELLINEN, TYYTYVÄINEN tai MIELISSÄNNE olette ollut viimeksi kuluneen viikon aikana?
   □ Erittäin onnellinen, en olisi voinut olla tyytyväisempi tai enemmän mielissäni
   □ Hyvin onnellinen suurimman osan ajasta
   □ Yleensä tyytyväinen ja mielissäni
   □ Joskus melko onnellinen ja joskus melko onneton
   □ Yleensä tyytymätön ja onneton
   □ Hyvin tyytymätön tai onneton suurimman osan ajasta tai koko ajan

10. Oletteko tuntenut itsenne riittävän TERVEEKSI tekemään asioita, joita haluatte tehdä tai Teidän on ollut pakko tehdä viimeksi kuluneen viikon aikana?
    □ Kyllä, ehdottomasti
    □ Suurimman osan ajasta
    □ Terveysongelmat ovat merkittävästi rajoittaneet minua
    □ Olen ollut vain niin terve, että olen voinut huolehtia itsestäni
    □ Olen tarvinnut jonkin verran apua itseni huolehtimisessa
    □ Olen tarvinnut toista henkilöä auttamaan itseäni seurauksena tai kaikissa asioissa, joita minun on täytynyt tehdä

11. Oletteko tuntenut itsenne niin SURULLISEKSI, LANNISTUNEEKSI tai TOIVOTTOMAKSI, että olette miettinyt, onko millään mitään merkitystä viimeksi kuluneen viikon aikana?
    □ Erittäin paljon – niin paljon, että olen ollut valmis luovuttamaan
    □ Hyvin paljon
    □ Melko lailla
    □ Jonkin verran – sen verran, että se on vaivannut minua
    □ Vähän
    □ En lainkaan
12. Oletteko herännyt PIRTEÄNÄ ja LEVÄNNEENÄ viimeksi kuluneen viikon aikana?

☐ En kertaakaan
☐ Muutaman harvan kerran
☐ Joitakin kertoja
☐ Aika monta kertaa
☐ Useimmiten
☐ Joka kerta

13. Oletteko ollut HUOLISSANNE tai LEVOTON TERVEYDESTÄNNE viimeksikuluneen viikon aikana?

☐ Erittäin paljon
☐ Hyvin paljon
☐ Melko paljon
☐ Jonkin verran, mutta en kovin paljon
☐ Käytännöllisesti katsoen en koskaan
☐ En lainkaan

14. Onko Teistä tuntunut siltä, että olisitte "MENETTÄMÄSSÄ JÄRKENNE" tai KONTROLLINNE siitä, miten TOIMITTE, PUTHETTE, AJATTELETTE, TUNNETTE tai MITÄ MUISTATTE viimeksi kuluneen viikon aikana?

☐ Ei lainkaan
☐ Vain vähän
☐ Jonkin verran, mutta ei niin paljon, että olisin ollut huolissani tai levoton siitä
☐ Jonkin verran ja olen ollut vähän huolissani
☐ Jonkin verran ja olen ollut melko huolissani
☐ Kyllä, hyvin paljon ja olen ollut hyvin huolissani
15. Päivittäinen elämäni on ollut TÄYNNÄ minua KIINNOSTAVIA ASIOITA viimeksi kuluneen viikon aikana?

☐ Ei lainkaan tänä aikana
☐ Vain pienen osan tästä ajasta
☐ Joskus
☐ Melkoisen osan tästä ajasta
☐ Suurimman osan tästä ajasta
☐ Koko ajan

16. Oletteko tuntenut itsenne AKTIIVISEKSI/TARMOKKAAKSI tai TYLSÄKSI/VELTOKSI viimeksi kuluneen viikon aikana?

☐ Hyvin aktiiviseksi/tarmokkaaksi joka päivä
☐ Enimmäkseen aktiiviseksi/tarmokkaaksi – en koskaan tylsäksi/veltoksi
☐ Melko aktiiviseksi/tarmokkaaksi – harvoin tylsäksi/veltoksi
☐ Melko tylsäksi/veltoksi – harvoin aktiiviseksi/tarmokkaaksi
☐ Enimmäkseen tylsäksi/veltoksi – en koskaan aktiiviseksi/tarmokkaaksi
☐ Hyvin tylsäksi/veltoksi joka päivä

17. Oletteko ollut HUOLESTUNUT, HARMISSANNE tai AHDISTUNUT viimeksi kuluneen viikon aikana?

☐ Erittäin paljon – niin paljon, että olen tuntenut itseni melkein sairaaksi huolestuneisuudesta
☐ Hyvin paljon
☐ Melko lailla
☐ Jonkin verran – sen verran, että se on vaivannut minua
☐ Vähän
☐ En lainkaan
18. Olen tuntenut itseni TASAPAINOISEKSI ja VARMAKSI viimeksi kuluneen viikon aikana?
☐ En lainkaan tänä aikana
☐ Pienen osan tästä ajasta
☐ Joskus
☐ Huomattavan osan tästä ajasta
☐ Suurimman osan tästä ajasta
☐ Koko ajan

19. Oletteko tuntenut itsenne LEVOLLISEKSI/HUOJENTUNEEEKSI vai PINGOTTUNEEEKSI/KIREÄKSI
viimeksi kuluneen viikon aikana?
☐ Olen tuntenut itseni levolliseksi ja huojentuneeksi koko viikon
☐ Olen tuntenut itseni levolliseksi ja huojentuneeksi suurimman osan ajasta
☐ Yleensä olen tuntenut itseni levolliseksi, mutta ajoittain olen tuntenut itseni melko
pingottuneeksi
☐ Yleensä olen tuntenut itseni pingottuneeksi, mutta ajoittain olen tuntenut itseni melko
levolliseksi
☐ Olen tuntenut itseni pingottuneeksi/kireäksi suurimman osan ajasta
☐ Olen tuntenut itseni hyvin pingottuneeksi/kireäksi koko ajan

20. Olen tuntenut itseni ILOISEKSI/HUOLETTOMAKSI viimeksi kuluneen viikon aikana?
☐ En lainkaan tänä aikana
☐ Pienen osan tästä ajasta
☐ Joskus
☐ Melkoisen osan tästä ajasta
☐ Suurimman osan tästä ajasta
☐ Koko ajan
21. Olen tunenut itseni VÄSYNEEKSI ja LOPPUUN KULUNEEKSI viimeksi kuluneen viikon aikana?

☐ En lainkaan tänä aikana
☐ Pienen osan tästä ajasta
☐ Joskus
☐ Melkoisen osan tästä ajasta
☐ Suurimman osan tästä ajasta
☐ Koko ajan

22. Oletteko tunnenut itsenne ”STRESSAANTUNEENKSI”, RASITTUNEENKSI tai PAINEEN ALAISEKSI viimeksi kuluneen viikon aikana?

☐ Kyllä, melkein enemmän kuin voin sietää tai kestää
☐ Kyllä melko lailla
☐ Kyllä, jonkin verran – enemmän kuin tavallisesti
☐ Kyllä, jonkin verran – kuten tavallisesti
☐ Kyllä, vähän
☐ En lainkaan

TARKISTAKAA, ETTÄ OLETTE VASTANNUT KAikkiN KYSymyKiSiIN!
KIITOS HYVÄstä YHTEISTYÖSTÄ.
Gastrointestinal Symptoms in Celiac Disease Patients on a Long-Term Gluten-Free Diet

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Abstract: Experience suggests that many celiac patients suffer from persistent symptoms despite a long-term gluten-free diet (GFD). We investigated the prevalence and severity of these symptoms in patients with variable duration of GFD. Altogether, 856 patients were classified into untreated (n = 128), short-term GFD (1–2 years, n = 93) and long-term GFD (≥3 years, n = 635) groups. Analyses were made of clinical and histological data and dietary adherence. Symptoms were evaluated by the validated GSRS questionnaire. One-hundred-sixty healthy subjects comprised the control group. Further, the severity of symptoms was compared with that in peptic ulcer, reflux disease, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Altogether, 93% of the short-term and 94% of the long-term treated patients had a strict GFD and recovered mucosa. Untreated patients had more diarrhea, indigestion and abdominal pain than those on GFD and controls. There were no differences in symptoms between the short- and long-term GFD groups, but both yielded poorer GSRS total score than controls (p = 0.03 and p = 0.05, respectively). Furthermore, patients treated 1–2 years had more diarrhea (p = 0.03) and those treated >10 years more reflux (p = 0.04) than controls. Long-term treated celiac patients showed relatively mild symptoms compared with other gastrointestinal diseases. Based on our results, good response to GFD sustained in long-term follow-up, but not all patients reach the level of healthy individuals.

Keywords: celiac disease; gastrointestinal diseases; symptoms; gluten-free diet

1. Introduction

The only current treatment for celiac disease is a life-long gluten-free diet. Commencement of a strict diet usually results in prompt relief of clinical symptoms, while recovery of small-bowel mucosal damage may take even years [1,2]. Although mucosal healing is the ultimate goal of the dietary treatment [3], from the patient’s perspective alleviation of self-perceived clinical symptoms is usually the most rewarding outcome. A good clinical response in the early stages of dietary treatment further motivates to maintain a strict diet, which consequently facilitates mucosal recovery. There is some evidence that after the initial enthusiasm has faded, many patients experience ongoing symptoms while maintaining an apparently strict gluten-free diet [4–7]. Such persistence of symptoms despite burdensome dietary restriction is frustrating and may even predispose to poor dietary adherence and
thus further worsen the situation. Hitherto, however, neither the prevalence nor the severity of the persistent symptoms in celiac disease patients on a gluten-free diet has been well characterized, let alone their impact on patients’ daily life. Data on these aspects would be necessary in order to optimize the follow-up of patients and, in the future, to develop interventions on top of the gluten-free diet.

The aim of the present nationwide study was to define the prevalence and severity of gastrointestinal symptoms in a large cohort of long-term dietary treated adult celiac disease patients and to compare these with those seen in untreated and short-term treated patients and in healthy controls. Further, symptom severity was compared with other common gastrointestinal diseases based on a literature search.

2. Materials and Methods

2.1. Study Design and Participants

The large cross-sectional study was carried out at Tampere University Hospital and the University of Tampere. The celiac disease patients were collected from our prospectively maintained research database, in which the patients have been recruited via newspaper advertisements and via local and national celiac disease associations from different parts of Finland. Exclusion criteria for the present study were age under 15 years and uncertain diagnosis of celiac disease (not based on biopsy). The final study cohort comprised 856 consecutive subjects with confirmed celiac disease. All celiac disease patients had received professional dietary counseling and were placed on a strict gluten-free diet soon after the diagnosis was confirmed.

Clinical data were collected systematically from the medical records. Further, all study subjects were interviewed by an experienced physician or a study nurse in the study clinic and asked about demographics, clinical presentation of the disease at the time of diagnosis, family history of celiac disease, duration of gluten-free diet, and adherence to dietary treatment. The main mode of presentation of celiac disease at diagnosis was further classified into gastrointestinal symptoms (e.g., indigestion, diarrhea and signs of malabsorption), extraintestinal symptoms (e.g., dermatitis herpetiformis, dental enamel defects and neurological symptoms) and patients detected by screening at-risk groups (celiac disease in family, type I diabetes, thyroid disease, Sjögren’s syndrome, Addison’s disease and IgA nephropathy). The results of serum endomysial antibody (EmA) measurements and small-bowel mucosal biopsy sampling were collected systematically.

In order to compare differences in the presence and severity of persistent gastrointestinal symptoms between subjects dieting for different periods of gluten-free diet the celiac disease patients were further divided into three groups based on the duration of the gluten-free diet as follows: (i) newly diagnosed patients (no diet); (ii) patients with short-term treatment (diet 1–2 years) and (iii) patients with long-term treatment (diet ≥ 3 years). For a more detailed analysis the long-term treatment group was further divided into subjects who had been on a gluten-free diet either 3–5 years, 6–10 years or > 10 years.

One-hundred-and-sixty healthy individuals (72% females, median age 55 (range 23–87) years) with no first-degree relatives with celiac disease served as a control group in the comparison of gastrointestinal symptoms.

The Regional Ethics Committee of the Tampere University Hospital District approved the study protocol and all participants gave written informed consent.

2.2. Celiac Disease Serology and Small-Bowel Mucosal Histology

Serum IgA class EmA were measured by an indirect immunofluorescence method with human umbilical cord as substrate [8], and a dilution of 1: ≥5 was considered positive. Positive samples were further diluted up to 1:4000 until negative. In cases of selective IgA deficiency, the corresponding IgG class antibodies were measured. Serum transglutaminase 2 antibodies had also been measured in most of the patients, but since test methods and reference values had varied during the study period
these readings were not used here. The degree of small-bowel mucosal damage was systematically measured from several well-orientated duodenal biopsy samples and reported by quantitative villous height crypt depth ratio (VH/CrD) as previously described [9]. Here VH/CrD < 2.0 was considered to indicate active celiac disease [9].

2.3. Gastrointestinal Symptoms

For the systematic evaluation of current gastrointestinal symptoms, participants in each group filled a self-administered, structured Gastrointestinal Symptom Rating Scale (GSRS) questionnaire. This is a validated questionnaire used widely in research on celiac disease and other gastrointestinal disorders [10–15]. The questionnaire measures five sub-dimensions of gastrointestinal symptoms: Indigestion, diarrhea, abdominal pain, reflux and constipation. It comprises altogether 15 separate items. Values for each of the five sub-dimension scores were calculated as a mean of the respective items and the total GSRS score as a mean of all 15 items. The scoring is based on a Likert scale from 1 to 7 points, where 1 point signifies minimal gastrointestinal symptoms and 7 points the most severe symptoms.

To further identify patients with persistent gastrointestinal symptoms, a cut-off for significantly worsened symptoms was set in the GSRS total and sub-dimension scores. This was the case when the subject’s GSRS total score or subscore was higher than 1 standard deviation (SD) compared with the corresponding mean score or subscore of the healthy controls [4,16–18].

Besides between study groups, the GSRS scores in untreated and long-term treated celiac disease patients were compared with those seen in subjects with common gastrointestinal disorders, namely peptic ulcer, gastro-esophageal reflux disease (GER), inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) as established by literature search [11,13–15]. In all diseases the GSRS scores were from untreated patients at diagnosis except for IBD, where the subjects were on treatment.

2.4. Adherence to the Gluten-Free Diet

Based on dietary interview, a subject was considered to be adherent to the gluten-free diet in the case of minor inadvertent gluten intake a few times a year or less. In addition, an objective estimation of dietary adherence was carried out by measuring the percentage of EmA-positive subjects in each treatment group. Positivity for EmA was considered to represent non-adherence when detected after two years on a gluten-free diet [19].

2.5. Statistics

Categorical data were described using percentages and quantitative data using either medians with range or means with 95% confidence intervals. Cross-tabulation with Pearson’s χ² test was used to analyze differences between categorical variables. To compare means between study groups, one-way ANOVA with Bonferroni post hoc analysis was used in normally distributed variables and Kruskal-Wallis test in non-parametric variables. To investigate correlation between variables, correlation coefficient (r) was calculated using Spearman’s correlation. To take account of the effect of age, a covariance analysis was used. Analyses were made with the whole study cohort and also separately for males and females. A p-value < 0.05 was considered statistically significant. All data were analyzed by SPSS (Statistical Package for the Social Sciences) Statistics Version 21 (IBM Corporation, Armonk, NY, USA).

3. Results

The median age of all 856 celiac disease patients was 54 years (range 15–85 years) and 75% were females. In 64% the reasons for celiac disease suspicion were gastrointestinal symptoms and in 18% extraintestinal symptoms; 18% were detected by screening. There were no significant differences between the celiac disease groups in either gender, median age at time of study, clinical presentation at diagnosis or celiac disease in the family (Table 1). Among patients on a gluten-free diet the long-term
treated cohort contained lower percentage of EmA-positive subjects than the short-term treated, while there were no differences in self-reported dietary adherence or VH/CrD.

**Table 1.** Demographic characteristics and selected celiac disease-associated data on untreated, short-term (1–2 years) treated and long-term (>3 years) treated celiac patients and healthy controls.

| Celiac Patients on a GFD n = 728 |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                               | Untreated Patients n = 128   | Short Treatment n = 93       | Long Treatment n = 635       |
| Females, %                    | 76                            | 72                            | 75                            | 72                            |
| Current age, median (range)   | 47 (15–72)                    | 51 (16–80)                    | 55 (17–85)                    | 55 (23–87)                    |
| GFD, median (range), years.   | 0                             | 1 (1–2)                       | 12 (3–48)                     | 0                             |
| Mode of presentation at diagnosis, % |                                |                                |                                |                                |
| Gastrointestinal              | 66                            | 63                            | 64                            | 0                             |
| Extraintestinal a             | 12                            | 16                            | 19                            | 0                             |
| Screen-detected b             | 23                            | 20                            | 17                            | 0                             |
| Celiac disease in family, %   | 47                            | 54                            | 61                            | 0                             |
| Self-reported strictness of GFD, (%) |                                |                                |                                |                                |
| Strict diet                   | 0                             | 93                            | 94                            | 0                             |
| Occasional gluten             | 0                             | 7                             | 6                             | 0                             |
| No diet                       | 100                           | 0                             | 0                             | 100                           |
| Positive EMA, %               | 93                            | 8 < 0.001                     | 3 < 0.001                     | 0                             |
| VH/CrD, mean (95% CI)         | 0.5 (0.4–0.6)                 | 2.7 (2.5–2.9) c,f             | 2.8 (2.6–2.9) c,d             | 3.2 (3.0–3.3) h |

a Dermatitis herpetiformis, aphthous ulcerations, enamel defects, elevated liver enzymes, neurological and musculoskeletal symptoms, psychiatric symptoms, infertility or early menopause; b Family history of celiac disease, type I diabetes, thyroidal disease, Sjögren’s syndrome, Addison’s disease, IgA nephropathy; c p < 0.001 compared with untreated patients; d p = 0.028 compared with short treatment group; e–h Data available on e 50 subjects, f 20 subjects, g 191 subjects and h 35 subjects. GFD, gluten-free diet; EMA, endomysial antibodies; VH/CrD, small-bowel mucosal villous height crypt depth ratio; CI, confidence interval.

Untreated celiac patients had significantly higher (more symptoms) GSRS scores on indigestion, diarrhea, abdominal pain and total scores than those on a gluten-free diet and healthy controls, whereas there was no difference in any of the scores between long-term and short-term treated patients (Figure 1). Long-term treated patients yielded higher GSRS reflux scores, and short-term treated higher diarrhea and total scores compared with the healthy controls. In more detailed analysis reflux was seen particularly in patients treated >10 years (data not shown). None of the gastrointestinal symptoms correlated with VH/CrD levels either in the whole cohort (r varying between −0.013 and −0.220) or in the different durations of gluten-free diet (r varying between −0.146 and 0.142).

When analyzing the occurrence of significantly increased (by definition, GSRS score > 1 SD compared with healthy controls) gastrointestinal symptoms, the untreated celiac disease patients again showed significant overrepresentation in all GSRS scores except constipation compared with the other study groups (Table 2). In addition, both long- and short-term treatment groups evinced more reflux and total gastrointestinal symptoms than controls; the short-term treated patients also reported more diarrhea (Table 2). In treatment groups the mean VH/CrD levels did not differ between patients with and without increased symptoms in any of the GSRS sub-groups (data not shown). In separate analysis, long-term treated women had higher GSRS total scores (2.0 vs. 1.8, p = 0.001) and indigestion scores (2.5 vs. 2.3, p = 0.034) than men, whereas short-term treated men had higher diarrhea scores (2.4 vs. 1.7, p = 0.003). The overrepresentation of increased (>1 SD) reflux and total scores seen in the long-term treatment group in both genders combined remained significant in women (p = 0.002 and p = 0.015, respectively) but not in men. The GSRS diarrhea scores were also increased in short-term treated men and long-term treated women compared with healthy controls (2.4 vs. 1.5, p < 0.001 and 1.7 vs. 1.5, p = 0.042, respectively).
Figure 1. Gastrointestinal Symptom Rating Scale (GSRS) total (A) and sub-dimension (B–F) scores in untreated, short-term treated and long-term treated patients compared with healthy controls. Values are expressed as means with 95% confidence intervals (CI) and gray bars denote 95% CIs of controls. There were significant differences between the groups as follows: (A) Untreated patients and all other groups ($p < 0.001$), and short-term treated patients and healthy controls ($p = 0.03$); (B) Untreated and all other groups ($p < 0.001$); (C) Untreated and short-term treated ($p = 0.015$) and long-term treated ($p < 0.001$) patients, and short-term treated patients and healthy controls ($p = 0.010$); (D) Untreated patients and all other groups ($p < 0.001$); (E) Healthy controls and untreated ($p < 0.001$) and long-term treated ($p = 0.013$) patients.
Table 2. Presence (%) of increased gastrointestinal symptoms\textsuperscript{a} in untreated, short-term (GFD 1–2 years) treated and long-term (GFD $\geq$ 3 years) treated celiac disease patients and in healthy controls.

<table>
<thead>
<tr>
<th>Celiac Patients on a GFD</th>
<th>n = 728</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSRS Score</td>
<td>Untreated Patients n = 128</td>
</tr>
<tr>
<td>Total score</td>
<td>48\textsuperscript{b}</td>
</tr>
<tr>
<td>Indigestion</td>
<td>41\textsuperscript{b}</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>47\textsuperscript{b}</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>43\textsuperscript{b}</td>
</tr>
<tr>
<td>Reflux</td>
<td>34\textsuperscript{b}</td>
</tr>
<tr>
<td>Constipation</td>
<td>16</td>
</tr>
</tbody>
</table>

GSRS, Gastrointestinal Symptom Rating Scale; GFD, gluten-free diet. \textsuperscript{a} Defined as GSRS scores $> 1$ SD compared to mean values of healthy controls; \textsuperscript{b} $p < 0.05$ compared with short and long treatment groups and with controls; \textsuperscript{c} $p < 0.05$ compared with healthy controls; \textsuperscript{d} $p < 0.05$ compared with long treatment group.

Comparisons of GSRS scores between celiac disease patients in the present study (untreated and long-term treated) and those with other gastrointestinal disorders can be seen in Figure 2. In general, untreated celiac patients suffered from a wider spectrum of symptoms compared with other gastrointestinal disease groups, the most severe being indigestion, diarrhea and abdominal pain (Figure 2). However, in long-term treated patients the gastrointestinal symptoms were clearly milder.

![Figure 2](image_url)

**Figure 2.** The mean Gastrointestinal Symptom Rating Scale (GSRS) sub-dimension scores of untreated and long-term treated patients (present study) compared with other gastrointestinal diseases [11,13–15]. Ulcer, peptic ulcer disease; Reflux, gastroesophageal reflux disease; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome.

4. Discussion

The main finding in the present study was that both short-term and long-term dietary treated celiac disease patients have more symptoms than non-celiac controls. However, although the majority of gastrointestinal symptoms are alleviated well on a strict gluten-free diet, not all patients reach the level of the general population even in long-term follow-up.

Here, the majority of the celiac disease patients showed rapid relief of symptoms during the first year on a gluten-free diet. This is in accord with previous studies investigating short-term responses, where the diet has also alleviated typical gastrointestinal symptoms within the first few months after diagnosis [20–22]. The only exception here was diarrhea, which, although alleviated on a long-term diet, remained fairly common in short-term-treated patients. This raises the question
whether the alleviation of diarrhea requires more complete histological recovery than other symptoms. However, in a recent study we observed no differences in symptoms or quality of life between patients evincing full histological recovery and those with ongoing mucosal damage after one year [22]. Hence, incomplete mucosal recovery would not appear to explain slow recovery from diarrhea in our patients. The few previous studies investigating this issue have obtained somewhat contradictory results. In accord with our observations, Pulido and colleagues showed very slow resolution of diarrhea on treatment within five or more years [7], whereas a group under Murray observed improvement of diarrhea already within six months [20]. The reason for these considerable variations between the studies remains unclear, but might for example involve differences in interpretation and definition of symptoms. Obviously different study designs and populations may also have an effect, as we had demographic characteristics and design similar to those of Pulido and colleagues [7], whereas Murray and group [20] investigated mainly short-term responses to a gluten-free diet in one well-defined geographical region.

In contrast to the well-documented short-term outcome [2], the long-term response to a gluten-free diet has thus far been poorly investigated. Judging from our results, in most patients with good adherence and recovered villi the good initial response to the diet remains after several years, demonstrating that it is not only based on a short-term “honeymoon” effect. Notwithstanding this long-lasting positive effect, we found even long-term dietary treated patients to have more symptoms than healthy controls. Such ongoing symptoms may in the long run discourage patients from adhering to what is a socially restrictive and expensive treatment mode if they consider it ineffective. In such cases, it is particularly important for physicians to urge patients to persist with a strict gluten-free diet in order to prevent disease-associated complications [2]. In addition to the increased GSRS total score, particularly reflux symptoms showed a tendency to persist for several years. Gastroesophageal reflux is common in general populations [23] and in earlier studies it has appeared to be approximately as common in celiac patients [24]. Then again, initiation of a gluten-free diet has often reduced reflux symptoms rapidly [25–27], and the response has also persisted in the long term [27]. In some studies reflux symptoms have been suggested to be more common in aged people [28], but this is controversial and did not explain the difference in the present study. Altogether, the reason for the increase in reflux symptoms in long-term treated celiac disease remains unclear and needs to be clarified in future studies.

We observed long-term treated celiac disease women to experience more symptoms than men. Previously Hallert and associates has reported similar findings in Swedish women [6,29,30], and Pulido and colleagues observed more symptoms in both undiagnosed Canadian women and those on dietary treatment [7]. One plausible explanation for the gender difference might be the higher prevalence of concomitant functional gastrointestinal disorders in women, which have also been shown to be exacerbated by psychological distress such as that involved in following a burdensome dietary treatment [31–33]. Women may also find the inevitable social restrictions caused by the gluten-free diet harder to cope with [29]. Other possible reasons could be differences in fiber intake and the symptom-modifying effect of gonadal hormones [34–36]. In any case, physicians should acknowledge the higher risk of persistent symptoms in women and provide adequate support if needed.

In comparison with the other common gastrointestinal disorders as reported in the literature [11,13–15], we observed that untreated celiac patients evince a fairly wide range of symptoms. In line with this, other recent studies have reported that nowadays only a minority of patients present with classical symptoms, such as diarrhea and malabsorption, but, instead, suffer from a plethora of “atypical” symptoms or have no symptoms at all [4,19,37]. As well as in the diagnostic workout, this heterogeneous clinical presentation also needs to be taken into account when evaluating the long-term dietary response. In particular, although the specific GSRS scores here were mostly fairly low, physicians should remember that suffering from multiple, even if moderate, symptoms simultaneously may constitute a substantial burden in individual patients.
The most common reason for the persistence of symptoms in celiac disease has been ongoing gluten consumption [5,38]. However, in agreement with our previous studies [19,39,40], more than 90% of the patients here were strictly adherent and even in the few who reported lapses these were only occasional. This conception was further confirmed by the well-recovered histology and low EmA-positivity among patients on the diet. Thus, although gluten intake should always be excluded [41], other explanations for persistent symptoms must be sought in patients with proven strict adherence. These include for example small-intestinal bacterial overgrowth or some other concomitant disorder such as IBD and microscopic colitis, or refractory celiac disease [5,41]. An interesting new research topic related to this issue is dysbiosis of the intestinal microbiota [42]. We have recently shown that celiac patients suffering from persistent symptoms on a gluten-free diet had an altered balance and reduced richness of duodenal microbiota [43]. The intestinal microbiota affects the complex gut-brain axis along with the enteric nervous system, immune system and external environment, and alterations in this axis may predispose to chronic pain in functional gastrointestinal disorders and perhaps also in celiac disease [44]. A deeper understanding of these mechanisms would be important in order to make the development of new pharmacological interventions possible.

Several novel adjunct therapies to improve the treatment of celiac disease are currently under development [45]. In these circumstances evaluating long-term symptoms in treated celiac disease patients becomes more and more important. For now, gluten-free diet remains the gold standard treatment for celiac disease and thus every new therapeutic approach needs to be compared with the response to the diet. The present study provides solid information of the response of celiac individuals to gluten-free diet and could thus be used as baseline to the future pharmacological trials.

Strengths of the present study were the large and nationwide cohort of clinically representative celiac disease patients and the use of well-validated and structured symptom questionnaire. A major limitation was the retrospective cross-sectional study design, this, however, being offset by the fact that patients with different durations of gluten-free diet were comparable regarding most of the clinical and demographic parameters and were diagnosed and treated similarly according to nationwide guidelines. Another limitation was that the majority of the participants were recruited via celiac disease associations, which may cause some selection bias. Finally, celiac disease was not excluded among all healthy controls, a few of whom might have been suffering unrecognized.

5. Conclusions

In conclusion, we showed that the good initial clinical response to a gluten-free diet is sustained also in the long run. However, it is important for physicians to realize that one year might not be long enough for all symptoms to abate, and that some patients may continue to have mild or moderate gastrointestinal symptoms despite long-term and strict dietary treatment. A fuller understanding of the factors behind persistent symptoms in celiac disease would provide new treatment possibilities in the future.

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Author Contributions: Pilvi Laurikka, Teea Salmi, Pekka Collin, Heini Huhtala, Markku Mäki, Katri Kaukinen and Kalle Kurppa conceived and designed the study; Pekka Collin and Markku Mäki contributed to the acquisition of data; Pilvi Laurikka, Heini Huhtala, Katri Kaukinen and Kalle Kurppa analyzed the data; Pilvi Laurikka and Kalle Kurppa drafted the manuscript and Teea Salmi, Pekka Collin, Heini Huhtala, Markku Mäki and Katri Kaukinen revised the manuscript for important intellectual content.

Conflicts of Interest: The authors declare no conflict of interest. The sponsors had no role in the study design; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.
References


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Predictors of persistent symptoms and reduced quality of life in treated coeliac disease patients: a large cross-sectional study

Pilvi Paarlahti1, Kalle Kurppa1, Anniina Ukkola1, Pekka Collin2, Heini Huhtala3, Markku Mäki1 and Katri Kaukinen2,4*

Abstract

Background: Evidence suggests that many coeliac disease patients suffer from persistent clinical symptoms and reduced health-related quality of life despite a strict gluten-free diet. We aimed to find predictors for these continuous health concerns in long-term treated adult coeliac patients.

Methods: In a nationwide study, 596 patients filled validated Gastrointestinal Symptom Rating Scale and Psychological General Well-Being questionnaires and were interviewed regarding demographic data, clinical presentation and treatment of coeliac disease, time and place of diagnosis and presence of coeliac disease-associated or other co-morbidities. Dietary adherence was assessed by a combination of self-reported adherence and serological tests. Odds ratios and 95% confidence intervals were calculated by binary logistic regression.

Results: Diagnosis at working age, long duration and severity of symptoms before diagnosis and presence of thyroidal disease, non-coeliac food intolerance or gastrointestinal co-morbidity increased the risk of persistent symptoms. Patients with extraintestinal presentation at diagnosis had fewer current symptoms than subjects with gastrointestinal manifestations. Impaired quality of life was seen in patients with long duration of symptoms before diagnosis and in those with psychiatric, neurologic or gastrointestinal co-morbidities. Patients with persistent symptoms were more likely to have reduced quality of life.

Conclusions: There were a variety of factors predisposing to increased symptoms and impaired quality of life in coeliac disease. Based on our results, early diagnosis of the condition and consideration of co-morbidities may help in resolving long-lasting health problems in coeliac disease.

Keywords: Coeliac disease, Symptoms, Quality of life, Gluten-free diet, Adults

Background

At present, the only treatment for coeliac disease is a life-long gluten-free diet, i.e. exclusion of wheat-, rye- and barley-containing cereals and food products. Upon removal of gluten from the diet clinical symptoms are usually rapidly alleviated, while recovery of the duodenal mucosa may take several months or even years [1]. The treatment may also prevent many coeliac disease-associated complications such as intestinal malignancies [2]. Notwithstanding these benefits, the stigma of a chronic disorder and the need for major dietary restrictions increases the self-perceived burden of illness and may impair patients’ quality of life [3,4]. The symptoms may also remain despite a long-term and strict diet [5,6]. As a result, in some studies even well-treated coeliac patients have failed to attain well-being similar to that of the population in general [7,8], albeit that there are also contradictory results [9,10]. To improve the situation, knowledge of the factors underlying these persistent health concerns in coeliac patients is required. Thus far, only a limited number of studies have investigated this issue [11]; data are scant, particularly in screen-detected patients and in subjects with extraintestinal presentation [9,12].
The aim of this large nationwide study was to find predictors of persistent gastrointestinal symptoms and reduced health-related quality of life in long-term treated adult coeliac patients. Particular attention was devoted to aspects such as duration and severity of symptoms before diagnosis and presence of coeliac disease-associated and other co-morbidities.

Methods

Study design and participants

The trial was conducted at the Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital. Adult volunteers were recruited by a nationwide search using newspaper advertisements and via national and local coeliac disease societies. The study cohort comprised a total of 596 adults (age > 18 years) with biopsy-proven coeliac disease. All coeliac diagnoses and other relevant medical data (see below) were confirmed from the patients’ medical records. Subjects who had been on a gluten-free diet less than one year, had biopsy-proven refractory coeliac disease, or whose coeliac disease diagnosis could not be verified were excluded from the study.

All eligible subjects filled self-administered, validated and structured gastrointestinal symptom and corresponding health-related quality of life questionnaires. Next, to reveal factors associated with persistent symptoms and poor quality of life, the participants were interviewed by a physician or study nurse with expertise in coeliac disease. They were asked to report their demographic data and family history of coeliac disease, clinical presentation at diagnosis, time and place of diagnosis (primary, secondary or tertiary health care), dietary counseling and regular follow-up, adherence to the gluten-free diet, possible consumption of gluten-containing products and coeliac disease serology. Participants were rated as a sum of the items in each sub-dimension in which higher scores indicate better quality of life. The Psychological General Well-Being (PGWB) questionnaire [12,13,15,21] was used to measure health-related quality of life of the participants were evaluated by structured and well-validated questionnaires widely applied in coeliac disease research [7,12-15]. The Gastrointestinal Symptom Rating Scale (GSRS) [16,17] was used to evaluate gastrointestinal symptoms. The questionnaire comprises 15 separate items covering five different sub-dimensions: diarrhea, indigestion, constipation, abdominal pain and reflux. The scoring is based on a 7-grade Likert scale in which 1 point indicates no symptoms and 7 points the most severe gastrointestinal symptoms. Values for each sub-dimension score are calculated as a mean of the relevant items. The total GSRS score is calculated as a mean value of all 15 items and may thus also gain values between 1 to 7 points. Patients with a score higher than 1 standard deviation (SD, 0.66 points) compared to the control mean were considered to have increased gastrointestinal symptoms [18-20]. The threshold score for increased symptoms was therefore 2.55 points.

The Psychological General Well-Being (PGWB) questionnaire [12,13,15,21] was used to measure health-related quality of life. This survey consists of 22 separate items covering six different sub-dimensions: anxiety, depression, well-being, general health and vitality. The scoring is based on a 6-grade Likert scale in which higher scores indicate better quality of life. The value of the total PGWB score may range from a minimum of 22 to maximum 132. The sub-scores are calculated as a sum of the items in each sub-dimension in question. Subjects with a score less than 1 SD (12.1 points) compared to the control mean were considered to have reduced quality of life [18-20], so the threshold score was 93.1 points.

Adherence to the gluten-free diet

The possible consumption of gluten-containing products was assessed by a combination of self-reported adherence and coeliac disease serology. Participants were rated strictly adherent if they reported being on a strict gluten-free diet in dietary interview and were found to be negative for serum endomysial (EmA) and transglutaminase 2 (TG2-ab) antibodies.

Serum IgA class EmA were measured by an indirect immunofluorescence method with human umbilical cord
as substrate [22] and serum IgA class TG2-ab by enzyme-linked immunosorbent assay (QUANTA Lite h-tTG IgA, INOVA Diagnostics, San Diego, CA, USA). A dilution of 1: ≥5 for EmA was considered positive and positive samples were further diluted 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000 and 1:4000. In TG2-ab unit values ≥30 U/l were considered positive. In cases known to involve selective IgA deficiency, the corresponding IgG-class antibodies were applied.

Statistics
Categorical data were described using percentages and quantitative data using medians with range. Binary logistic regression analysis was used to identify factors associated with reduced quality of life. Results are presented as odds ratios (OR) with 95% confidence intervals (CI). A p-value ≤ 0.05 was considered statistically significant. T-test was used to compare the GSRS and PGWB mean scores between coeliac patients and healthy controls.

If a patient failed to answer on one or two items in the PGWB or GSRS questionnaire, the missing answer was replaced by the mean value of the other scores for the same subject. If more than two answers were missing the questionnaire was disqualified. All statistical data were analyzed by the Predictive Analytic SoftWare for Windows version 19 (IBM Corporation, Armonk, NY, USA).

Results
Clinical characteristics of the study cohort are shown in Table 1. In total, 69% of the subjects were diagnosed on the basis of classical gastrointestinal presentation and 31% on the basis of extraintestinal symptoms or by screening in at-risk coeliac disease groups. Altogether 88% of the subjects were strictly adherent; none reported totally unrestricted gluten consumption and 82% used oats as a regular part of their diet. Lactose intolerance was present in 17%, food allergy in 5% and fructose intolerance in none of the participants. Further, 6% of the subjects had reflux disease, 4% gastritis, 2% diverticulosis and 2% cholelithiasis. The most common psychiatric disorder was depression, with a prevalence of 2% (78% of subjects with psychiatric diagnosis).

The mean total GSRS score was 2.1 (range 1.0- 4.8) in the study group and 1.9 (range 1.0- 4.2) in the controls. The score was higher than 1 SD compared to the control mean in 25% of the participants. There was no significant association between the persistent gastrointestinal symptoms and gender or current age, but patients diagnosed between the age of 25 and 60 years had more persistent symptoms than those diagnosed at younger or older age (Table 2). Further, subjects who had suffered from moderate or severe symptoms at the time of the diagnosis or had had symptoms more than 10 years before it had more current gastrointestinal symptoms than initially asymptomatic patients. Patients diagnosed on extraintestinal symptoms had fewer current symptoms than those with gastrointestinal presentation (Table 2). The presence of thyroid disease, non-coeliac food intolerance, other gastrointestinal disease and any coeliac disease-related co-morbidity also increased the risk of persistent symptoms. None of the other variables examined, including dietary non-adherence and consumption of oats, was associated with persistent symptoms. The GSRS mean total score was significantly higher in the study participants than in control subjects (p= 0.003).

The mean PGWB total score was 102.8 (range 29.0- 132.0) in the study group and 105.3 (range 65.0-126.0) in the controls. Altogether 25% of the participants had the score lower than 1 SD compared to the control mean. Reduced health-related quality of life was seen in patients with manifest symptoms prior to diagnosis compared with asymptomatic subjects; the difference was evident in particular among those with symptoms for more than 10 years. Further, subjects with any psychiatric co-morbidity had reduced quality of life (Table 3). A similar trend was observed in patients with neurological disease and gastrointestinal co-morbidity.

### Table 1 Demographic data and clinical characteristics of the 596 coeliac disease patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td>452 (76)</td>
</tr>
<tr>
<td>Current age, median (range), years</td>
<td>55 (19-92)</td>
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<tr>
<td>Age at diagnosis, median (range), years</td>
<td>44 (1-82)</td>
</tr>
<tr>
<td>Site of diagnosis, n (%)</td>
<td></td>
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<tr>
<td>Primary care</td>
<td>149 (25)</td>
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<tr>
<td>Secondary or tertiary care</td>
<td>446 (75)</td>
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<tr>
<td>Presenting symptom at time of diagnosis, n (%)</td>
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<tr>
<td>Gastrointestinal</td>
<td>410 (69)</td>
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<tr>
<td>Extraintestinal</td>
<td>95 (16)</td>
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<tr>
<td>Screen-detected</td>
<td>91 (15)</td>
</tr>
<tr>
<td>Coeliac disease in family, n (%)</td>
<td>372 (62)</td>
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<tr>
<td>Coeliac disease-associated disorders, n (%)</td>
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<tr>
<td>Type 1 diabetes mellitus</td>
<td>14 (2)</td>
</tr>
<tr>
<td>Thyroid disease</td>
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<tr>
<td>Gluten-free diet, n (%)</td>
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<tr>
<td>Strict</td>
<td>520 (88)</td>
</tr>
<tr>
<td>Occasional gluten</td>
<td>76 (12)</td>
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<tr>
<td>No diet</td>
<td>0</td>
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<tr>
<td>Consumption of oats, n (%)</td>
<td>487 (82)</td>
</tr>
<tr>
<td>Duration of gluten-free diet, median (range), years</td>
<td>10 (1-53)</td>
</tr>
<tr>
<td>Coeliac disease in relatives, presence of autoimmune disorder.</td>
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</tr>
</tbody>
</table>

1Stomach pain, diarrhea, constipation, heartburn, flatulence, swelling, anaemia, malabsorption.
2Dermatitis herpetiformis, tiredness, joint pains, neurologic symptoms, gynaecologic problems.
3Coeliac disease in relatives, presence of autoimmune disorder.

![image](https://via.placeholder.com/150)
<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Symptoms, %</th>
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<th>95% CI</th>
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<td>25-60</td>
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but the results in question were not statistically significant (Table 3). Of note, patients with persistent gastrointestinal symptoms were also more likely to have reduced quality of life (OR 4.66, CI 3.01-7.00, p < 0.001). The coeliac patients had lower mean PGWB scores than healthy subjects (p = 0.05).

Discussion
The results of our nationwide study showed that up to 25% of adult coeliac disease patients suffer from persistent gastrointestinal symptoms despite a strict gluten-free diet. These findings show an excess of gastrointestinal complaints in treated coeliac patients, although to a somewhat lesser extent than noted in some previous studies [23,24]. Yet it needs to be remembered that comparison between studies is difficult because both the methods to measure and definitions used for persistent symptoms may differ. Further, it is important to distinguish primary non-responsive patients from those investigated in the present study; common reasons for non-responsive coeliac disease are inadvertent gluten intake, refractory coeliac disease and concomitant diseases such as malignancies, microscopic colitis and pancreatic insufficiency [5,25,26]. Patients with any such concomitant condition or refractory coeliac disease were excluded from the present study. Furthermore, although no histological evaluation was undertaken here, judging from both dietary interview and serological testing the majority of participants were adhering strictly to the diet. It would thus seem unlikely that inadvertent gluten intake or presence of the aforesaid adjunct disease would explain the ongoing gastrointestinal symptoms.

In the present study, coeliac patients diagnosed at working age had more current symptoms than those diagnosed at an either younger or older age. Although we cannot confirm the reason for this age dependency of the symptoms, it is possible that changes in established dietary habits are more difficult to cope with simultaneously with other challenges encountered in daily work and family life. Likewise, for some unexplained reason, irritable bowel syndrome (IBS) often emerges in early adulthood [27]. Interestingly, also severe and long-lasting symptoms prior to diagnosis predispose coeliac patients to persistent symptoms. It has been suggested that such long-lasting abdominal complaints result in a chronic cycle of pain in consequence of changes in the gut-brain axis [28,29]. Of note, similar chronic alterations have been hypothesized to be involved in the pathogenesis of IBS, which often coexists with coeliac disease [30-32]. There is also evidence suggesting that the symptoms in IBS might be caused by continuous low-grade small-bowel mucosal inflammation [30,31,33], a condition which may persist in coeliac disease despite a strict gluten-free diet [34,35]. Finally, small-intestinal bacterial overgrowth may account for symptoms in both coeliac disease and IBS and is also accompanied by mucosal inflammation [33,36,37]. Whatever mechanisms lie behind the persistent symptoms, our results indicate that they could be ameliorated by diagnosing coeliac disease as early as possible.

The results obtained showed the presence of a coeliac disease-related co-morbidity, another gastrointestinal disorder or non-coeliac food intolerance to predispose to persistent symptoms. It is possible that concomitant gastrointestinal co-morbidity further reinforces the impact of the aforementioned long-lasting changes in the gut-brain axis. Interestingly, a particular association was seen between persistent symptoms and concomitant thyroid disease, which when untreated is known to may cause abdominal complaints, these usually recovering on treatment [38]. However, some patients evince persistent clinical symptoms and reduced health-related quality of life even while on treatment and euthyroid [39]. The pathogenic mechanisms underlying this remain unclear, but clearly the possible connection between thyroid disease and persistent gastrointestinal symptoms in coeliac disease calls for further investigation.

As was the case with gastrointestinal symptoms, a substantial proportion of the coeliac patients here showed reduced health-related quality of life while on a gluten-free diet. Again, long duration of symptoms before diagnosis was a predisposing factor. In contrast, there was no association between clinical manifestations of coeliac disease at diagnosis and current quality of life. This is in line with our previous results showing similar health-related quality of life in screen-detected and symptom-detected coeliac patients on a gluten-free diet [12,13]. These findings constitute further evidence that early detection and dietary treatment of coeliac disease is beneficial even in screen-detected patients with mild or atypical symptoms at diagnosis.
### Table 3 Factors associated with persistently reduced quality of life* in treated coeliac disease patients

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</table>
There was a significant association between reduced quality of life and the presence of psychiatric co-morbidities in our study patients. This is in accord with some previous findings and there is also evidence that such coeliac patients might benefit from intensified psychological counseling [8,18,40-42]. Consequently, health-care professionals should pay particular attention to this patient group, who may need special support with their dietary treatment [42]. Since in the present study 78% of participants with a psychiatric disorder suffered from depression, the role of other psychiatric disorders needs further investigation. Although not statistically significant, there was a trend suggesting that the presence of certain other co-morbidities, for example neurological disorders, other gastrointestinal diseases or non-coeliac food intolerance are factors predisposing to reduced quality of life in coeliac disease. Further studies are warranted to clarify the significance of these issues.

A major strength of our study was a large study cohort with well-defined coeliac disease diagnoses and clinical data. In addition, there were a substantial number of screen-detected patients and subjects with extraintestinal presentation of coeliac disease. A possible limitation, on the other hand, was that a substantial proportion of the participants were recruited through local or national coeliac disease associations, which might have caused some selection bias. Furthermore, our participants showed excellent adherence to the gluten-free diet, this however being in accord with our earlier findings [12]. Although good adherence is beneficial for the patient, in this study it might have concealed the association between poor adherence and reduced quality of life. As a consequence, results may be different in countries where adherence to a gluten-free diet is lower. Although the threshold value of 1 SD to define increased symptoms and reduced quality of life has also been used in other studies, it is somewhat artificial. The fact that healthy controls were not precisely matched with the study participants was also a limitation.

The majority of participants used oats as a regular part of their diet, and this was not associated with increased gastrointestinal symptoms or reduced quality of life. This is important, since the use of oat-containing products in coeliac disease has hitherto remained controversial [43]. Based on our previous results [44] and the findings here, purified oats could be a regular part of a gluten-free diet. One important issue which should be considered in evaluating gastrointestinal symptoms in coeliac patients is that commercial gluten-free products may have marked qualitative differences, for example in the use of additives and preservatives and the amount of fibre [45].

**Conclusions**

As a conclusion, we showed that many coeliac disease patients suffer from persistent gastrointestinal symptoms and reduced quality of life despite strict dietary treatment. In particular, long-lasting and severe symptoms before the diagnosis and concomitant thyroid, gastrointestinal and psychiatric co-morbidities were significant risk factors for these ongoing health concerns. The results emphasize the importance of early diagnosis and careful follow-up in coeliac disease.

**Competing interests**

The authors declare that they have no competing interests.

**Authors' contributions**

PP: Study concept and design; analysis and interpretation of data; statistical analysis; writing of the manuscript. KK: Study concept and design; analysis and interpretation of data; statistical analysis; drafting of the manuscript; critical revision of the manuscript for important intellectual content. AU: Study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content. PC: Study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content. MP: Study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content. HT: Study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content. MM: Study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content. AU: Study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content. ME: Study concept and design; acquisition of data; critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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**Table 3 Factors associated with persistently reduced quality of life* in treated coeliac disease patients (Continued)**

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*Defined as a score >1 standard deviation from the mean Psychological General Well-Being total score of healthy controls; CI, confidence interval; OR, odds ratio defined by bivariate logistic regression.
References
Altered Duodenal Microbiota Composition in Celiac Disease Patients Suffering from Persistent Symptoms on a Long-term Gluten-free Diet

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Short title: Microbiota in Treated Celiacs with Persisting Symptoms

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Abstract

OBJECTIVES: A significant fraction of celiac disease patients suffer from persistent symptoms despite a long-term gluten-free diet (GFD) and normalized small bowel mucosa. Commonly suggested reasons, such as inadvertent gluten-intake or presence of other gastrointestinal disease, do not explain the symptoms in all these patients. Recently, alterations in intestinal microbiota have been associated with autoimmune disorders, including celiac disease. This led us to test a hypothesis that abnormal intestinal microbiota may be associated with persisting gastrointestinal symptoms in treated celiac disease patients.

METHODS: Duodenal microbiota was analyzed in 18 GFD-treated patients suffering persistent symptoms and 18 treated patients without symptoms by the 16S rRNA gene pyrosequencing. The celiac disease patients had been following a strict gluten-free diet for several years and had restored small bowel mucosa and negative celiac autoantibodies. Their symptoms on GFD were assessed with Gastrointestinal Symptom Rating Scale.

RESULTS: The results of several clustering methods showed that the treated celiac disease patients with persistent symptoms were colonized by different duodenal microbiota in comparison to the patients without symptoms. The treated patients with persistent symptoms had a higher relative abundance of Proteobacteria (p=0.04), and a lower abundance of Bacteroidetes (p=0.01) and Firmicutes (p=0.05). Moreover, their microbial richness was reduced. The results indicated intestinal dysbiosis in the patients with persistent symptoms even while adhering to a strict GFD.

CONCLUSION: Our findings indicate that the dysbiosis of microbiota is associated with persistent gastrointestinal symptoms in treated celiac disease patients and open new possibilities to treat this subgroup of patients.
STUDY HIGHLIGHTS:

1. WHAT IS CURRENT KNOWLEDGE
   - Intestinal microbiota is suggested to have a role in several gastrointestinal disorders, including celiac disease.
   - Many otherwise healthy celiac disease patients suffer from persistent gastrointestinal symptoms despite a strict long-term gluten-free diet and normalized small bowel mucosa.
   - Role of microbiota in treated celiac patients with persistent symptoms is not known.

2. WHAT IS NEW HERE
   - Treated celiac disease patients with persistent symptoms had altered duodenal microbiota composition and reduced microbial richness in comparison to patients without symptoms, indicating dysbiosis of the intestine.
   - We propose that untreated celiac disease may lead to dysbiotic intestinal microbiota and consequently predispose to persistent symptoms in celiac disease patients on a gluten-free diet.
Introduction

In celiac disease, the initiation of a gluten-free diet (GFD) usually results in a rapid improvement of the clinical symptoms followed by a slower recovery of the small bowel mucosal damage. Nevertheless, there is evidence that many patients suffer from persistent symptoms despite long-term dietary treatment (1, 2). The most common reason for such ongoing symptoms is continuous intentional or inadvertent gluten intake. Alternative causes are, for example, the presence of another unrecognized gastrointestinal disease, constipation due to low fiber content in gluten-free products, or refractory celiac disease (1-3). However, in many cases reason for persistent symptoms in otherwise healthy celiac patients with a strict GFD and restored intestinal mucosa cannot be explained (4). In this respect, the rapidly advancing research focusing on the association of gut microbiota with gastrointestinal disorders is of great interest. There are several studies reporting imbalances in the intestinal microbiota of celiac disease patients (5-7). For instance, abnormal microbiota composition characterized by decreased numbers of Bifidobacterium spp. and increased numbers of Bacteroides spp. have been found in duodenal biopsies of untreated patients (5, 6). In addition, the composition of duodenal microbiota has been shown to vary depending on the clinical presentation of celiac disease (8). Interestingly, it appears that dysbiosis of intestinal microbiota does not completely recover even after the commencement of GFD and normalization of the small bowel mucosal morphology (5, 6, 9). This led us to hypothesize that sustained intestinal dysbiosis might be associated with the inadequate clinical response and persistent gastrointestinal symptoms often seen in treated celiac disease. To address this question, we compared the duodenal microbiota composition between well-defined cohorts of celiac disease patients with and without persistent symptoms while they were on long-term GFD and had normalized intestinal mucosa.
Methods

Patients and study design

The patient recruitment and sampling were carried out at the Tampere University Hospital and University of Tampere. The enrollment was executed by inviting volunteer long-term treated (GFD ≥ 3 years), adult (age ≥ 18 years) celiac disease patients to attend a health survey. Inclusion criteria were that patients were strictly adherent to GFD, and the diagnosis had to be based on the combination of positive celiac disease autoantibodies and subtotal or total small bowel mucosal villous atrophy. In order to minimize confounding factors and to increase the probability to detect differences in microbiota, only subjects with gastrointestinal symptoms (e.g. indigestion, diarrhea, abdominal pain) as an initial presentation of celiac disease were included (8). Patients with extraintestinal symptoms or asymptomatic patients at the time of diagnosis were excluded, as well as those with recent or current use of medications that could remarkably affect bowel function, such as antibiotics, opioids, laxatives or anti-diarrheal drugs. All voluntary participants (n=177) underwent a thorough clinical examination, dietary assessment, gastrointestinal endoscopy with duodenal biopsies and measurements of celiac disease serology, and genetics and basic laboratory parameters. After the analyses, subjects with negative celiac antibodies and normal small bowel mucosa (n=164) were selected for further assessment of current gastrointestinal symptoms by Gastrointestinal Symptom Rating Scale (GSRS, see below in detail) and, based on the results, altogether 18 subjects with the highest GSRS total score (Persistent symptoms group) and 18 subjects with the lowest total score (No symptoms group) were chosen for the final study cohort for intestinal microbiota analyses.
Small bowel mucosal biopsies

Upon upper gastrointestinal endoscopy, a minimum of seven forceps biopsy specimens were taken from the duodenum. Three bowel biopsy specimens were freshly embedded in an optimal cutting temperature compound (Tissue-Tec, Miles Elkhart, Indiana), snap-frozen in liquid nitrogen and stored at -70°C until used for immunohistochemical stainings and for microbial DNA extraction. The remaining biopsies were stained with hematoxylin-eosin (H&E) and used for morphological analyses. The small bowel mucosal villous height/crypt depth ratio (Vh/CrD) was determined from multiple well-orientated H&E stained biopsy samples as previously described in detail (10). Densities of CD3+ and γδ+ intraepithelial lymphocytes (IEL) were analyzed from the frozen samples with immunohistochemistry as follows: first, 5 μm thick biopsy cuttings were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA) for CD3+ IEL and with T-cell-receptor-γ antibody (Endogen, Woburn, MA) for γδ+ IEL. Next, the number of positive IELs was counted with a 100 x flat-field light microscope objective throughout the surface epithelium. A minimum of 30 fields measuring 1.6 mm in epithelial length were calculated. IEL density was expressed as cells in mm of epithelium (11). The reference values were considered to be 37 cells/mm for CD3+ and 4.3 cells/mm for γδ+ IELs (11). During the endoscopy, biopsy samples were also taken from the corpus and antrum of the stomach for routine histological assessment and Helicobacter pylori (H. pylori) staining.

Clinical and dietary evaluation
Besides routine clinical investigations, all the patients were interviewed concerning demographic data, initial presentation of celiac disease, duration of GFD, family history for celiac disease, presence of other autoimmune or gastrointestinal diseases and current use of medications including non-steroid anti-inflammatory drugs (NSAID) and protein-pump inhibitors (PPI). An experienced dietitian analyzed the strictness of the gluten-free diet, uses of purified oats and gluten-free wheat starch and consumption of fiber (g/ day) by means of a four-day food diary and personal interview. Body mass index (BMI) was calculated as kg/m$^2$.

The severity of current gastrointestinal symptoms was assessed quantitatively by GSRS, which is a structured and validated questionnaire widely used in the research of gastrointestinal diseases (12-14). The survey covers five different gastrointestinal symptoms: indigestion, diarrhea, constipation, abdominal pain, and reflux. The scoring is based on a 7-degree Likert scale, which ranges from 1 (minimal symptoms) to 7 (maximal symptoms) points. In our previous studies, the mean GSRS total score has been approximately 2.7 in untreated celiac disease patients, between 1.8 and 2.0 in patients on GFD and 1.8 in healthy non-celiac population (15, 16).

**Laboratory parameters and celiac disease genetics**

Serum IgA class endomysial antibodies (EmA) were investigated by an indirect immunofluorescence method using human umbilical cord as substrate as previously described in detail (17). A titer of $1: \geq 5$ for EmA was considered positive. Serum IgA class transglutaminase 2 antibodies (TG2ab) were measured by an enzyme-linked immunosorbent assay (Celikey; Phadia, Freiburg, Germany), according to the manufacturer’s instructions. TG2ab values of $\geq 5.0$ U were
considered positive. Blood hemoglobin (reference values: men 134-167 g/l; women 117-155 g/l), serum total iron (reference values 9-34 μmol/l), and erythrocyte folic acid (reference values 200-700 nmol/l) were measured using standard laboratory methods. The presence of celiac disease-associated human leukocyte antigen (HLA)-DQB1*02 and DQB1*0302 alleles (DQ2 and DQ8) was investigated by using either the DELFIA Celiac Disease Hybridization Assay (PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or the SSP DQB1 low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden/Qiagen Vertriebs GmbH, Vienna, Austria), according to the manufacturer’s instructions. Both tests were available in our laboratory at the time of study and were equally appropriate to test celiac disease genetics.

**DNA extraction**

The total DNA was extracted from the frozen biopsy samples using mechanical lysis of bacterial cells combined with the QIAamp® Mini Kit (Qiagen, Valencia, CA). Briefly, the biopsy samples were lysed by incubating the sample in ATL lysis buffer with proteinase K over-night at 56 °C and following mechanical lysis with Fastprep® instrument (MP Biomedicals, Carlsbad, CA) for 1 min at the level of 6.0 m/s, purified with spin columns, and eluted with 400 μl of buffer AE. The DNA concentrations were determined with NanoDrop 1000 (Thermo Scientific, Wilmington, DE). The extracted DNA was stored at -20°C.

*The 16S rRNA gene pyrosequencing*
The bar-coded pyrosequencing method was used to profile duodenal microbiota. The V4-V6 region of the 16S rRNA gene was PCR amplified in three replicates using a bacterial primer pair (F515 5'-TGYCAGCMGCNGTGGCR-3'; 1061R 5'-TCACGRCACGAGCTGACG-3'). The V4-V6 variable region was chosen for this study based on pretesting results of PCR amplification. In the pretesting, the primers amplifying V4-V6 region showed significantly better amplification (PCR products analyzed by agarose gel electrophoresis) in comparison to the other tested primer pair for V1-V3. Nested PCR protocol would have been necessary for proper amplification with the primer pair for V1-V3 region, which would have caused more bias to the results due to larger number of amplification cycles. The PCR products were purified, quantified, and pooled in equal amounts and sequenced using a Genome sequencer FLX Titanium (Roche) in the Eurofins MWG (Ebersberg, Germany). The raw sequences were trimmed using Mothur v.1.31.2 (18). The sequences that had an averaged quality score of over 25 within a 50 bp window, length of over 250 bases, maximum 2 mismatches to barcode tags and the forward primer, no ambiguous bases, no homopolymers longer than 8 bp, and were non-chimeric according to the Uchime (Robert C. Edgar, http://drive5.com/uchime) were included in the analysis. Two samples with a low sequence gain were excluded from the further analysis. The high-quality sequences were aligned using Greengenes as the reference database (May 2013 release, http://greengenes.secondgenome.com/).

To avoid the bias of varying sequencing coverage in samples, 960 sequences were randomly subsampled from each sample using Mothur. This subsampled dataset (32,640 sequences) was used in the further analysis. The sequences were binned into operational taxonomic units (OTUs) applying a sequence similarity of over 97%. Additionally, the sequences were classified into bacterial taxa using the Wang approach in Mothur and SILVA, release 111, as a reference database (July 2012) (19), and threshold of certainty over 60%.
Statistics

In the clinical, serological, and histological analyses, quantitative data were expressed as medians with ranges or means with 95% confidence intervals. Normality of the variables was analyzed by Kolmogorov-Smirnov test and differences between mean values of the study groups were analyzed with independent sample T-test in normally distributed variables or with Mann-Whitney U test in non-parametric variables. Cross-tabulation with Pearson’s $\chi^2$ test or Fisher’s exact test was used to assess differences between categorical variables.

Inverse Simpson and Shannon microbial diversity indices, which reflect the number, abundance and evenness of species, and species richness (number of detected microbial species) were calculated using R version 3.0.2 (20) and its extension package vegan (21). Average microbial richness was compared by c2m randomization test with 9,999 permutations using R package rich (22). The difference in mean diversity and in the relative abundance of taxa between the study groups was assessed by ANOVA in R. Sequence coverage was estimated using a rarefaction analysis and Good’s coverage calculation in Mothur. Redundancy analysis (RDA) based on Hellinger transformed data and multidimensional scaling (MDS) based on Bray-Curtis distances were assessed in R and its extension package Vegan. ANOVA for RDA clustering was performed with a full model using 999 permutations in R. Weighted and Unweighted Unifrac (23) and hierarchical clustering applying a parsimony method with 1,000 replications was calculated as implemented in Mothur. To exclude the possible confounding effect of other gastrointestinal diseases, all the analyses were repeated with a dataset containing only samples of the patients (16
in No symptoms group and 9 in Persistent symptoms group), who did not report any other gastrointestinal disease than celiac disease.

Ethical considerations

The study protocol was approved by the Regional Ethics Committee of Tampere University Hospital District. All study participants gave written informed consent.

Results

Clinical data

Altogether 18 patients with persistent gastrointestinal symptoms and 18 with no current symptoms were selected for intestinal microbiota analyses. Microbiota profiling was successful for all samples except one sample from each study group, and the final results were thus available for 34 patients. There were no differences between the two groups in any demographic, clinical, serological, or histological parameters except that the patients with persistent symptoms were younger and had, by definition, higher GSRS scores than the patients with no symptoms (Table 1). The age difference was mainly caused by two young patients (27 and 31 years old) in the Persistent symptoms group. Celiac disease-associated genotypes were tested for 22 out of the 34 patients, all of whom had the HLA DQ2 or DQ8. In the Persistent symptoms group, eight patients reported to suffer from gastrointestinal disorders which were previously diagnosed by health care providers: four with gastro-esophageal reflux (GER), one with diverticulosis, two with lactose intolerance, and one with irritable bowel syndrome (IBS). One patient in the No symptoms group
had a previous diagnosis of GER. In each case the disease was considered mild by the patient, and there were no differences in the use of NSAIDs or PPIs between the groups (Table 1). None of the participants were found to have *H. pylori* infection. Controlled hypothyroidism was reported in four patients with persistent symptoms and three patients with no current symptoms. No other gastrointestinal or autoimmune diseases, e.g. motility disorder or diabetes, were reported by the patients or found in medical records.

*Mucosal microbiota composition*

The microbiota analysis indicated that the celiac disease patients with persistent symptoms on GFD had altered duodenal microbiota composition in comparison to the patients without symptoms. Microbiota compositional differences were assessed by several clustering methods, which all gave very congruent results at the genera level and mostly similar at the OTU level. According to an constrained clustering method, RDA, microbiota composition based on bacterial genera (p=0.01) or OTUs (p=0.03) differed significantly between the groups (Figure 1 and Supplementary Figure 1A). An unconstrained clustering method, MDS, also supported the detected differences between the study groups (Figure 2 and Supplementary Figure 1B). Similarly, the intestinal microbiota structure differed between the study groups by Weighted Unifrac based on genera (p<0.001) or OTUs (p<0.001). According to Unweighted Unifrac, the membership of duodenal bacterial genera (p=0.02), but not OTUs (p=0.25), varied statistically significantly between the study groups. There was a similar trend in hierarchical clustering based on the parsimony analysis of genera but this was not significant (p=0.07). Hierarchical clustering of OTUs was not statistically significant (p=0.71).
A further analysis of microbiota showed that the celiacs suffering from persistent symptoms had a lower relative abundance of Bacteroidetes (15% vs. 25%, p=0.01) and Firmicutes (33% vs. 46%, p=0.05) and a higher relative abundance of Proteobacteria (40% vs. 21%, p=0.04) compared with patients with no symptoms on GFD (Figure 3). The difference in microbiota composition was also detected at the genus level in altogether seven taxa, including the highly abundant genus *Prevotella* (Table 2). The most abundant bacterial genera in both study groups are presented in Supplementary Table 2.

The duodenal microbiota richness, measured as a number of detected genera or OTUs, was reduced in the patients with persistent symptoms on GFD. On average 32 genera and 72 OTUs per sample (in total 117 genera and 721 OTUs) were detected in patients in the Persistent symptoms group, whereas the No symptoms group had on average 37 genera and 106 OTUs per sample (in total 131 genera and 1016 OTUs). Further, the microbiota richness based on genera (p=0.05) or OTUs (p=0.007) was lower in symptomatic compared with non-symptomatic patients assessed by c2m randomization test (Figure 4 and Supplementary Figure 1C). Similarly, rarefaction curves based on OTUs and genera indicated lower richness in the patients with persistent symptoms (Supplementary Figures 1D and 2), although the difference was significant only for OTUs. No difference was detected on inverse Simpson or Shannon diversity indices, which take account both the richness (number of species) and the abundances and evenness of species. As the richness is part of diversity estimates, they usually correlate well. Thus, the lack significance in diversity between the groups indicates that the abundance of the dominating species in Persistent symptom
group was getting more event (their evenness was increasing) together with the decrease of richness. This suggests that the abundance of different species was affected differently in subjects with persistent symptoms. A decreasing rate of genera and OTUs at the end of the rarefaction curves (Supplementary Figures 1D and 2) and Good’s coverage for genera of over 97.8% in all samples demonstrated that the sequencing effort was adequate to capture most of the bacterial diversity.

To study the possible contribution of the additional gastrointestinal diseases on observed microbiota alterations, microbiota analyses were repeated by excluding the nine patients with a diagnosis of other gastrointestinal disease from the analyses. The results of this smaller dataset (n=25 including 9 patients from the Persistent symptoms and 16 patients from the No symptoms groups) were mostly in accordance with those seen in all 34 patients (Supplementary Figure 3). The relative abundances of Firmicutes and Bacteroidetes were lower and the relative abundance of Proteobacteria higher in the patients with persistent symptoms, although only the abundance of Bacteroidetes (p=0.03) was statistically significant. Also, similarly to the whole dataset, microbiota composition differed between the groups by RDA (p=0.06) (Supplementary Figures 3A and B) and by MDS, but not by Unifrac and parsimony analyses. Based on the OTUs, microbial richness was reduced in the Persistent symptoms group, even after exclusion of the patients with other gastrointestinal diseases (Supplementary Figure 3D). Richness based on genera (p=0.21) did not differ between the groups in analysis excluding celiac disease patients with an additional gastrointestinal disease (Supplementary Figure 3C).
Discussion

Aberrations in intestinal microbiota have been associated with a variety of gastrointestinal diseases, such as IBS (24, 25), inflammatory bowel disease (IBD) (26), and celiac disease (5-7). These associations demonstrate the importance of intestinal homeostasis for well-being and health. In the present study, we assessed the duodenal microbiota of the treated celiac disease patients and found that the patients with persistent symptoms had altered, Proteobacteria-rich intestinal microbiota composition and lower microbial richness compared with the patients whose symptoms disappeared on GFD. All patients, including those with persistent symptoms, had been committed to a strict GFD and showed restored small bowel mucosa and negative celiac disease auto-antibodies demonstrating good histological and serological response to the treatment with no signs of inadvertent gluten consumption. Normal villous morphology also rules out refractory celiac disease as a cause of the symptoms, since this condition is defined by persistent mucosal atrophy despite strict GFD (27). It must also be emphasized that all patients of the present study were voluntarily participating into a health survey, and did not visit a clinic for their symptoms. This indicates that many treated celiac disease patients are forced to accept persistent symptoms as an inevitable part of their daily life. Our findings suggest that altered duodenal microbiota composition and reduced microbial richness are associated with the persistent gastrointestinal symptoms occurring in some treated celiac disease patients. Previous results have shown that persistent gastrointestinal symptoms are common among adult celiac disease patients, even while on a strict GFD (4). Further, we have shown that the health-related quality of life is lower in celiac disease patients with persistent symptoms (2). Understanding the causes behind the persistent symptoms would provide a basis for the development of applications to alleviate the symptoms, subsequently also improving the quality of life of these patients.
Intestinal microbiota composition in healthy adults is relatively stable and can tolerate normal stress in the intestine caused by e.g. daily changes in diet. However, after strong perturbation, such as antibiotics therapy (28), microbiota may undergo an incomplete recovery, stabilizing into an altered state. This reformed but stable and possibly dysbiotic state of intestinal microbiota has been suggested to contribute to chronic diarrhea or inflammation (29). We propose that untreated celiac disease may disrupt a stable intestinal microbiota community which, in some patients, could then reform in a dysbiotic state, subsequently contributing to the persistent gastrointestinal symptoms.

In line with our hypothesis, treated celiac disease patients have been shown to differ in their intestinal microbiota from healthy subjects in pediatric studies (5-7), indicating that GFD may not completely restore microbiota in patients with major dysbiosis. Furthermore, in the present study, duodenal microbiota in the Persistent symptom group showed reduced microbial richness and Proteobacteria-rich microbiota, which both have been associated with intestinal dysbiosis, for example, in IBD (30,31, 32). In healthy subjects, Proteobacteria, a phylum containing many pathobionts and pathogens, usually comprise a minor fraction of small bowel microbiota (33, 34).

In the treated patients here with persistent symptoms, the Proteobacteria dominating, Bacteroidetes and Firmicutes–poor microbiota actually resembles the microbiota of untreated celiac patients with gastrointestinal symptoms (8), even if GFD seems to diminish the dysbiosis. The reduced richness and enrichment of Proteobacteria supports the hypothesis that microbiota in patients with persistent symptoms on GFD has not fully recovered, although the causality of this phenomenon in relation to the symptoms remains to be shown. Interestingly, we have recently shown that delayed diagnosis is associated with the occurrence of persistent symptoms in celiac disease (2).

Likewise, a long duration of gastroenteritis is a major risk factor for persistent intestinal symptoms.
in IBS. It has been suggested that prolonged initial illness causes more serious mucosal injury and inflammation, resulting in persistent changes to immunological cells and intestinal microbiota (35, 36). Accordingly, it is possible that also long-time untreated celiac disease enhances intestinal dysbiosis, thus predisposing to persistent symptoms even on GFD. Another interesting issue possibly affecting intestinal microbiota composition is the so-called host genetic gardening (37). Indeed, based also in our earlier results, which showed association of FUT2 with intestinal microbiota (38, 39), the effect of host genes would have been interesting to investigate in here also. However, the number of subjects was too small for reliable analyses (39) and this remains an issue for further studies.

Small intestinal bacterial overgrowth (SIBO) is one of the factors suggested as a cause of persistent symptoms in treated celiac disease patients (40, 41). SIBO is defined as \( \geq 1 \times 10^3 \) colony-forming units per ml of proximal jejunal aspiration (42) and characterized by an increase in colonic bacteria in the small bowel in general, rather than overgrowth of single bacterial strain (43, 44). Thus, SIBO could also be considered as a specific dysbiotic state of the small bowel. We did not perform diagnostics targeted at SIBO, such as culturing of jejunal aspirates or hydrogen breath test, which may be considered as a drawback (45). On the other hand, the interpretation of SIBO tests is very challenging and there is no consensus on the best diagnostic method (40, 46). The participants had no medical conditions predisposing to SIBO, such as previous abdominal surgery, diabetes or neurological disorder (46). The subjects with persistent symptoms were colonized with microbiota typical for the small bowel, that is Streptococcus and Veillonella spp. (9, 47), and the seven genera significantly differing between the groups (Table 2) did not include the colonic bacteria commonly detected in SIBO e.g. Escherichia, Enterococcus, Klebsiella and Proteus spp. (40). It is also
noteworthy that in previous studies exploring the role of SIBO in non-responsive celiac disease mostly patients with villous atrophy had been investigated, while all subjects in here had normal mucosal morphology on GFD (45). Therefore, it is unlikely that SIBO plays a major role in causing persistent symptoms in our study.

The celiac disease patients with persistent symptoms on GFD had previous symptom-based diagnoses of other mild gastrointestinal diseases, such as GER and lactose intolerance, more often than the patients with no symptoms on GFD. Nevertheless, there were no differences between the study groups in the small bowel mucosal morphology or degree of inflammation. Moreover, the separate microbiota analysis, when repeated without patients with additional gastrointestinal diseases, gave in most part similar differences in microbiota composition and richness. A diagnosis of IBS could also be considered in subjects with persistent symptoms in here. It can be difficult to distinguish symptoms of IBS from celiac disease as there are no pathognomonic signs or definite test for IBS. However, the patients attending the present study were considering themselves healthy and only one patient had previous diagnosis for IBS and had fulfilled the Rome III criteria. We consider it unlikely that significant part of the persistent symptoms would be explained by undiagnosed IBS. Another clinical explanation for the persistent symptoms might be disorders in gastrointestinal motility. Although this was not systematically investigated, none of the participants had signs of severe motility disorder or predisposing conditions such as diabetes. In theory, temporary acid suppression with PPI may cause false negative results in H. pylori testing, but we did not observe any differences in the use of PPI medication between the groups. Further, the relationship between uncomplicated H. pylori infection and recurrent abdominal symptoms is controversial (48). Some of the patients had thyroidal disease, which in theory could cause
intestinal symptoms but the prevalence of the condition did not differ between the groups and all of these patients received treatment for the disease. Thus, it is unlikely that the concomitant gastrointestinal maladies or other disorders would explain the detected differences in symptoms between the study groups.

The median age was significantly lower in the treated patients with persistent symptoms. Although ageing has been suggested to alter intestinal microbiota (49, 50), the microbiota is relatively stable throughout adulthood. A recent study showed that intestinal microbiota was rather similar in young adults and the aged, and in fact the major changes of microbiota in adulthood do not occur until after the age of 75-80 years (51). The difference in the median ages in our study (54 vs. 63 years) was caused mostly by the presence of two young patients in the Persistent symptoms group. The microbiota richness of these subjects varied from low (17 genera) to one of the highest (36 genera) among the patients with persistent symptoms. In addition, the two young patients did not differ from the other patients in MDS clustering (Figure 2). Thus, the age difference between the study groups is unlikely to have had a major effect on the findings. Long-term dietary habits and medications may also have an influence on intestinal microbiota (52). In the present study, all patients were on a strict GFD, and there was no difference between the groups in the consumption of oats, starch or fiber. In addition, the use of PPI and NSAID was comparable, and none of the patients had medications affecting bowel function. Nevertheless, we had no long-term information on the history of antibiotic use, and their effect on the results cannot be fully excluded. According to our study design only subjects with no symptoms and most symptomatic were investigated and thus it is possible that the results would have been different if patients with subtle symptoms had been included. Further, to minimize confounding factors, only patients with gastrointestinal
symptoms were included, since we have previously shown that microbial composition varies between celiac disease phenotypes (8). It is possible that symptoms fluctuate by time, but the present symptomatic patients had a history of long-lasting complaints. It is also true that the present study was not able to show causality or distinguish the effects of different bacteria to the persistent symptoms, and further studies are needed in this area.

Our hypothesis that dysbiotic microbiota is associated with persistent symptoms in treated celiac disease patients raises an intriguing question on their treatment. One possibility could be the intestinal microbiota modulation by probiotics. Although probiotic intervention studies have reported symptom alleviation in IBS, IBD, and celiac disease, it still remains to be shown whether probiotics modify dysbiotic microbiota towards a stable and disease-free state (53). Additional possibility for the modification of intestinal microbiota is fecal transplantation, which has been successful in treating recurrent *C. difficile* infection (54). The pioneering studies on the use of fecal transplantation as treatment for a variety of gastroenterological diseases, including Crohn's disease and IBS, have been encouraging (55).

In conclusion, our results showed that celiac disease patients suffering from persistent symptoms on GFD had altered Proteobacteria-rich duodenal microbiota and reduced richness of bacteria, indicating intestinal dysbiosis. We propose that this altered microbiota is associated with persistent symptoms in celiac patients with strict GFD and small-bowel mucosal recovery. Further studies are warranted to confirm our results and possibly to find applications for alleviating the symptoms of this specific patient subgroup by intestinal microbiota modulation.
Conflict of interest/Study support:

- Guarantor of the article: Kalle Kurppa
- Specific author contributions: Wacklin: Design of the study, acquisition, analysis and interpretation of the data, and writing of the article. Laurikka: Analysis and interpretation of the data, drafting of the article, revision of the article critically for important intellectual content. Lindfors: Interpretation of the data, drafting of the article, revision of the article critically for important intellectual content. Collin, Salmi and Lähdeaho: Recruitment of patients and sample collection, revision of the article critically for important intellectual content. Saavalainen: Acquisition and analysis of the genomic data, revision of the article critically for important intellectual content. Mäki: Acquisition of the data, revision of the article critically for important intellectual content. Mätö: Acquisition of the data, revision of the article critically for important intellectual content. Kurppa: Sample collection, acquisition and interpretation of the data, drafting the article, revision of the article critically for important intellectual content. Kaukinen: Conception and design of the study, sample collection, acquisition, analysis and interpretation of the data, revision of the article critically for important intellectual content. All the authors approved the final draft of submitted manuscript.
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Savolainen’s fund allocated for the development of cancer treatment, the Finnish Medical Foundation, the Foundation for Pediatric Research, and the Finnish Coeliac Society.

- Potential competing interests: None.
References


Table 1. Comparison of clinical and histological data of the 34 treated celiac disease patients divided into Persistent symptoms and No symptoms groups by GSRS total score. Numeric variables, excluding age and the duration of the gluten-free diet (GFD), are expressed as means with 95% confidence interval.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Persistent symptoms on a GFD, n=17</th>
<th>No symptoms on a GFD, n=17</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSRS total score</td>
<td>2.8 (2.6-3.0)</td>
<td>1.3 (1.2-1.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, median (range), yr</td>
<td>54 (27-72)</td>
<td>63 (42-75)</td>
<td>0.010</td>
</tr>
<tr>
<td>Females, %</td>
<td>94</td>
<td>71</td>
<td>0.175</td>
</tr>
<tr>
<td>Duration of GFD, median (range), yr</td>
<td>10 (3-23)</td>
<td>8 (4-35)</td>
<td>0.433</td>
</tr>
<tr>
<td>Celiac disease in family, %</td>
<td>29</td>
<td>53</td>
<td>0.163</td>
</tr>
<tr>
<td>Use of NSAIDs, %</td>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
<td>1.000</td>
</tr>
<tr>
<td>Use of PPIs, %</td>
<td>19</td>
<td>13</td>
<td>1.000</td>
</tr>
<tr>
<td>Oats in diet, %</td>
<td>87</td>
<td>76</td>
<td>0.659</td>
</tr>
<tr>
<td>Wheat starch in diet, %</td>
<td>86</td>
<td>100</td>
<td>0.224</td>
</tr>
<tr>
<td>Use of fiber, g/day</td>
<td>15.7 (13.4-18.0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.6 (14.6-24.5)</td>
<td>0.144</td>
</tr>
<tr>
<td>Blood hemoglobin, g/l</td>
<td>138 (133-143)</td>
<td>141 (133-149)</td>
<td>0.546</td>
</tr>
<tr>
<td>Serum total iron, μmol/l</td>
<td>16.5 (13.8-19.2)</td>
<td>17.8 (15.9-19.8)</td>
<td>0.399</td>
</tr>
<tr>
<td>Erythrocyte folate, nmol/l</td>
<td>559 (448-670)</td>
<td>600 (470-729)</td>
<td>0.533</td>
</tr>
<tr>
<td>Body mass index, kg/ m²</td>
<td>25.3 (23.5-27.2)</td>
<td>26.2 (24.8-27.5)</td>
<td>0.442</td>
</tr>
<tr>
<td></td>
<td>Value</td>
<td>Value</td>
<td>p-value</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>Positive EmA, %</td>
<td>0</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>TG2ab, units/ml</td>
<td>0.3 (0.0-0.6)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.8 (0.1-1.4)</td>
<td>0.318</td>
</tr>
<tr>
<td>Vh/CrD</td>
<td>3.2 (2.9-3.4)</td>
<td>3.0 (2.8-3.2)</td>
<td>0.336</td>
</tr>
<tr>
<td>CD3+ IEL, cell/mm</td>
<td>54 (44-64)</td>
<td>53 (37-70)</td>
<td>0.969</td>
</tr>
<tr>
<td>γδ+ IEL, cell/mm</td>
<td>16.6 (10.4-22.7)</td>
<td>14.8 (9.0-20.5)</td>
<td>0.655</td>
</tr>
</tbody>
</table>

NSAID, non-steroid anti-inflammatory drugs; PPI, protein pump inhibitor; EmA, serum endomysium antibodies; TG2ab, serum transglutaminase antibodies; Vh/CrD, small bowel mucosal villous height crypt depth ratio; IEL, intraepithelial lymphocytes; GSRS, Gastrointestinal Symptom Rating Scale. Data available in each variable ≥ 80% of the subjects except in <sup>b</sup>9 subjects and <sup>c</sup>13 subjects. <sup>a</sup>Two-tailed T-test was used in normally distributed numeric variables and Mann-Whitney U test in non-parametric variables. Pearson’s χ<sup>2</sup> test or Fisher’s exact test was used in categorical variables.
Table 2. Average abundance of bacterial taxa significantly differing in celiac disease patients with and without persistent symptoms on a strict gluten-free diet (GFD).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>No of sequences</th>
<th>Persistent symptoms on a GFD</th>
<th>No symptoms on a GFD</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prevotella</em></td>
<td>4887</td>
<td>10.56 %</td>
<td>19.38 %</td>
<td>0.02</td>
</tr>
<tr>
<td>Uncl. <em>Lactobacillales</em></td>
<td>164</td>
<td>0.28 %</td>
<td>0.72 %</td>
<td>0.01</td>
</tr>
<tr>
<td>Uncl. <em>Lachnospiraceae</em></td>
<td>100</td>
<td>0.12 %</td>
<td>0.50 %</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Megasphaera</em></td>
<td>48</td>
<td>0.07 %</td>
<td>0.22 %</td>
<td>0.05</td>
</tr>
<tr>
<td>Uncl. <em>Veillonellaceae</em></td>
<td>37</td>
<td>0.05 %</td>
<td>0.18 %</td>
<td>0.01</td>
</tr>
<tr>
<td><em>Bergeriella</em></td>
<td>29</td>
<td>0.04 %</td>
<td>0.13 %</td>
<td>0.05</td>
</tr>
<tr>
<td>Uncl. <em>Firmicutes</em></td>
<td>29</td>
<td>0.05 %</td>
<td>0.13 %</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*Uncl.*, unclassified. <sup>a</sup>P-values are indicated for ANOVA.
Figures:

Figure 1. Redundancy analysis (RDA) plot indicating alterations of duodenal microbiota composition in the celiac disease patients with persistent symptoms (closed circles) in comparison to the patients without symptoms on gluten-free diet (open circles). RDA was based on observed genera. Triangles indicate centroids of the study groups.
Figure 2. Multidimensional scaling (MDS) plot indicating differences on duodenal microbiota composition in the gluten-free diet treated celiac disease patients with persistent symptoms (closed symbols) and the treated patients without symptoms (open symbols). The celiac disease patients with an additional gastrointestinal disease are indicated by squares and the patients without other gastrointestinal symptoms are indicated by circles. The arrows indicate the samples of two youngest patients (see text for details). MDS was based on observed genera.
Figure 3. The differences in average abundances of bacterial phyla in the gluten-free diet treated celiac disease patients suffering persistent symptoms and the treated patients without symptoms.

Figure 4. The reduced richness of duodenal bacterial genera in the gluten-free diet treated celiac patients suffering persistent symptoms in comparison to the treated patients without symptoms.
Supplementary Table 1. Most abundant bacterial taxa in the celiac disease patients with persistent symptoms and without symptoms on GFD. The taxa, which had abundance over 3% in either group are presented.

<table>
<thead>
<tr>
<th>Taxa</th>
<th>No of sequences</th>
<th>Persistent symptoms on a GFD</th>
<th>No symptoms on a GFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus</td>
<td>3014</td>
<td>18 %</td>
<td>27 %</td>
</tr>
<tr>
<td>Prevotella</td>
<td>1724</td>
<td>11 %</td>
<td>19 %</td>
</tr>
<tr>
<td>Phyllobacterium</td>
<td>1664</td>
<td>10 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Veillonella</td>
<td>1112</td>
<td>7 %</td>
<td>10 %</td>
</tr>
<tr>
<td>Micrococcineae</td>
<td>1028</td>
<td>6 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Herbaspirillum</td>
<td>985</td>
<td>6 %</td>
<td>1 %</td>
</tr>
<tr>
<td>Haemophilus</td>
<td>705</td>
<td>4 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Variovorax</td>
<td>678</td>
<td>4 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Neisseria</td>
<td>645</td>
<td>4 %</td>
<td>5 %</td>
</tr>
<tr>
<td>Gemella</td>
<td>590</td>
<td>4 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>539</td>
<td>3 %</td>
<td>2 %</td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>435</td>
<td>3 %</td>
<td>3 %</td>
</tr>
<tr>
<td>other</td>
<td>3201</td>
<td>20 %</td>
<td>16 %</td>
</tr>
</tbody>
</table>

Supplementary Table 2. Comparison of Gastrointestinal Symptom Rating Scale (GSRS) sub-dimension scores of the celiac disease patients with or without persistent symptoms on a strict gluten-free diet (GFD). Variables are expressed as means with 95% confidence interval.

<table>
<thead>
<tr>
<th></th>
<th>Persistent symptoms on a GFD, n=17</th>
<th>No symptoms on a GFD, n=17</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigestion</td>
<td>3.3 (3.0-3.6)</td>
<td>1.5 (1.3-1.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>2.1 (1.6-2.6)</td>
<td>1.1 (1.0-1.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Constipation</td>
<td>3.5 (2.7-4.4)</td>
<td>1.0 (1.0-1.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pain</td>
<td>2.5 (2.1-2.8)</td>
<td>1.4 (1.2-1.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Reflux</td>
<td>2.1 (1.6-2.7)</td>
<td>1.3 (1.1-1.6)</td>
<td>0.014</td>
</tr>
<tr>
<td>GSRS total score</td>
<td>2.8 (2.6-3.0)</td>
<td>1.3 (1.2-1.4)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Supplementary Figure 1. Composition and richness analyses of duodenal microbiota based on operational taxonomic units (OTU). Sequences sharing similarity over 97% were clustered into same OTU. A) RDA plot indicating significant differences on microbiota composition between the treated celiac patients with persistent symptoms (closed symbols) and without symptoms (open symbols). Triangles show centroids of the study groups. B) MDS plot indicating alterations of microbiota composition in the treated patients with (closed symbols) and without (open symbols) persistent symptoms. The celiac disease patients having additional gastrointestinal disease are indicates by squares and the patients without other gastrointestinal symptoms by circles. The arrows indicate the samples of two youngest patients (see text for details). C) The reduced richness of OTUs in the treated celiac patients suffering persistent symptoms in comparison to the treated patients without symptoms. D) Rarefaction curves indicating differences on OTU richness between the celiac disease patients with
persistent symptoms (red) and without symptoms on GFD (blue). The bold lines indicate average rarefaction curve for the groups and the thin lines indicate 95% confidence intervals.

Supplementary Figure 2. Rarefaction curves indicating differences on richness of duodenal bacterial genera between the celiac disease patients with persistent symptoms (red) and without symptoms on GFD (blue). The bold lines indicate average rarefaction curve for the groups and the thin lines indicate 95% confidence intervals.
Supplementary Figure 3. Composition and richness analyses of duodenal microbiota performed with only the patients, which did not have other gastrointestinal disease in addition to celiac disease. RDA plots based on genera (A) and OTUs (B) indicate significant differences in duodenal microbiota composition in the celiac disease patients suffering persistent symptoms on GFD (closed circles) in comparison to that in the patients without symptoms in GFD (open circles). Triangles show centroids of the study groups. Richness of bacterial genera (C) did not differ between the patients with and without persistent symptoms on GFD. Richness of OTUs (D) was significantly reduced in the patients with persistent symptoms.