TUIRE ILUS

Non-Responsive Coeliac Disease

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ACADEMIC DISSERTATION
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University of Tampere, School of Medicine
Tampere University Hospital,Department of Gastroenterology and
Alimentary Tract Surgery
Finland

Supervised by
Professor Katri Kaukinen
University of Tampere
Finland
Docent Pekka Collin
University of Tampere
Finland

Reviewed by
Docent Perttu Arkkila
University of Helsinki
Finland
Professor Markku Voutilainen
University of Turku
Finland

Cover design by
Mikko Reinikka

Distributor:
kirjamyynti@juvenes.fi
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Medicine is a science of uncertainty and an art of probability.

-Sir William Osler

ABSTRACT

According to the recent literature, complete histological normalization of the small-intestinal mucosa is achieved in approximately only one fifth of coeliac patients after initiation of a gluten-free diet (GFD). Even while villous atrophy resolves, intraepithelial lymphocytosis may persist for years indicating some degree of ongoing small-intestinal mucosal inflammation. Some patients may develop refractory coeliac disease (RCD), defined by persistent or recurrent villous atrophy and continuous symptoms and signs of malabsorption despite a strict GFD for a minimum of 6-12 months in the absence of other causes of non-responsive coeliac disease and overt malignancy. RCD involves a risk of severe complications, enteropathy-associated T cell lymphoma (EATL) being the most serious. Apart from EATL, an increased risk of other malignancies of the gastrointestinal tract has been documented in coeliac disease, but in contrast, the risk of breast and lung cancer has been decreased. The incidence of overall malignancy in coeliac disease varies from equal to slightly increased compared to that of general population.

This dissertation comprises three studies. In Study I, small-intestinal mucosal recovery was assessed in 177 volunteer coeliac patients adhering to a strict GFD. This study concentrated on factors contributing to a persistent small-intestinal inflammation in coeliac disease and its clinical relevance. In Study II, the prevalence of RCD was investigated in 11 hospital districts in Finland, where the number of diagnosed coeliac patients and adult inhabitants was known. Aiming at the identification of risk factors for forthcoming RCD, clinical characteristics present at the primary diagnosis of coeliac disease were compared to those of 866 controls with uncomplicated coeliac disease. Study III evaluated the risk of malignancies in a large, population-based cohort including 32,439 adult biopsy-proven coeliac patients. This cohort was obtained from the Social Insurance Institution of Finland, which has kept a nationwide register of coeliac patients eligible for reimbursement of dietary costs since October 2002. This register was collated with the Finnish Cancer Registry, which covers over 98% of diagnosed malignancies. The risk for the most common malignancies was estimated by comparing observed cancer cases in coeliac patients to those expected, based on incidence figures of the whole population.

Study I revealed that only 4% of long-term treated coeliac patients evinced villous atrophy. Among patients with improved villous atrophy, 56% had persistent intraepithelial lymphocytosis and 44% had completely normal small-intestinal mucosa. Consumption of oats was the only factor found to contribute to the persistent inflammation of the small-intestinal mucosa when over 30 intraepithelial lymphocytes (IELs) per 100 enterocytes were regarded as increased. With a lower cut-off value of 25 IELs per 100 enterocytes, consumption of oats was no longer associated with ongoing inflammation of the small-intestinal mucosa. Comorbidities, such as *H. pylori* gastritis; drugs or wheat starch-containing diet had no effect on the ongoing inflammation. The clinical outcome of patients with persistent small-intestinal inflammation was not different from those with completely healed mucosa in any measured aspect: signs of malabsorption, incidence of malignancies, gastrointestinal symptoms and quality of life. This implies that the prognosis of patients with persistent intraepithelial lymphocytosis is good, and the association with the consumption of oats may have been coincidental.

Study II revealed that the prevalence of RCD was 0.31% among patients with diagnosed coeliac disease and 0.002% in Finnish general population. Comparison of 44 enrolled patients with RCD to 886 controls with uncomplicated disease revealed that at the primary diagnosis of coeliac disease the RCD patients were significantly older, more often males and coeliac antibody-negative; and had more severe symptoms. All these factors may be associated with a long diagnostic delay of coeliac disease and constitute a risk for future RCD.

In Study III, the risk of any malignancy in clinically diagnosed coeliac disease was decreased, mainly due to the decreased risk of breast and lung cancer. However, the risks of small-intestinal cancer and non-Hodgkin's lymphoma (including EATL) was increased, but to a lesser extent than reported in earlier studies.

This dissertation demonstrated that histological non-responsiveness, RCD and malignancies are rare in clinically diagnosed coeliac disease patients. Persistent villous atrophy, RCD and malignancies are reportedly much more prevalent in countries with a lower prevalence of clinically diagnosed coeliac disease than Finland. Therefore active screening for coeliac disease in risk groups leading to early commencement of a GFD and appointed duodenal control biopsies taken to confirm mucosal recovery on GFD; both well-established policies in the Finnish health-care system, may contribute to a good clinical outcome in coeliac disease.

TIIVISTELMÄ

Kirjallisuuden mukaan ohutsuolen limakalvon histologia normaalistuu täysin vain noin viidesosalla keliaakikoista gluteenittoman dieetin myötä. Vaikka villusatrofia korjaantuukin, intraepiteliaaliset lymfosyytit (IEL:t) voivat jäädä koholle vuosiksi viitaten jonkinasteisen inflammaation jatkumiseen ohutsuolen limakalvolla. Osalle potilaista voi kehittyä refraktaarikeliakia, joka määritellään jatkuvaksi tai uusiutuvaksi villusatrofiaksi sekä jatkuvan malabsorption oireiksi ja löydöksiksi 6-12 kuukauden tiukasta gluteenittomasta dieetistä huolimatta, kun muut hoidolle reagoimattoman taudin syyt ja syöpätaudit on poissuljettu. Refraktaarikeliakiaan liittyy riski saada vakavia komplikaatioita, joista vaikein on enteropatiaan assosioituva T-solulymfooma (EATL). Myös muiden ruuansulatuskanavan syöpien riskin on todettu suurentuneen keliakiassa, kun taas rinta- ja keuhkosyövän riskin on todettu pienentyneen. Syöpätautien riskin yleensä on keliakiassa todettu olevan joko muun väestön tasolla tai hieman suurentuneen.

Tämä väitöskirja koostuu kolmesta osatyöstä. Osatyössä I tutkittiin ohutsuolen limakalvovaurion korjaantumista 177 vapaaehtoisella tiukkaa gluteenitonta dieettiä noudattaneella keliaakikolla. Tutkimuksessa keskityttiin löytämään syitä jatkuvalle tulehdusreaktiolle ohutsuolen limakalvolla ja arvioimaan sen kliinistä merkitystä. Tutkimuksessa II selvitettiin refraktaarikeliakian esiintyvyyttä 11 Suomen sairaanhoitopiirissä, joissa kliinisesti todettujen keliaakikkojen ja aikuisväestön määrä oli tiedossa. Jotta löydettäisiin kehittyvälle refraktaarikeliakialle altistavia tekijöitä jo siinä vaiheessa kun varsinainen keliakia todetaan, vertailtiin taudin kliinisiä piirteitä 866:een komplisoitumatonta keliakiaa sairastavaan verrokkiin. Tutkimuksessa III arvioitiin syöpäriskiä suuressa väestöpohjaisessa kohortissa, johon kuului 32 439 ohutsuolikoepalalla varmistettua aikuista keliakiapotilasta. Kohortti kerättiin Kansaneläkelaitoksen erityiskorvausrekisteristä, johon on liitetty lokakuusta 2002 lähtien kaikki ruokavaliokorvaukseen oikeutetut keliaakikot. Tämä rekisteri yhdistettiin Suomen Syöpärekisteriin, joka kattaa yli 98 % todetuista syövistä. Tärkeimpien syöpäsairauksien riskiä tutkittiin vertaamalla keliaakikoilla todettujen syöpätapauksien määrää odotettuihin väestöpohjaisiin ilmaantuvuuslukuihin.

Tutkimus I paljasti, että vain 4 %:lla pitkään gluteenittomalla dieetillä olleista keliaakikoista esiintyi jatkuvaa villusatrofiaa. Potilaista, joiden villusatrofia oli

korjaantunut, 56 %:lla esiintyi jatkuvaa intraepiteliaalista lymfosytoosia ja 44 %:lla limakalvo oli korjaantunut täysin. Kauran käyttö osoittautui ainoaksi ohutsuolen jatkuvalle tulehdusreaktiolle altistavaksi tekijäksi, kun lisääntyneen intraepiteliaalisen lymfosytoosin raja-arvona pidettiin 30 IEL:ä 100 enterosyyttiä kohti. Kun raja-arvoksi valittiin 25 IEL:ä 100 enterosyyttiä kohti, kauran käyttö ei assosioitunutkaan enää jatkuvaan ohutsuolen tulehdusreaktioon. Liitännäissairauksilla, kuten *H. pylorin* aiheuttamalla gastriitilla; lääkkeiden käytöllä tai vehnätärkkelystä sisältävällä ruokavaliolla ei ollut merkitystä. Potilaiden kliiniseen tilanteeseen ei vaikuttanut se, oliko ohutsuolen limakalvo korjaantunut täysin vai jatkuiko tulehdusreaktio limakalvolla kun asiaa tarkasteltiin malabsorption merkkien, syöpien ilmaantuvuuden, ruuansulatuskanavan oireiden tai elämänlaadun kannalta. Tämän perusteella potilaiden, joilla jatkuvaa intraepiteliaalista lymfosytoosia ohutsuolen limakalvolle todetaan, ennuste on hyvä, ja yhteys kauran käyttöön saattoi olla sattumaa.

Tutkimuksessa II todettiin refraktaarikeliakian esiintyvyydeksi 0.31 % kliinisesti diagnosoiduilla keliaakikoilla ja 0.002 % suomalaisessa väestössä yleensä. Kun verrattiin 44 tutkimukseen otettua refraktaarikeliaakikkoa 886 komplisoitumatonta keliakiaa sairastavaan verrokkiin, todettiin että refraktaarikeliaakikot olivat merkittävästi iäkkäämpiä, useammin miehiä ja keliakiavasta-aineiden suhteen negatiivisia, ja heillä oli vaikeampia oireita jo varsinaisen keliakian toteamisen yhteydessä. Kaikki nämä tekijät saattavat viitata pitkään diagnostiseen viiveeseen ja ovat riskitekijöitä kehittyvän refraktaarikeliakian suhteen.

Tutkimuksessa III todettiin kokonaissyöpäriskin olevan diagnosoiduilla keliaakikoilla vähäisemmän kuin suomalaisessa väestössä yleensä, johtuen lähinnä selkeästi alentuneesta rinta- ja keuhkosyövän riskistä. Kuitenkin ohutsuolen syövän ja non-Hodgkinin lymfooman (sisältäen EATL:n) riski oli lisääntynyt, mutta huomattavasti vähäisemmässä määrin kuin aikaisemmissa tutkimuksissa on todettu.

Tämä väitöskirjatutkimus osoitti, että kliinisesti diagnosoiduilla keliaakikoilla limakalvovaurion korjaantumattomuus, refraktaarikeliakia ja syöpätaudit ovat harvinaisia. Koska jatkuvan villusatrofian, refraktaarikeliakian sekä syöpätautien ilmaantuvuus on tutkimusten perusteella huomattavasti yleisempää maissa, joissa kliinisesti diagnosoidun keliakian esiintyvyys on alhaisempi kuin Suomessa, on mahdollista että Suomessa hyvin käytäntöön omaksuttu keliakian seulontamalli riskiryhmistä ja hoitovasteen kontrollointi uudella ohutsuolikoepalalla saattavat edesauttaa hyvän hoitotuloksen saavuttamista keliakiassa.

Contents

ABBREVIATIONS

AGA	antigliadin antibodies
AIDS	acquired immune deficiency syndrome
AN-PEP	propyl endopeptidase from *Aspergillus niger*
APC	antigen presenting cell
ARA	antireticulin antibodies
ASCT	autologic stem cell transplantation
BMI	body mass index
CD	cluster of differentiation
CI	confidence interval
CT	computed tomography
DH	dermatitis herpetiformis
DP-AGA	deamidated gliadin peptide antibodies
EATL	enteropathy-associated T cell lymphoma
ELISA	enzyme-linked immunosorbent assay
EMA	endomysium antibodies
ESPG(H)AN	European Society of Pediatric Gastroenterology, (Hepatology), and Nutrition
^{18}F-FDG-PET	^{18}F-fluorodeoxyglucose positron emission tomography
GFD	gluten-free diet
GSRS	Gastrointestinal Symptom Rating Scale
Hb	haemoglobin
HLA	human leukocyte antigen
IEL	intraepithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MCV	mean corpuscular volume
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
MYO9B	myosin gene IX B
NHL	non-Hodgkin's lymphoma
NK	natural killer
NSAID	non-steroidal anti-inflammatory drug
OR	odds ratio
PGWB	Psychological General Well-Being
PPI	proton pump inhibitor
RCD	refractory coeliac disease

RR	risk ratio
SC-PEP	propyl endopeptidase from *Sphingomonas capsulata*
SII	Social Insurance Institution
SIR	standardized incidence ratio
TCR	T cell receptor
TG	transglutaminase
TG2-ab	transglutaminase 2 antibodies
TGF	transforming growth factor
TNF	tumour necrosis factor
UK	United Kingdom
USA	United States of America
Vh/CrD	villous height/crypt depth ratio

LIST OF ORIGINAL PUBLICATIONS

The dissertation is based on the following original publications, referred to in the text by the Roman numerals I-III:

I Ilus T, Lähdeaho M-L, Salmi T, Haimila K, Partanen J, Saavalainen P, Huhtala H, Mäki M, Collin P and Kaukinen K (2012): Persistent duodenal intraepithelial lymphocytosis despite a long-term strict gluten-free diet in coeliac disease. Am J Gastroenterol 107;1563-9.

II Ilus T, Kaukinen K, Virta LJ, Huhtala H, Mäki M, Kurppa K, Heikkinen M, Heikura M, Hirsi E, Jantunen K, Moilanen V, Nielsen C, Puhto M, Pölkki H, Vihriälä I and Collin P (2014): Refractory coeliac disease in a country with a high prevalence of clinically diagnosed coeliac disease. Aliment Pharmacol Ther 39:418-25.

III Ilus T, Kaukinen K, Virta LJ, Pukkala E and Collin P (2014): Incidence of malignancies in diagnosed coeliac patients. A population-based estimate. Am J Gastroenterol, doi 10.1038/ajg2014.194

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INTRODUCTION

Coeliac disease is a chronic autoimmune disorder triggered by ingestion of gluten in genetically predisposed persons (Green and Cellier 2007). The characteristic histological lesion on small-intestinal mucosa gradually develops from increased intraepithelial lymphocytosis to villous atrophy and crypth hyperplasia (Marsh 1990). Elimination of gluten from the diet usually leads to recovery of the villous structure, but intraepithelial lymphocytosis may persist for years (Kutlu et al. 1993, Järvinen et al. 2003, Koskinen et al. 2010, Lähdeaho et al. 2011) and complete mucosal recovery is achieved in only 8-20% of adults (Ciacci et al. 2002, Wahab et al. 2002, Lee et al. 2003, Tursi et al. 2006, Bardella et al. 2007, Lanzini et al. 2009, Hutchinson et al. 2010, Sharkey et al. 2013). The most common reason for non-responsive coeliac disease is ongoing gluten ingestion, which is often inadvertent (Abdulkarim et al. 2002, Leffler et al. 2007b, Dewar et al. 2012, Sharkey et al. 2013). Nevertheless, in Finland about 90% of coeliac patients maintain a strict diet, labelling of gluten-free products is strictly controlled by the legislation and public awareness of the disease is good; all implying that dietary regressions are rare (Kurppa et al. 2012). Persistent villous atrophy, even without symptoms, is not a harmless condition, but is associated with increased risk of osteoporosis and malignancies, including non-Hodgkin´s lymphoma (NHL) (Kaukinen et al. 2007b, Lebwohl et al. 2013b). However, the clinical relevance of persisting intraepithelial lymphocytosis with recovered villous atrophy has remained obscure. It is known that small-intestinal mucosal lymphocytosis is an unspecific finding, and apart from coeliac disease, may be caused by other autoimmune diseases, drugs and *Helicobacter pylori* gastritis (Mahadeva et al. 2002, Kakar et al. 2003, Memeo et al. 2005, Aziz et al. 2010). The precipitating factors of persistent intraepithelial lymphocytosis in coeliac disease are unknown.

Refractory coeliac disease (RCD) should be considered when there are continuous symptoms and signs of malabsorption in addition to persistent villous atrophy despite a 6-12 months' strict gluten-free diet (GFD) and other reasons for non-responsive disease and overt malignancies have been excluded (Biagi and Corazza 2001, Rubio-Tapia and Murray 2010, Malamut et al. 2012). RCD can further be classified as type I when the phenotype of intraepithelial lymphocytes (IELs) is normal and indistinguishable from active coeliac disease, and type II when abnormal, monoclonal IELs have emerged from immature lymphatic precursor cells, and are characterized by loss of normal surface markers and a gain of monoclonal T cell receptor rearrangements (Cellier et al. 1998, Rubio-Tapia and Murray 2010, Malamut et al. 2012). While type I RCD usually follows a relatively benign course; severe complications such as enteropathy-associated T cell

lymphoma (EATL) frequently occurs in type II (Cellier et al. 2000, Al-toma et al. 2007a, Daum et al. 2009, Malamut et al. 2009, Rubio-Tapia et al. 2009b). Although persistent villous atrophy is common in treated coeliac disease, genuine RCD affects probably 1.5-10% of diagnosed coeliac patients (Wahab et al. 2002, West 2009, Rubio-Tapia and Murray 2010, Roshan et al. 2011, Arguelles-Grande et al. 2013). Nevertheless, the exact prevalence of RCD is unknown as the current literature involves only tertiary centres.

Increased risk of EATL in coeliac patients has been acknowledged since 1960s (Gough et al. 1962), and increased risk of other malignancies of the gastrointestinal tract has also been verified in earlier studies (Askling et al. 2002, Green et al. 2003, Silano et al. 2007, Elfström et al. 2012). Conversely, a decreased risk of breast and lung cancer has been reported (Askling et al. 2002, Card et al. 2004, West et al. 2004, Silano et al. 2007, Grainge et al. 2012, Ludvigsson et al. 2012c). Although the overall risk of cancer and the risk of NHL have decreased over time, in a recent study these were still elevated to 1.4-fold and 12-fold respectively (Grainge et al. 2012). Many studies estimating the risk of cancer in coeliac disease have been conducted in countries where the prevalence of clinically detected coeliac disease is low, implying that many patients with mild symptoms have probably not been diagnosed, and not contributed to the cancer risk estimates with a diluting effect (West et al. 2003, Lohi et al. 2007, Kane et al. 2011, Rubio-Tapia et al. 2012). In Finland a high prevalence of clinically diagnosed coeliac disease (0.6%) has been reached by active screening of risk groups and individuals with mild or atypical symptoms (Collin et al. 2007, Virta et al. 2009). A study conducted in this country, covering a wider spectrum of coeliac disease, should give a more realistic projection of the actual risk of cancer in coeliac disease.

This dissertation aimed to estimate the prevalence of the whole spectrum of non-responsive coeliac disease from persistent intraepithelial lymphocytosis and villous atrophy to RCD; and estimate the risk of malignancies from the perspective of high prevalence of clinically diagnosed coeliac disease and good dietary compliance. The specific aims were to identify the predisposing factors and clinical relevance of persistent intraepithelial lymphocytosis, to describe the clinical features of RCD in Finland and to identify risk factors present at the primary coeliac disease diagnosis for evolving RCD.

1. Definition of coeliac disease

Coeliac disease is a chronic inflammatory disorder that develops in genetically susceptible individuals following the ingestion of dietary gluten. Genetic susceptibility is associated with major histocompatibility complex (MHC) class II molecules human leukocyte antigen (HLA) DQ2 and DQ8. The disorder gradually develops from increased intraepithelial lymphocytosis to crypth hyperplasia and finally villous atrophy in the small-intestinal mucosa, but affects other organ systems as well. Immunotoxic gluten peptides are mainly derived from dietary gliadin in wheat, but homologous sequences exist in rye secalins and barley hordeins. (Green and Cellier 2007, Husby et al. 2012, Ludvigsson et al. 2013b).

Dermatitis herpetiformis (DH) is an extraintestinal manifestation of coeliac disease, characterized by itching and blistering rash on the elbows, knees and buttocks (Collin and Reunala 2003). Although gastrointestinal symptoms may be absent, most patients with DH evince villous atrophy or marked intraepithelial lymphocytosis in their small-intestinal mucosa (Gawkrodger et al. 1984, Reunala et al. 1984, Savilahti et al. 1992).

2. Pathogenesis of coeliac disease

Dietary gluten consists of α-, γ- and ω-gliadins and glutenins. Gluten peptides contain 30% glutamine and 15% proline and the high proline content renders it resistant to degradation by gastrointestinal enzymes (Shan et al. 2002). In active coeliac disease the permeability of the small-intestinal mucosa is increased as a consequence of impaired function of tight junctions regulated by zonulin enabling paracellular entry of large immunogenic peptides into the mucosa (Fasano et al. 2000, Drago et al. 2006). Gluten transport also occurs via epithelial transcytosis by binding of the peptides with secretory immunoglobulin A (IgA) to the transferrin receptor cluster of differentiation (CD) 71 (Matysiak-Budnik et al. 2008).

There is a variety of intraepithelial lymphocytes (IELs) in the small-intestinal mucosa, most of them being CD8+ T cell receptor (TCR) αβ+ T cells. Most of these TCR+ cells also express natural killer (NK) cell receptors which act by lowering the threshold for T cell activation at stressful times (Bauer et al. 1999). The α-gliadin p31-43 causes IELs to express high levels of activating NK-receptors: CD94/NKG2C and NK2GD (Meresse et al. 2004). Simultaneously epithelial cells increase their expression of MICA and HLA-E ligands, and the interaction of the receptors with their ligands promotes the production of proinflammatory cytokines and cytolytic enzymes leading to mucosal damage. An important cytokine in the process of IELs acquiring an activating NK-receptor repertoire is interleukin 15 (IL-15) (Meresse 2004). Transforming growth factor β (TGF β)-secreting γδ+ IELs partially function as regulatory cells also suppressing the cytotoxity of αβ+ IELs by expressing inhibitory CD94-NKG2A-receptor (Bhagat et al. 2008).

In lamina propria, transglutaminase 2 (TG2) deamidates the glutamine residues on gliadin peptides to glutamate, which increases the ability of DQ2 and DQ8 molecules on the surface of antigen presenting cells (APCs) to bind these deamidated gliadin peptides (Molberg et al. 1998). TG2 is a mainly intracellular enzyme, but under tissue-damageable conditions, such as gastrointestinal infections the tolerance to native gluten peptides breaks and TG2 is released (Siegel et al. 2008). APCs present the deamidated gliadin peptides to CD4+ T cells, which in turn produce proinflammatory cytokines such as interferon γ (IFN-γ) that enhance epithelial cell cytolysis by activated effector cells (Sollid et al. 1997). CD4+ T cells

also activate B cells to produce autoantibodies against gliadin and TG2 (Sollid et al. 1997).

Antibodies targeted against TG2 have been assumed to be pathogenic, as they have been shown to increase intestinal cell proliferation and epithelial permeability an also to activate monocytes in the lamina propria (Zanoni et al. 2006, Barone et al. 2007). TG2 antibodies have also been shown to disrupt several steps of angiogenesis and, by impairing the function of local capillaries, they may be involved in the extraintestinal manifestations of coeliac disease (Myrsky et al. 2008). Supporting this, TG2-antibody deposits have been found in the skeletal muscle and liver of untreated coeliac disease patients (Korponay-Szabo et al. 2004). In patients lacking overt small-intestinal involvement, autoantibodies have been found targeting other transglutaminase isozymes, namely TG3 in DH and TG6 in gluten ataxia (Sardy et al. 2002, Hadjivassiliou et al. 2010).

3. Genetic background of coeliac disease

HLA-DQA1*05-DQB1*02 (DQ2) or HLA-DQA1*03-DQB1*0302 (DQ8) positivity is a prerequisite for coeliac disease, but constitutes no more than 40% of the genetic risk (Bevan et al. 1999). The disease association is stronger with DQ2 (Sollid et al. 1989, Karell et al. 2003) than DQ8 (Spurkland et al. 1992). Usually α- and β-chains of the DQ2 heterodimer are encoded together in cis-position on DRB1*03 (DR3) haplotype, but they may also be encoded in trans-position with DQA1*05 allele on DRB1*11 (DR5) and DQB1*02 allele on DRB1*07 (DR7) haplotype in different chromosomes (Karell et al. 2003). DQ2 homozygotes have a five-fold risk for coeliac disease compared to heterozygotes (Murray et al. 2007, Koskinen et al. 2009). DQ2 homozygocity, especially for the DQB1*0201 allele, is also associated with more severe phenotype of coeliac disease and increased risk of complications (Al-toma et al. 2006a, Karinen et al. 2006). This is explained by the capability of APCs on DQ2 homozygotes to induce a stronger T cell proliferation and IFN-γ production by presenting a wider repertoire of gluten peptides to T cells, referred to as a gene dose effect (Vader et al. 2003). In DQ8 positive patients only 1α-gliadin peptides induce T cell response and as these peptides are not in the proline-rich areas, they are more easily digested by intestinal enzymes, and the risk of coeliac disease is lower (Henderson et al. 2007). Approximately 25% of Europeans are DQ2 positive (Sollid et al. 1989), but only 3% will ever develop coeliac disease (Wolters et al. 2008), indicating that in-risk HLA genotypes are albeit necessary, but not sufficient to cause the disease. Non-HLA gene loci are currently investigated, and T-lymphocyte regulatory genes CD28, CTLA4 and ICOS located in the genomic region 2q33 have shown linkage to coeliac disease in multiple populations (van Heel et al. 2005). With genome-wide association studies, altogether 25 additional gene variants have been identified predisposing to the disease, all containing genes involved in immune response (Dubois et al. 2010).

4. Diagnosis of coeliac disease and dermatitis herpetiformis

4.1 Diagnostic criteria of coeliac disease

The first diagnostic criteria for coeliac disease were proposed in 1969 by the European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) and included: 1) structurally abnormal jejunal mucosa on a gluten containing diet, 2) clear improvement of villous structure on a GFD and 3) deterioration of the mucosa during gluten challenge (Meeuwisse 1970). These criteria were reviewed by ESPGAN in 1990, and a gluten challenge was no longer required for the diagnosis except for children under two years old (Walker-Smith et al. 1990). In 2001 a working group of the United European Gastroenterology Week stated that the diagnosis of coeliac disease should be based on a duodenal biopsy showing typical villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis while on a gluten-containing diet and that the lesion normalizes on a GFD (UEGW 2001). Circulating coeliac antibodies were stated to support the diagnosis, but not to be essential for it. Possessing the HLA-DQ2 or HLA-DQ8 genotype was considered circumstantial evidence (UEGW 2001).

Since then, antibody tests with a higher specificity for coeliac disease have been developed and the condition is now comprehended as a continuum from latent disease with positive serology but normal villous structure to a classic disease with villous atrophy. Today, the diagnosis of coeliac disease should be based on a combination of compatible medical history, physical signs, serology and endoscopic markers as well as histological analysis of multiple biopsies from the duodenum (Rubio-Tapia et al. 2013).

For children, the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) has proposed that the diagnosis of coeliac disease could be set without an intestinal biopsy when the following criteria are met: 1) characteristic symptoms of coeliac disease, 2) TG2-antibody (TG2-ab) levels over ten times the upper limit of normal with positive endomysium antibodies (EMA) in a different blood sample and 3) positive HLA DQ2/DQ8 (Husby et al. 2012). Currently these recommendations are being validated in children and in adults.

4.2 Serology

The first coeliac antibodies against reticulin fibres (ARAs) were discovered in the 1970s and were detected by indirect immunofluorescence using rodent tissues as antigens. ARA were present in the sera of 38% of adult coeliac patients (Seah et al. 1973). In the 1980s antibodies against wheat gliadin (AGAs) and endomysium of the smooth muscle in monkey oesophagus (EMAs) were discovered. AGA were measured by enzyme-linked immunosorbent assay (ELISA) and reached 93% sensitivity in adults with untreated coeliac disease. However, AGAs were not specific to coeliac disease and high titers were found in patients with other gastrointestinal disorders (Hill et al. 1991). EMAs were detected by indirect immunofluorescence and human umbilical cord soon substituted the originally used monkey oesophagus as an antigen (Ladinser et al. 1994). Although the method requires microscopic evaluation and may be subject to interobserver variability, it is considered the reference standard for coeliac disease-specific antibody detection (Husby et al. 2012). In the late 1990s TG2 was identified as an autoantigen of the disease (Dietrich et al. 1997) and an ELISA method was established to detect antibodies targeted against it (Sulkanen et al. 1998). This method is now widely available and inexpensive compared to EMAs (Husby et al. 2012). Although ARAs and AGAs have vanished from clinical practice due to their low sensitivity and specificity for coeliac disease, a new ELISA test recognizing deamidated gliadin peptides (DP-AGA) has been developed (Prince 2006). This test has a sensitivity of 91% and a specificity of 98% for coeliac disease (Kaukinen et al. 2007a), and it may be useful in distinguishing non-responsive patients with persistent mucosal damage on GFD with a sensitivity of 87% and a specificity of 89% (Spatola et al. 2014). Unfortunately this test cannot differentiate patients with RCD from other non-responding patients (Spatola et al. 2014).

Coeliac disease can be screened in risk groups using TG2-ab or EMA. Class IgA antibodies are usually sufficient, but in the case of IgA deficiency, IgG antibodies should be used instead. Selective and partial IgA deficiency is present in only 2% of coeliac disease patients and the need for routine measurement of total serum IgA in every patient is questionable, since most adults with partial IgA deficiency are still able to produce IgA class TG2-abs (Chow et al. 2012). The sensitivity of EMA in detecting coeliac disease varies from 86% to 100% and the specificity from 90% to 100% (Hill 2005). The sensitivity of TG2-ab is 77-100% and the specificity 91-100% (Hill 2005). Rapid point-of-care tests have also been developed to measure TG2-ab with a reported sensitivity of 78% and specificity of 100% (Korponay-Szabo et al. 2007).

Coeliac antibodies are usually detected in the sera of coeliac patients consuming gluten in their diet, and the antibody levels decrease within months once a GFD is introduced (Midhagen et al. 2004). However, seroconversion correlates poorly with mucosal recovery and negative serology cannot replace control biopsies from the duodenal mucosa in estimating the responsiveness to a GFD (Dickey et al. 2000b, Kaukinen et al. 2002a). Nor can coeliac serology be used to exclude dietary regressions, as traces of gluten in the diet or even minor voluntary lapses do not always provoke positive antibody reactions (Leffler et al. 2007a). Notwithstanding, persistent EMA-negativity occurs in 10-20% of patients with biopsy-confirmed coeliac disease on a gluten-containing diet (McMillan et al. 1991, Dickey et al. 2000a, Salmi et al. 2006) and may be related to a lesser degree of villous atrophy (Abrams et al. 2004). TG2-ab negativity, on the other hand, may indicate a long diagnostic delay, as TG2-ab are proven to acquire a better avidity to small-intestinal mucosa in a long-standing disease resulting in undetectable antibodies in the sera (Salmi et al. 2006).

4.3 Histology

The characteristic features of coeliac disease in the duodenal mucosa include villous atrophy, crypt hyperplasia and intraepithelial lymphocytosis (Marsh 1990). In the subsequent Marsh-Oberhuber classification, class I comprises marked intraepithelial lymphocytosis with no signs of villous atrophy or crypt hyperplasia. Class II is defined by crypt hyperplasia without villous atrophy. Class IIIa relates to partial, IIIb subtotal and IIIc total villous atrophy with crypt hyperplasia (Oberhuber et al. 1999). A count of 25-29 IELs per 100 epithelial cells is considered borderline and over 30/100 represents pathological intraepithelial lymphocytosis (Villanacci et al. 2011). Villi architecture is considered normal when the villous height/crypt depth (Vh/CrD) ratio is over 3:1 (Villanacci et al. 2011). Pitfalls in the histological analysis are poorly orientated biopsy specimens over- or underestimating Vh/CrD ratio and inadequate number of biopsy specimens, as typical coeliac lesions may be patchy on the duodenal mucosa (Collin et al. 2005).

Although duodenal villous atrophy is typical for coeliac disease, other aetiologies do exist: small-intestinal bacterial overgrowth, autoimmune enteropathy, hypogammaglobulinaemia, drug-associated enteropathy, Whipple´s disease, collagenous sprue, Crohn´s disease, eosinophilic enteritis, intestinal lymphoma, infectious enteritis, graft versus host disease and acquired immune deficiency syndrome (AIDS) enteropathy (Rubio-Tapia et al. 2013).

An early phenomenon in the development of coeliac disease is marked intraepithelial lymphocytosis, which abates on a GFD, but returns rapidly when there is re-exposure to dietary gluten (Marsh 1990, Kutlu et al. 1993, Lähdeaho et al. 2011). However, intraepithelial lymphocytosis is an unspecific reaction of innate immunity to various luminal contents of the small intestine: bacterial antigens, drugs and gluten peptides (Mahadeva et al. 2002, Kakar et al. 2003, Aziz et al. 2010, Shmidt et al. 2014). Intraepithelial lymphocytosis can be found in 2-7% of routinely taken duodenal biopsies and coeliac disease is diagnosed in 9-40% of these (Mahadeva et al. 2002, Kakar et al. 2003, Walker et al. 2010, Shmidt et al. 2014). Positive coeliac antibodies increase the likelihood of coeliac disease. High count of γδ-IELs or increased γδ/CD3-ratio detected by immunohistochemical analysis may improve the specificity for coeliac disease diagnosis (Järvinen et al. 2003). Antibodies against TG2 are produced in the small-intestinal mucosa, where they can be detected deposited on the extracellular TG2 by direct immunofluorescence even before they can be measured in the serum (Kaukinen et al. 2005). These TG2-specific IgA deposits and also high density of villous tip IELs are highly specific for coeliac disease (Järvinen et al. 2004, Kaukinen et al. 2005, Salmi et al. 2006, Koskinen et al. 2010).

Other autoimmune diseases may cause intraepithelial lymphocytosis; most frequently hypothyreosis, rheumatoid arthritis, psoriasis, glomerulonephritis, primary biliary cirrhosis and multiple sclerosis (Kakar et al. 2003). *Helicobacter pylori* and other infections, such as giardiasis, may cause intraepithelial lymphocytosis as well as bacterial overgrowth in the small intestine (Memeo et al. 2005, Aziz et al. 2010, Shmidt et al. 2014). Drugs, mostly non-steroidal anti-inflammatory drugs (NSAIDs), can induce intraepithelial lymphocytosis (Kakar et al. 2003, Aziz et al. 2010, Shmidt et al. 2014). Intolerance to dietary proteins other than gluten, selective IgA- and other primary immunodeficiency, Crohn´s disease, irritable bowel syndrome, eosinophilic gastroenteritis and autoimmune enteropathy are other reported aetiologies (Aziz et al. 2010, Villanacci et al. 2011).

4.4 Genetics

Testing of the compatible HLA DQ2/DQ8 routinely in the initial diagnosis of coeliac is unreasonable, since the positive predictive value of the test is only about 8% (Hadithi et al. 2007b). However, HLA DQ-typing can rule out the disease in patients who have started a GFD before proper diagnostics, in patients with discrepant serology towards histology and in patients with suspected RCD when the original diagnosis of coeliac disease remains in question; because the negative

predictive value of the test is almost 100% (Kaukinen et al. 2002b, Al-toma et al. 2007a, Rubio-Tapia and Murray 2010).

4.5 Diagnosis of dermatitis herpetiformis

The diagnosis of dermatitis herpetiformis is based on a typical rash, especially on the knees, elbows, buttocks and scalp; and pathognomonic granular IgA deposits in the papillary dermis detected by direct immunofluorescence in a biopsy from the uninvolved skin (Fry and Seah 1973). The majority of DH patients have small-intestinal mucosal changes consistent with coeliac disease: 80-90% have villous atrophy and the rest intraepithelial lymphocytosis (Gawkrodger et al. 1984, Reunala et al. 1984, Savilahti et al. 1992). 79 % of DH patients have TG2-ab and 74 % EMA (Kumar et al. 2001).

5. Clinical features of coeliac disease

5.1 Classic symptoms

The clinical manifestation of coeliac disease is probably determined by genetic background and environmental factors (Karinen et al. 2006, Tack et al. 2010a). DQ2 homozygotes present with more severe symptoms, but neither the degree of villous atrophy nor the extent of the mucosal lesion correlate with the clinical presentation (Karinen et al. 2006, Brar et al. 2007b, Murray et al. 2008). The classic signs and symptoms of coeliac disease are abdominal pain and distention, persistent diarrhoea, failure to thrive, muscle wasting, steatorrhoea, vomiting and weight loss, which are also the predominant symptoms in children (Green and Cellier 2007). The mechanism of gastrointestinal symptoms in active coeliac disease may be multifactorial, but most importantly the gluten-induced damage to the small-intestinal mucosa results in malabsorption of nutrients, especially sugars and fats resulting in diarrhoea, increased flatulence and weight loss. Abnormalities of serotonin metabolism may result in functional disorders and accompanying bacterial overgrowth, pancreatic insufficiency and microscopic colitis may also contribute to diarrhoea (Brar et al. 2007b).

The clinical presentation of coeliac disease has changed in recent decades with increasing percentage of patients now presenting with atypical disease, including osteoporosis and iron-deficiency anaemia (Collin et al. 1997, Bottaro et al. 1999, Rambertap et al. 2006, Hurley et al. 2012, Ludvigsson et al. 2013a). This atypical clinical picture of coeliac disease is also more common in adults and in the elderly (Green and Cellier 2007). Heightened awareness of the disease and wider use of serological tests have resulted in more individuals with non-specific gastrointestinal symptoms such as bloating and flatulence being investigated and diagnosed with coeliac disease (Hurley et al. 2012).

5.2 Extraintestinal manifestations

Extraintestinal manifestations may lead to a diagnosis of coeliac disease in as many as 24% of cases (Collin et al. 1997). The most common extraintestinal

manifestations are osteoporosis, dermatitis herpetiformis, neurological disorders, arthritis, aphtous stomatitis and dental enamel defects (Collin et al. 1997, Tack et al. 2010a). Neurological disorders compatible with coeliac disease are peripheral neuropathy, cerebellar ataxia, epilepsy, encephalopathy, myelopathy and myopathy (Hadjivassiliou et al. 2010). Coeliac disease can cause infertility and recurrent spontaneous abortions (Özgör and Selimoglu 2010). Other reproductive disorders include delayed menarche, amenorrhoea, early menopause, low birth-weight and preterm delivery (Özgör and Selimoglu 2010, Santonicola et al. 2011). In past decades liver enzymes were elevated in up to 40% of patients with untreated coeliac disease, but nowadays they are mainly within the reference values (Rubio-Tapia and Murray 2007, Korpimäki et al. 2011). Mild elevations of transaminases with non-specific hepatic histological changes and even severe hepatic failures have been reported to reverse with the initiation of a GFD (Kaukinen et al. 2002c, Rubio-Tapia and Murray 2007).

5.3 Associated diseases and risk groups

Approximately 20-30% of coeliac disease patients have another autoimmune disorder (Sategna-Guidetti et al. 2001, Viljamaa et al. 2005a, Cosnes et al. 2008, Elli et al. 2012). The most common concomitant autoimmune diseases are type I diabetes mellitus, autoimmune thyreoiditis, Sjögren´s syndrome, rheumatoid arthritis, sarcoidosis and Addison´s disease (Collin et al. 1994, Viljamaa et al. 2005a). Coeliac disease is associated with microscopic colitis (Matteoni et al. 2001, Green et al. 2009, Koskela et al. 2011, Stewart et al. 2011) and autoimmune liver diseases such as primary biliary cirrhosis, autoimmune hepatitis and primary sclerosing cholangitis (Rubio-Tapia and Murray 2007). Coeliac disease is also frequent in Down´s and Turner´s syndrome and in myasthenia gravis. Alopecia areata, atrophic gastritis, dermatomyositis, scleroderma, systemic lupus erythematosus, psoriasis and vitiligo have also been reported in coeliac patients (Tack et al. 2010a). The increased risk of concomitant autoimmune disease is largely explained by the common genetic background (Collin et al. 1994, Viljamaa et al. 2005a). It remains debatable whether GFD protects against a future autoimmune disease, or even has a beneficial effect on its clinical course (Sategna-Guidetti et al. 2001, Viljamaa et al. 2005a, Cosnes et al. 2008, Elli et al. 2012, Metso et al. 2012).

Patients diagnosed with the above-mentioned autoimmune diseases constitute a risk group for coeliac disease, and by serological screening of these individuals, numerous patients with mild or no symptoms can be detected (Collin et al. 1997).

Another risk group for coeliac disease consists of first degree relatives of coeliac patients, and they ought to be offered serological screening, too (Rubio-Tapia et al. 2013).

5.4 Health-related quality of life

Depression and anxiety are frequent in untreated coeliac patients and may be associated with defective monoamine metabolism or local hypoperfusion of the brain (Hernanz and Polanco 1991, Addolorato et al. 2001, Addolorato et al. 2004). The low tryptophan-concentrations associated with depression have been shown to resolve on a six-months GFD (Pynnönen et al. 2005), although depressive mood has not been alleviated in all coeliac patients on a GFD (Addolorato et al. 2001). The quality of life in long-treated coeliac patients may be impaired by physical and mental co-morbidities, problems with adhering to a GFD and dissatisfaction with doctor-patient communication (Häuser et al. 2007, Paarlahti et al. 2013), but in general it is on the same level as that of the population in general (Viljamaa et al. 2005b, Roos et al. 2006, Nachman et al. 2009, Kurppa et al. 2010, Ukkola et al. 2011). Although gastrointestinal symptoms usually alleviate rapidly after commencing a GFD, coeliac women in particular experience more indigestion, diarrhoea, constipation and abdominal pain than population in general (Midhagen and Hallert 2003) and are also more likely to have impaired quality of life (Paarlahti et al. 2013).

Recently diagnosed patients may experience problems with adjusting to a chronic illness, as the diagnosis of coeliac disease has been reported to be a shock to 6%, but on the other hand, a relief to 40% of patients (Ukkola et al. 2012). GFD may impose social restrictions, but on the whole, the quality of life has been shown to improve in most coeliac patients when commencing a GFD, including screen-detected patients (Viljamaa et al. 2005b, Nachman et al. 2009, Ukkola et al. 2011). Only completely asymptomatic patients experience a fall in their quality of life when commencing a life-long GFD (Ukkola et al. 2011).

6. Epidemiology

6.1 Epidemiology of coeliac disease

The serologic prevalence of coeliac disease varies from 0.2 to 1.2% in most European and North American countries (Table 1). The highest prevalence of coeliac antibodies (5.6%) has been reported among children of the Saharawi population in Algeria (Catassi et al. 1999). In Australia and New Zealand, the serologic prevalence varies from 0.3 to 0.9% (Table 1). In South American countries the serological prevalence has been reasonably low at 0.2-0.6% (Table 1). There are reports from India, Iran, Israel, Tunisia and Turkey with 0.1-1.3% prevalence of coeliac antibodies among healthy blood donors (Table 1). Coeliac disease is very rare in Japan and China (Cummins and Roberts-Thompson 2009). In Finland the estimated prevalence of coeliac disease is 1% in children, 2% in adults and 2.7% in the elderly, combining figures of previously clinically diagnosed patients with newly screen-detected (Mäki et al. 2003, Lohi et al. 2007, Vilppula et al. 2009).

The prevalence of clinically diagnosed coeliac disease remains low, less than 0.1% in most countries (Table 1), meaning that most patients are never actually diagnosed. In Finland, an efficient case-finding policy has been adopted advocating serological screening in risk groups: family members of coeliac patients, patients with concomitant autoimmune diseases and patients suffering from mild gastrointestinal symptoms and extraintestinal manifestations (Collin et al. 2007, Virta et al. 2009). For that reason, the prevalence of clinically diagnosed coeliac disease in Finland is 0.6%, which is the highest reported world-wide (Virta et al. 2009).

The incidence of coeliac disease is increasing over the world (Hurley et al. 2012, Ludvigsson et al. 2013a), and in Finland it has almost doubled in twenty years (Lohi et al. 2007). Improved serological methods, intensified screening of risk groups and better availability of endoscopic resources certainly account for this, but the real increase in the prevalence of the disease is rather attributable to environmental changes (Rambertap et al. 2006, Lohi et al. 2007, Hurley et al. 2012, Ludvigsson et al. 2013a), which have been suggested to include increased consumption of wheat and improved level of hygiene (Kondrashova et al. 2008,

Ludvigsson et al. 2013a). Nevertheless, coeliac disease seems to remain widely under-diagnosed (Mäki et. al 2003, Hurley et al. 2012, Ludvigsson et al. 2013a).

As in many autoimmune diseases, coeliac disease is more common in females, the ratio to males being between 2:1 and 3:1 (Virta et al. 2009, Hurley et al. 2012, Ludvigsson et al. 2013a). Most of the diagnoses are set in early childhood or between ages 30 and 50 (Tack et al. 2010a), but the disease may develop at any age, also in the elderly (Vilppula et al. 2009, Catassi et al. 2010).

Table 1. Prevalence of screen-detected, clinically diagnosed and combined adult coeliac disease (CD)

Study	Country	Study population (n)	Prevalence of screen-detected CD	Prevalence of clinically diagnosed CD	Combined prevalence of CD
Corazza et al. 1997	Italy	2237	0.2%		
Not et al. 1998	USA	2000	0.4%		
Hovdenak et al. 1999	Norway	2096	0.3%		
Ivarsson et al. 1999	Sweden	1894	0.4%	0.1%	0.5%
Rostami et al. 1999	Netherlands	1000	0.3%		
Cook et al. 2000	New Zealand	1064	0.9%	0.3%	1.2%
Gandolfi et al. 2000	Brazil	2045	0.2%		
Riestra et al. 2000	Spain	1170	0.3%		
Gomez et al. 2001	Argentina	2000	0.6%	0.05%	0.6%
Hovell et al. 2001	Australia	3011	0.3%	0.07%	0.4%
Volta et al. 2001	Italy	3483	0.5%		
Shamir et al. 2002	Israel	1571	0.6%		
Sanders et al. 2003	UK	1200	1.0%		
Shahbazkhani et al. 2003	Iran	2000	0.6%		
West et al. 2003	UK	7550	1.2%	0.05%	1.2%
Schweizer et al. 2004	Netherlands	50760	0.4%	0.02%	0.4%
Tatar et al. 2004	Turkey	2000	1.3%		
Gursoy et al. 2005	Turkey	906	1.0%		
Akbari et al. 2006	Iran	2799	1.0%		
Bdioui et al. 2006	Tunisia	1418	0.1%		
Melo et al. 2006	Brazil	3000	0.3%		
Menardo et al. 2006	Italy	1002	1.0%	0.04%	1.1%
Pereira et al. 2006	Brazil	2086	0.2%		
Remes-Troche et al. 2006	Mexico	1009	2.7%		
Lohi et al. 2007	Finland	8028	1.5%	0.5%	2.0%
Oliveira et al. 2007	Brazil	3000	0.5%		
Roka et al. 2007	Greece	2230	0.2%		
Chin et al. 2009	Australia	3011	0.6%		

Johansson et al. 2009	Iceland	813	0.7%		
Bahari et al. 2010	Iran	1600	0.9%		
Alencar et al. 2012	Brazil	4000	0.4%		
Kochhar et al. 2012	India	1610	0.6%		
Lillemäe et al. 2012	Estonia	891	0.3%		
Rubio-Tapia et al. 2012	USA	7798	0.4%	0.08%	0.5%
Kratzer et al. 2013	Germany	2157	0.4%		

UK United Kingdom, USA United States of America

6.2 Epidemiology of dermatitis herpetiformis

The highest prevalence of 0.08% in DH has been reported in Finland (Salmi et al. 2011). In previous studies the prevalence of DH has varied from 0.01 to 0.04% in Europe and North America (Table 2). The incidence of DH is decreasing, which can be related to the increased recognition of subclinical coeliac disease; since early commencement of GFD may prevent from the skin involvement (Salmi et al. 2011, Hurley et al. 2012). Unlike in coeliac disease, most of the patients suffering from DH are males and currently DH is diagnosed at the mean age of 43 years (Salmi et al. 2011).

Table 2. Prevalence of dermatitis herpetiformis (DH)

Study	Country	Prevalence of DH
Reunala and Lokki 1978	Finland	0.01 %
Gawkrodger et al. 1984	United Kingdom	0.01%
Mobacken et al. 1984	Sweden	0.02 %
Moi 1984	Sweden	0.04 %
Christensen et al. 1986	Sweden	0.02 %
Smith et al. 1992	United States of America	0.01 %
Salmi et al. 2011	Finland	0.08 %

7. Treatment of coeliac disease

7.1 Gluten-free diet

The normal western diet contains about 15-20 grams of gluten daily (van Overbeek et al. 1997, Pinier et al. 2010). About 100 milligrams of gluten per day is enough to cause mucosal damage, and 10-30 milligrams is considered to be safe (Catassi et al. 1993, Collin et al. 2004b, Gibert et al. 2006, Catassi et al. 2007). However, tolerance of gluten traces in the diet is individual (Lähdeaho et al. 2011). The current international Codex Alimentarius defines gluten-free products as having less than 20 milligrams of gluten per kilogram. Industrially purified wheat starch has been proven safe in the GFD (Kaukinen et al. 1999, Peräaho et al. 2003). GFD can reduce beneficial bacteria in normal gut flora (Pozo-Rubio et al. 2012) and often constains less fibre and more fat than the normal diet (Wild et al. 2010). Deficiencies of iron, folic acid, vitamins B12 and D, zinc and copper are more frequent on a GFD than on a normal diet (Wild et al. 2010).

7.2 Oats

The percentage of proline and glutamine that are abundant in the toxic regions of other prolamins is low in the avenin of oats (Arentz-Hansen et al. 2004). Pure oats have been proven safe in the treatment of coeliac disease providing by definition that they are not contaminated by gluten traces from other cereals (Janatuinen et al. 2002, Collin et al. 2004b, Holm et al. 2006, Sey et al. 2011). However, some coeliac disease patients may develop an immunological reaction towards avenins in oats and this may be related to a variation in the toxicity of different oat cultivares (Lundin et al. 2003, Arentz-Hansen et al. 2004, Comino et al. 2011).

In Finland oats have been permissible in a GFD since 1997 for adult coeliac patients and since 2000 in children. In 2004, 70% of Finnish coeliac patients were consuming oats because they liked the taste, found them easy to use and inexpensive. They also felt that oats diversified their diet (Peräaho et al. 2004a), which might also serve to increase their compliance with the diet (Störsrud et al. 2003a). The safety of oats has been evaluated in numerous studies and the

consumption of oats has not damaged the villous structure of small-intestinal mucosa in coeliac patients even when used long-term and in large quantities (Janatuinen et al. 1995, Janatuinen et al. 2002, Störsrud et al. 2003b, Högberg et al. 2004, Peräaho et al. 2004b, Holm et al. 2006, Srinivasan et al. 2006, Sey et al. 2011, Cooper et al. 2013, Kaukinen et al. 2013). Nevertheless, in a prospective study the density of IELs was higher in the small-intestinal mucosa of patients consuming oats than that of patients on a traditional diet (Peräaho et al. 2004b) and the oats-consuming patients also had more gastrointestinal symptoms (Peräaho et al. 2004b).

Consumption of oats has not been reported to impair the nutritional status or increase coeliac antibody levels in compliant patients (Janatuinen et al. 1995, Janatuinen et al. 2002, Störsrud et al. 2003b, Högberg et al. 2004, Peräaho et al. 2004b, Holm et al. 2006, Cooper et al. 2012, Kaukinen et al. 2013). On the contrary, oats can improve the nutritional value of the diet by increasing the intake of fibre, thiamin, magnesium and iron (Störsrud et al. 2003a).

7.3 Dietary compliance

Dietary non-compliance is often associated with inferior taste and higher price or inadequate labelling of gluten-free products (Vahedi et al. 2003, Butterworth et al. 2004, Green and Cellier 2007). GFD may impose social restrictions and be inconvenient while dining out and travelling. Currently, 88% of Finnish coeliac disease patients are following a strict diet (Kurppa et al. 2012). World-wide rates for strict adherence range from 42-91% (Vahedi et al. 2003, Leffler et al. 2007a, Hall et al. 2009). Strict dietary compliance is essential in achieving mucosal recovery, and lack of adherence is the main reason for poorly controlled disease (Ciacci et al. 2002, Lanzini et al. 2009, Hutchinson et al. 2010, Rubio-Tapia et al. 2010b, Sharkey et al. 2013, Lebwohl et al. 2014).

7.4 Clinical and mucosal recovery on GFD

Although a GFD usually alleviates symptoms associated with coeliac disease within a few weeks (Murray et al. 2004), a complete remission of gastrointestinal symptoms was reported in 75% and of extra-intestinal symptoms in 68% of coeliac patients during a mean of 16 months on a GFD (Lanzini et al. 2009). Complete histological normalization of the small-intestinal mucosa was achieved in 8-60% of

adult coeliac patients and the median recovery time varied from two to four years (Table 3). Slow recovery can be expected when the mucosal damage is severe at diagnosis, usually correlating with a long history of gluten exposure and a long diagnostic delay (Tursi et al. 2006, Rubio-Tapia et al. 2010b, Lebwohl et al. 2014).

Mucosal non-recovery has been reported to be associated with increased all-cause mortality; the most frequent causes of death being malignancies, infections and refractory coeliac disease (Rubio-Tapia et al. 2010b). Contrasting results were reported in a recent study, which demonstrated that persistent villous atrophy was not associated with increased mortality (Lebwohl et al. 2013a). This notwithstanding, in the same cohort, the risk of lymphoproliferative malignancies was higher among patients with mucosal non-recovery than in patients with healed mucosa (Lebwohl et al. 2013b). Persistent villous atrophy also predisposes to osteoporosis (Kaukinen et al. 2007b).

Table 3. Mucosal recovery in coeliac disease according to the duration of gluten-free diet (GFD)

Study	Patients (n)	Duration of GFD, mean (range); years	Normal histology (Marsh 0)	Intraepithelial lymphocytosis (Marsh I)	Villous atrophy and crypt hyperplasia (Marsh II-III)
Sharkey et al. 2013	391	1	26%	30%	44%
Lanzini et al. 2009	465	1.3 (1-9)	8%	65%	27%
Tursi et al. 2006	42	2	60%	16%	24%
Bardella et al. 2007	114	2 (1-23)	18%	20%	62%
Rubio-Tapia et al. 2010b	165	2	35%		
Hutchinson et al. 2010	284	2.9 (1-8)	39%	17%	44%
Wahab et al. 2002	158	5	41%	24%	35%
Ciacci et al. 2002	390	6.9 (2-22)	44%	9%	47%
Lee et al. 2003	39	8.5 (1-45)	21%		78%

7.5 Novel therapies

Currently GFD is the only available treatment for coeliac disease. However, even patients maintaining a strict diet may inadvertently be exposed to small amounts of gluten sufficient to cause gastrointestinal symptoms and small-intestinal mucosal damage (Ciacci et al. 2002, Lee et al. 2003, Midhagen and Hallert 2003). Therefore, a GFD alone may not result in full remission in every coeliac patient, and some may benefit from additional drug therapy. Notwithstanding, as GFD is safe, reasonably inexpensive and results in a good outcome in most coeliac patients; any novel therapy should not have serious adverse events and be economically highly unviable.

As wheat accessions differ in their content of immunotoxic peptides for coeliac patients, the development of strains that lack these proteins has become intriguing (Spaenij-Dekking et al. 2005). Selecting, breeding and genetically modifying wheat in a way that deletes toxic gluten sequences could result in varieties suitable for coeliac patients (Stoven et al. 2012). However, as gluten plays a major role in the desirable baking quality of wheat and as genetic engineering will inevitably raise the cost of cereal products, these varieties may not be financially viable alternatives for industrial wheat and consumption of these products may be limited to coeliac patients (Schuppan et al. 2009, Kaukinen et al. 2014). One alternative is sourdough fermentation, as selected lactobacilli and fungal proteases can hydrolyze the most immunotoxic proline-rich peptides in wheat gliadin (Di Cagno et al. 2004). Sourdough baked products proved to be safe for coeliac patients in a short clinical trial (Greco et al. 2011).

Various bacteria and fungi are also capable of degrading gluten. Propyl endopeptidases from *Aspergillus niger* (AN-PEP), *Flavobacter meningosepticum, Sphingomonas capsulata* (SC-PEP) and *Myxococcus xanthus* were resistant to breakdown by acid, pepsin, pancreatic proteases and membrane peptidases of the small-intestinal mucosa, and able to hydrolyze most of the immunotoxic gliadin peptides in biochemical analysis (Shan et al. 2004, Mitea et al. 2008). AN-PEP was safe and well-tolerated in a clinical trial; but as no clinical deterioration was observed upon gluten challenge in patients receiving placebo, the possible beneficial effect of AN-PEP was not demonstrable (Tack et al. 2013). Germinating seeds also contain proteases capable of degrading gluten. One of these enzymes is cysteine protease EP-B2, present in barley. As SC-PEP and EP-B2 degrade complementary gluten sequences, a combination of these enzymes was prepared and named ALV003 (Gass et al. 2007). In a clinical study it proved safe, well-tolerated and active in the mildly acid fed stomach (Siegel et al. 2012). However, in a placebo-controlled clinical trial, although the drug was able to diminish gluten-specific T cell responses

compared to placebo, gastrointestinal symptoms were reported equally in both groups during a gluten challenge (Tye-Din et al. 2010a). Furthermore, in a recent clinical trial, ALV003 attenuated gluten-induced mucosal deterioration as measured by change in the villous-crypth morphometry and IEL densities (Lähdeaho et al. 2014).

Probiotics can also hydrolyze the most immunotoxic gliadin peptides (De Angelis et al. 2006). They may also stabilize tight junctions by lowering zonulin release from epithelial cells (De Angelis et al. 2006). Numbers of bifidobacteria are lower in the intestinal mucosa of coeliac patients than in healthy mucosa (Poco-Rubio et al. 2012). However, in a placebo-controlled clinical trial administered *Bifidobacterium infantis* did not affect the abnormal intestinal permeability observed in untreated coeliac patients, but alleviated some gastrointestinal symptoms (Smecuol et al. 2013). However, *Bifidobacter lactis* was able to prevent cellular damage following gliadin administration in a cell culture (Lindfors et al. 2008).

High molecular weight polymers are commonly used to bind small molecules, such as potassium and bile salts in the gastrointestinal tract, thereby reducing their intestinal absorption. Polymeric binders can also sequester gluten in the small-intestinal lumen thereby decreasing the absorption of immunotoxic peptides (Pinier et al. 2009). Polyhydroxyethyl methacrylate-co-styrene sulfonate reduced gluten-induced secretion of proinflammatory tumour necrosis factor α (TNF-α) in human tissue (Pinier et al. 2012). It remains unresolved whether other dietary proteins can interact with the polymer leading to nutritional deficiencies (Kaukinen et al. 2014).

Gliadin activates the zonulin signalling cascade, which in turn opens the small-intestinal tight junctions and allows the passage of gluten peptides into the lamina propria. Larazotide acetate blocks the zonulin receptor and thus protects tight junction integrity (Drago et al. 2006). In a clinical trial, however, intestinal permeability was not affected, although gastrointestinal symptoms were less severe in patients receiving the drug compared to patients receiving placebo during a gluten challenge (Leffler et al. 2012). In addition to the paracellular route, gluten peptides may also pass through the epithelium by binding to transferrin receptor CD71 in a complex with secretory IgA thereby compromising the efficacy of larazotide acetate (Matysiak-Budnik et al. 2008).

TG2-inhibitors are intended to prevent the deamidation of gluten peptides, which is crucial to the adaptive immune response in coeliac disease (Molberg et al. 1998). Currently these compounds lack tissue specificity and may have deleterious effects outside the gastrointestinal tract (Rauhavirta et al. 2013). Innate immune reactions that native gluten peptides are able to trigger without deamidation make this approach also dubious (Schuppan et al. 2009). Adaptive immune responses

may also be prevented by blocking HLA-DQ2 and DQ8 molecules, but the risk of interfering with other HLA-class-2 responses, which are important to immunosurveillance, makes this therapeutic option risky (Kaukinen et al. 2014).

Interleukin 10 (IL-10) is an immunoregulatory cytokine which interferes with antigen presentation and results in the induction of hyporesponsive gliadin-specific T cells (Salvati et al. 2005). However, human recombinant IL-10 proved ineffective in a small clinical trial with RCD patients (Mulder et al. 2001). Other therapies targeted at cytokines have not functioned in coeliac disease, or adverse events have limited their use (Kaukinen et al. 2014).

Upon gluten-induced activation lymphocytes start to accumulate in the intestinal mucosa using chemokine receptor CCR9 in the homing process (Mora and von Andrian 2008). T cell responses in the intestinal mucosa can thus be reduced by blocking the chemokine receptor. However, this mode of therapy may predispose patients to microbial infections by preventing the chemotaxis of effector cells in a non-antigen-specific manner (Kaukinen et al. 2014).

Identification of major T cell epitopes in gluten has afforded an opportunity to induce tolerance to gluten by vaccination (Tye-Din et al. 2010b). However, there may be additional epitopes in gluten that are not covered by the peptides used in the current vaccine (Kaukinen et al. 2014).

Finally, parasite infections are thought to modulate the host´s immune system by skewing the type 1 T helper cell response to a less aggressive type 2 T helper cell or to a regulatory T cell response (Schuppan et al. 2009). In a clinical trial, coeliac patients were innoculated with a hookworm *Necator Americanus*, but the parasite did not prevent the deterioration of small-intestinal mucosal architecture caused by gluten challenge (Daveson et al. 2011).

Currently none of these novel therapies is available in clinical practice. These therapies are not designed to replace GFD, but to be used as an adjunctive therapy in situations where traces of gluten cause persistent symptoms or mucosal damage despite a strictly maintained diet.

8. Refractory coeliac disease (RCD)

8.1 Definition of RCD

RCD is defined by persistent or recurrent villous atrophy and malabsorptive symptoms despite a strict GFD for at least 6-12 months. Other causes of villous atrophy and non-responsive coeliac disease as well as malignant diseases must be excluded before the diagnosis of RCD can be established. The symptoms are often severe and require additional therapeutic intervention besides a GFD (Biagi and Corazza 2001, Rubio-Tapia and Murray 2010, Malamut et al. 2012, Ludvigsson et al. 2013b, Rubio-Tapia et al. 2013). The majority of RCD diagnoses are made due to recurrent symptoms after an initial clinical response to GFD (secondary RCD), but in some cases an early intervention is needed to control the symptoms due to an initial lack of response to GFD (primary RCD) (Malamut et al. 2009, Rubio-Tapia and Murray 2010). Although mucosal recovery often takes more than 12 months on a GFD, the clinical response is mostly observed within weeks in uncomplicated disease (Murray et al. 2004). RCD can be further divided into type I with a normal phenotype of IELs, and type II with abnormal, clonal IELs which have lost their normal surface markers CD3 and CD8 and express intracytoplasmic CD3 (Cellier et al. 1998, Rubio-Tapia and Murray 2010). Making the distinction between a slow response to a GFD, inadvertent gluten ingestion and RCD, especially type I may be difficult.

8.2 Pathogenesis of RCD

In RCD the immunologic reaction induced by gluten converts independent of the antigen stimulus and the inflammation persists despite a GFD (Malamut et al. 2012). IL-15 is the main contributor to the loss of homeostasis in the gut in RCD. Expression of IL-15 is increased in untreated as also in complicated coeliac disease and correlates with the severity of mucosal damage (Mention et al. 2003, Di Sabatino et al. 2006b). The over-expression of IL-15 leads to promoted cytotoxic activity and cytokine secretion of IELs (Meresse et al. 2004). It also leads to the expansion of IELs acquiring an NK-phenotype and the development of a

monoclonal cell population (Malamut et al. 2012). These monoclonal IELs escape from elimination under the anti-apoptotic influence of IL-15, and due to acquired new mutations finally turn into EATL (Mention et al. 2003). Gain of chromosome 1q has been found in monoclonal IELs and is claimed to be an early event in lymphomagenesis (Verkarre et al. 2003b).

Γδ-lymphocytes have a crucial role in immunosurveillance as well as mucosal repair in coeliac disease. The number of these cells is increased in untreated disease and although decreasing may be elevated for years in patients responding to a GFD (Kutlu et al. 1993, Järvinen et al. 2003). In RCD these cells are scarce, resulting in decreased production of TGF β, which in turn leads to impaired control of the cytotoxity of CD8+ lymphocytes (Verbeek et al. 2008a). If a response to medical treatment is achieved, the number of these cells again increases (Verbeek et al. 2008a). Due to IL-15 overexpression, IELs also become unresponsive to the regulatory effect of TGF β (Hmida et al. 2012).

8.3 Risk factors for RCD

HLA-DQ2 homozygous individuals are more susceptible to both types of RCD and EATL than are heterozygotes (Al-toma et al. 2006a, Malamut et al. 2009). This results from a gene dose effect, as DQ2-homozygotes produce a greater magnitude of gluten-specific T cell responses than do heterozygotes (Vader et al. 2003). Moreover, myosin IX B (MYO9B) gene T allele predisposes homozygous individuals to the development of RCD type II and EATL, but may rather be a marker of complicated disease than a causative mutation (Wolters et al. 2007).

Viral infections, especially chronic hepatitis B and C, have been detected in 20% of RCD type I patients and in 10% of type II patients (Malamut et al. 2012). It has been speculated that the antiviral immune response, especially interferons, can stimulate CD8+ T lymphocytes as well as NK cells and enhance the production of IL-12, which works synergistically with IL-15 (Malamut et al. 2012).

8.4 Epidemiology of RCD

The real prevalence of RCD is unknown, since the current literature involves only tertiary centres (Table 4). RCD has been diagnosed in 9-18% of coeliac patients suffering from ongoing symptoms (Abdulkarim et al. 2002, Leffler et al. 2007b, Dewar et al. 2012). In diagnosed coeliac disease, the reported prevalence of RCD is

0.4-10% (Table 5). The prevalence of ulcerative jejunitis was 0.7% in a coeliac cohort from Derby in United Kingdom (West 2009). In the Tampere region in Finland, 13 (1.9%) of coeliac disease patients had persistent villous atrophy despite a GFD and two of these patients developed symptomatic RCD and one died in EATL (Kaukinen et al. 2007b).

Table 4. Tertiary centres, numbers of recruited patients and length of follow-up in studies on refractory coeliac disease (RCD).

Study	Study population recruited from	RCD patients, all (n)	RCD patients, type I (n)	RCD patients, type II (n)	Follow-up, median (range), years
Cellier et al. 2000	56 referral centres in France	21	16	5	9.5 (1-27)
Maurino et al. 2006	Tertiary centre in Buenos Aires, Argentina	25	12	13	2.4
Al-toma et al. 2007a	Referral centre in Amsterdam, The Netherlands	93	43	50	5 (2-14)
Daum et al. 2009	Tertiary centre in Berlin, Germany	32	23	9	4.6 (1–31)
Malamut et al. 2009	6 referral hospitals in France	57	14	43	5.7 (2-10)
Rubio-Tapia et al. 2009b	Mayo Clinic in Rochester, USA	57	42	15	RCD I: 2.3 RCD II: 1.8
Roshan et al. 2011	Coeliac centre in Boston, USA	34	29	5	
Arguelles-Grande et al. 2013	Coeliac centre in New York, USA	73	67	6	1.8

USA United States of America

Table 5. Prevalence of refractory coeliac disease (RCD) in coeliac disease

Study	Coeliac patients (n)	Developed RCD (n)	Prevalence of RCD
Wahab et al. 2002	158	11	7%
Leffler et al. 2007b	603	10	1.7%
Rubio-Tapia and Murray 2010	204	3	1.5%
Roshan et al. 2011	844	34	4%
Arguelles-Grande et al. 2013	700	73	10%
Sharkey et al. 2013	391	2	0.4%

8.5 Diagnosis of RCD

The first step in the diagnosis of RCD is to confirm the primary coeliac disease diagnosis (Biagi and Corazza 2001). Supporting evidence for coeliac disease includes positive EMA or TG2-ab, presence of HLA-DQ2 or DQ8-haplotype and a family history of the disease (Rubio-Tapia and Murray 2010). Villous atrophy may be caused by autoimmune enteropathy, common variable immunodeficiency, eosinophilic gastroenteritis, Whipple´s disease, tuberculosis, giardiasis, Crohn´s disease, radiation therapy, bacterial overgrowth and AIDS enteropathy; which all should be taken into consideration in differential diagnosis (Daum et al. 2005).

The next step is to exclude dietary regressions, since overt or inadvertent gluten ingestion is the most common cause of non-responsiveness (Abdulkarim et al. 2002, Leffler et al. 2007b, Dewar et al. 2012). Positive coeliac serology may indicate dietary lapses, but is an unreliable marker for gluten contamination (Vahedi et al. 2003). To date, dietary evaluation made by an expert dietician is the gold standard for compliance with the diet (Leffler et al. 2007a).

A concomitant gastrointestinal disorder may cause persistent symptoms despite a strict diet. Reported aetiologies of persistent symptoms in coeliac patients are microscopic colitis, small-intestinal bacterial overgrowth, lactose intolerance, exocrine pancreatic insufficiency and irritable bowel syndrome (Abdulkarim et al. 2002, Leeds et al. 2007, Leffler et al. 2007b, Rubio-Tapia et al. 2009a, Dewar et al. 2012).

The last step in the diagnosis of RCD is to exclude malignancy (Biagi and Corazza 2001). Elevated body temperature, nocturnal sweating, unexplained weight loss, occult or visible gastrointestinal bleeding and abdominal pain are alarm symptoms for an evolved EATL (Gale et al. 2000). In laboratory evaluations,

lactate dehydrogenase and β2-microglobin are frequently elevated (Malamut et al. 2013). The risk of small-intestinal adenocarcinoma is also increased (Askling et al. 2002, Green et al. 2003). Capsule endoscopy, double-balloon enteroscopy and computed tomography (CT) or magnetic resonance imaging (MRI) enterography are useful in cancer screening (Daum et al. 2007, Hadithi et al. 2007a, Mallant et al. 2007, van Weyenberg et al. 2008, Atlas et al. 2011, van Weyenberg et al. 2011). ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG-PET) has been reported to be superior to normal CT scan in detecting EATL (Hadithi et al. 2006).

8.6 Differential diagnosis of RCD types I and II

Immunohistochemistry with CD3 and CD8 double-staining from paraffin-blocks is a widely available and inexpensive method for identifying abnormal IELs (Patey-Mariaud et al. 2000) (Picture 1). However, it cannot differentiate intracellular from extracellular expression of CD3, and in that sense is inaccurate in the differential diagnostics of RCD types I and II. Nevertheless, abnormal IELs (CD3+ CD8-) constituting over 50% of the IEL population are considered diagnostic for RCD type II (Rubio-Tapia and Murray 2010). A reduced number of CD8+ cells may give an erroneous impression of RCD type II, since most of the γδ-lymphocytes, which are abundant in active coeliac disease, are usually CD8- (Verbeek et al. 2008a).

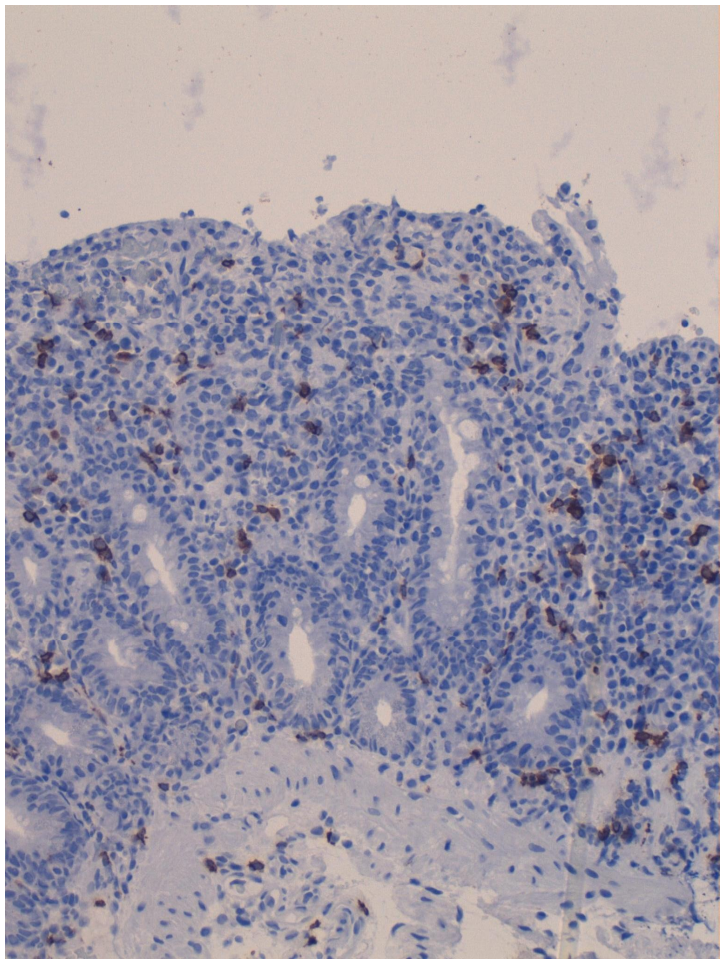
TCR γ-chain rearrangement detected by molecular analysis can improve the diagnostic accuracy of RCD type II, at least in predicting the risk of subsequent EATL (Cellier et al. 2000, Malamut et al. 2009, Rubio-Tapia and Murray 2010). The sensitivity of the method is 62-78% and the specificity 46% in predicting the risk of EATL (Daum et al. 2001, Verbeek et al. 2008b). Combining TCR β- with γ-chain rearrangement analysis may improve the recognition of monoclonal IEL-populations (Perfetti et al. 2012, Tack et al. 2012b). A problem with molecular analysis is the small amount of DNA used in the test, as a dominant clone may give an erroneous impression of monoclonality while there is actually oligoclonality in the specimen, or a small monoclone may be impeded in an oligoclonal sample (Woodward 2013).

Flow cytometry is a superior method in predicting the risk of a later EATL (Verbeek et al. 2008b). The method is able to differentiate between intracellular and extracellular expression of CD3, and abnormal cells constituting over 20% of the IEL population is considered diagnostic for RCD type II (Rubio-Tapia and Murray 2010). With the mentioned cut-off-value the method has a 100% negative predictive value and 100% sensitivity for the development of an EATL (Verbeek et

al. 2008b). Problems associated with this method are poor availability and need for a fresh biopsy specimen.

Monoclonal IELs may be present transiently during dietary lapses, while persistent monoclonality with over 80% of abnormal IELs predicts the development of EATL accurately, underlining the importance of continuous surveillance of the IEL population (Liu et al. 2010). Abnormal IELs can be found diffusely in the gastrointestinal tract including ventricle and colon as well as extra-intestinally in blood, bone marrow and skin (Verkarre et al. 2003a, Malamut et al. 2009, Verbeek et al. 2009).

Picture 1.



The number of CD8+ lymphocytes is low in RCD type II.

8.7 Clinical features of RCD and associated conditions

RCD mostly affects patients over 50 years of age (Table 6), but the condition has also been found in a child (Mubarak et al. 2011). Up to 76% of RCD patients are females (Table 7), which is consistent with uncomplicated coeliac disease as well as other autoimmune diseases (Green and Cellier 2007). Some genetic loci are gender-influenced and immunoregulation in general is subject to hormones, which may explain the difference (Tack et al. 2010a). However, the preponderance of women diminishes somewhat in RCD type II and EATL (Al-toma et al. 2007a, Malamut et al. 2009, Rubio-Tapia et al. 2009b).

In 36-48% of patients with RCD type I the condition is primary and in 52-64% secondary with a mean interval of 10.2 years between the diagnosis of coeliac disease and RCD (Malamut et al. 2009, Arguelles-Grande et al. 2013). In RCD type II, the condition was primary in 49% of patients, and the mean interval for the loss of response to GFD in secondary RCD type II was 7.4 years (Malamut et. al. 2009). The median interval from the diagnosis of coeliac disease to the diagnosis of RCD was 18 and 22 months in two studies from the United States not separating the primary and secondary forms of the disease (Rubio-Tapia et al. 2009b, Roshan et al. 2011).

Typical symptoms of RCD are abdominal pain, involuntary weight loss and persistent diarrhoea (Table 8). Steatohorrhoea, nausea, elevated body temperature and fatigue are also common. The risk of thromboembolic events is increased resulting from dehydration and decreased activity of proteins C and S in the inflammatory milieu (Daum et al. 2005). The risk of infectious diseases is increased as a consequence of malnutrition and hyposplenism (Daum et al. 2005). Other autoimmune diseases are common, affecting up to 50% of RCD patients (Daum et al. 2009). Ulcerative skin lesions may appear on the legs and facial area (Daum et al. 2005).

Signs of malabsorption are common in the laboratory evaluations including low haemoglobin and hypoalbuminaemia (Al-toma et al. 2007a, Rubio-Tapia et al. 2009b). Vitamin and mineral deficiencies are frequently found (Daum et al. 2005). Liver enzymes are elevated in up to 50% of patients (Malamut et al. 2009). Coeliac serology is often negative, reflecting good adherence to GFD, but may be positive in 19-30% of patients despite a strict diet (Table 9). Detectable antibody levels can be maintained by long-lived plasma cells, or T cell clones that have evolved antigen independence and continue to stimulate antibody-secreting plasma cells (Slifka and Ahmed 1998, Daum et al. 2005).

It is vital to visualize the whole small-intestinal mucosa by capsule endoscopy or enteroscopy to exclude EATL and ulcerative jejunitis (van Weyenberg et al. 2008).

Endoscopic lesions may resemble untreated coeliac disease, since the mosaic pattern of the mucosa, flattened villi, scalloping, nodularity and loss of the circular folds are typical in both conditions (Hadithi et al. 2007a). The extent of the involvement may be patchy in 92% of patients (Rubio-Tapia et al. 2009b). Radiological imaging may reveal thickening of the small-intestinal mucosal wall, intussusception and mesenterial lymphadenopathy in 50% of patients (Mallant et al. 2007). Cavitating mesenteric lymph node syndrome and atrophy of the spleen are typical findings in RCD (Hadithi et al. 2007a). EATL may present as multifocal ulcerations on the mucosa or as an isolated stricture or occluding mass (Hadithi et al. 2007a).

Hyposplenism may affect up to 80% of patients with complicated coeliac disease (Di Sabatino et al. 2006a). It predisposes to infections caused by encapsulated bacteria and usually resolves with a GFD. Mesenteric lymph node cavitation syndrome is described by central necrosis of the mesenterial lymph nodes and is not specific for coeliac disease, but may be associated with other diseases of the small intestine: yersinosis, mycobacterial infections and Whipple´s disease (Freeman 2010).

Ulcerative jejunitis has been defined as a premalignant manifestation of RCD type II with no current signs of EATL (UEGW 2001). Multifocal deep mucosal ulcerations can be seen in enteroscopy, located mainly in the jejunum and scarring of these ulcerations may cause stenosis of the small intestine (van Weyenberg et al. 2008). In biopsy specimens, the ulcerations penetrate deep in the mucosal layer and are accompanied with fibrosis, marked villous atrophy and intraepithelial lymphocytosis in the adherent mucosa (Biagi et al. 2000). Abnormal IELs can be found in the ulcerations an also diffusely in the mucosa (Ashton-Key et al. 1997). Ulcerative jejunitis may present as bleeding, occlusion and perforation of the small intestine (Jewell 1983, Biagi et al. 2000). Patients often suffer from steatohorrhoea, severe abdominal pain, weight loss and elevated body temperature (Jewell 1983).

EATL may present as multifocal ulcerations or more rarely as a separate tumour in the small-intestinal wall (Gale et al. 2000, Malamut et al. 2013). There are two types of the disease, and type 1 is associated with coeliac disease (Delabie et al. 2011). Tumour cells usually express CD3 and CD7, but rarely CD4, CD8, CD5 or CD56; and anaplastic cells may also express CD30 (Farstad et al. 2002, van de Vater et al. 2010). IELs far from the tumour may express the same monoclone explaining the rapid spread of the disease in the gastrointestinal tract (van de Vater et al. 2010).

Table 6. Median age at diagnosis of refractory coeliac disease (RCD)

Study	RCD all; years	RCD I; years	RCD II; years
Al-toma et al. 2007a		49	59
Daum et al. 2009	51	51	50
Malamut et al. 2009		49	53
Rubio-Tapia et al. 2009b		58	70
Roshan et al. 2011	53		
Arguelles-Grande et al. 2013	56	55	67

Table 7. Proportion of females among patients with refractory coeliac disease (RCD)

Study	RCD all	RCD I	RCD II
Cellier et al. 2000	76%		
Maurino et al. 2006	60%		
Al-toma et al. 2007a	67%	72%	62%
Daum et al. 2009	69%	74%	56%
Malamut et al. 2009	63%	79%	58%
Rubio-Tapia et al. 2009b	67%	69%	60%
Roshan et al. 2011	76%		
Arguelles-Grande et al. 2013	78%	79%	67%

Table 8. Frequency of symptoms in refractory coeliac disease (RCD)

Study	Weight loss	Diarrhoea	Abdominal pain
Cellier et al. 2000	52%	86%	48%
Abdulkarim et al. 2002	100%	55%	33%
Malamut et al. 2009		RCD I: 86% RCD II: 88%	RCD I: 64% RCD II: 54%
Rubio-Tapia et al. 2009b	100%	100%	RCD I: 45% RCD II: 93%
Roshan et al. 2011	77%	79%	44%

Table 9. Prevalence of positive coeliac serology (transglutaminase-2 or endomysium antibodies) in refractory coeliac disease (RCD)

Study	RCD all	RCD I	RCD II
Al-toma et al. 2007a	0%		
Malamut et al. 2009		29%	28%
Rubio-Tapia et al. 2009b	19%	21%	13%
Roshan et al. 2011	30%		
Arguelles-Grande et al. 2013	22%	22%	33%

8.8 Treatment of RCD I

Hospitalization is needed in severe cases to correct the nutritional status and dehydration (Rubio-Tapia and Murray 2010). Parenteral nutrition is required in up to 70% of cases to alleviate severe malnutrition (Malamut et al. 2009, Rubio-Tapia et al. 2009b). Vitamins and trace elements such as B12-vitamin, folic acid, zinc, copper and magnesium may be substituted as well as calcium and vitamin D to prevent osteoporosis. In deep hypoalbuminaemia, albumin substitution might be considered (Daum et al. 2005).

There are no randomized clinical trials on the treatment of RCD I, reflecting the rarity of the disease. However, a combination of a corticosteroid and azathioprine is usually sufficient to control the malabsorption (Rubio-Tapia and Murray 2010). Corticosteroids are efficient in inducing clinical remission and a histological response may also be seen in RCD type I (Table 10). In RCD type II, 75% of patients may respond clinically to corticosteroids, but histological responses are rare, and the treatment does not prevent EATL from evolving and may even mask the alarming symptoms of a lymphoma (Malamut et al. 2009). Azathioprine has also proved efficient in the treatment of RCD I, with an 80-100% clinical and 80% histological response (Maurino et al. 2002, Goerres et al. 2003). Infections caused by opportunistic bacteria may complicate the treatment (Maurino et al. 2002).

Of RCD type I patients 60% had both a clinical and a histological response to cyclosporine in a small open-label study (Wahab et al. 2000). Case reports on the successful treatment of RCD type I patients with infliximab have been published (Gillet et al. 2002, Chaudhary and Ghosh 2005, Turner et al. 2005, Constantino et al. 2008). Mesalamine and tioguanine have also been used to spare RCD I patients from the long-term use of steroids (Jamma et al. 2011, Tack et al. 2012a). A strict GFD should be maintained and a restricted diet aiming at avoiding the smallest

gluten traces may suffice alone to achieve remission (Hollon et al. 2013). An elemental diet may alleviate the symptoms of malabsorption (Olaussen et al. 2005).

8.9 Treatment of RCD II

RCD II is usually resistant to any known treatment and progresses inevitably to an EATL. Histological remission is rarely achieved (Table 10). Immunosuppressive treatment that does not eradicate the abnormal cell population may in fact facilitate lymphomagenesis (Goerres et al. 2003). Although there are no randomized controlled studies on the treatment of RCD II, cladribine has shown some efficacy in reducing the number of monoclonal IELs. Nevertheless, despite treatment, a substantial number of patients have progressed to EATL and died (Al-toma et al. 2006b, Tack et al. 2011). Alemtuzumab is a CD52 monoclonal antibody which has not proved efficient in the treatment of RCD type II, at least when used as a monotherapy, but may complement other chemotherapy (Verbeek et al. 2006, Vivas et al. 2006). Autologic stem cell transplantation (ASCT) has improved the prognosis in patients who respond to the treatment, but carries a substantial risk of complications (Al-toma et al. 2007b, Tack et al. 2010b). IL-15 monoclonal antibodies, currently under investigation in the treatment of rheumatoid arthritis, may at least theoretically offer an opportunity to eradicate the abnormal IELs by enhancing apoptosis, and thereby preventing progression to EATL (Waldmann 2013).

Table 10. Therapies used in refractory coeliac disease (RCD) with reported clinical and histological responses.

Therapy	Study	Patients treated (n)	Clinical response*	Histological response*†
Prednisolone	Cellier et al. 2000	15	73%	21%
	Malamut et al. 2009	RCD I: 10	90%	40%
		RCD II: 32	77%	33%
Budesonide	Daum et al. 2006	RCD I: 4	100%	25%
		RCD II: 3	33%	33%
	Brar et al. 2007a	RCD I: 24	76%	
		RCD II: 5		
Azathioprine	Maurino et al. 2002	7	71%	71%
	Goerres et al. 2003	RCD I: 10	94%	80%
		RCD II: 8		
	Malamut et al. 2009	RCD I: 1	100%	0%
		RCD II: 5	50%	0%
Tioguanine	Tack et al. 2012a	RCD I: 12	83%	78%
Mesalamine	Jamma et al. 2011	RCD I: 10	75%	
Interleukin-10	Mulder et al. 2001	RCD I: 10	30%	20%

Elemental diet	Olaussen et al. 2005	RCD I: 10	67%	89%
Infliximab	Malamut et al. 2009	RCD I: 1	100%	0%
		RCD II: 3	67%	33%
Cladribine	Al-toma et al. 2006b	RCD II: 17	36%	59%
	Malamut et al. 2009	RCD II: 2	50%	50%
	Tack et al. 2011	RCD II: 32	81%	47%
Autologic stem cell transplantation	Al-toma et al. 2007b	RCD II: 7	100%	
	Tack et al. 2010b	RCD II: 13	85%	38%
Methotrexate	Malamut et al. 2009	RCD II: 8	71%	29%
Anti-CD52	Malamut et al. 2009	RCD II: 2	100%	100%

* Response in combined RCD I and II, if not reported separately in the study
†Recovery of villous atrophy, not the number of abnormal intraepithelial lymphocytes

8.10 Prognosis of RCD

The prognosis of RCD type I is good with an 80-96% 5-year survival (Table 11). In type II RCD, the prognosis is much poorer, as only 45-58% of patients survive for five years. Progression to EATL has been reported in 14% of RCD I and in 14-52% of RCD II patients (Table 12). The 5-year survival in EATL is 8-20% (Gale et

al. 2000, Al-Toma et al. 2007a, Malamut et al. 2013). Factors associated with increased mortality in RCD overall are hypoalbuminaemia, anaemia, age over 65 years, total villous atrophy and presence of an abnormal T cell clone (Rubio-Tapia et al. 2009b).

Table 11. Five year survival in refractory coeliac disease (RCD)

Study	RCD I	RCD II
Maurino et al. 2006	58%	54%
Al-toma et al. 2007a	96%	58%
Daum et al. 2009	90%	53%
Malamut et al. 2009	93%	44%
Rubio-Tapia et al. 2009b	80%	45%

Table 12. Proportion of patients with different subtypes of refractory coeliac disease (RCD) whose disease progressed to enteropathy-associated T cell lymphoma (EATL), time interval from diagnosis of RCD to EATL, and proportion of patients with EATL who died.

	RCD I	RCD II	Time; years	Died
Cellier et al. 2000		14%	4-7	
Farstad et al. 2002		43%		
Maurino et al. 2006		12%		
Al-toma et al. 2007a		52%	4-6	88%
Daum et al. 2009		44%	0-5	75%
Malamut et al. 2009	14%	37%	5	
Rubio-Tapia et al. 2009b		67%	1.5	70%
Roshan et al. 2011		40%	2	
Dewar et al. 2012		57%		

8.11 Management of RCD

The routine management of asymptomatic patients with persistent villous atrophy has not been established. Dietary compliance should be addressed as

intensified counselling targeted at the removal of identifiable gluten sources and avoidance of gluten traces led to the resolution of persisting villous atrophy in 50% of coeliac patients (Sharkey et al. 2013). If no contamination was found, a supersensitive diet excluding wheat starch, barley malt extract and pure oats resulted in mucosal recovery in further 62% of cases (Sharkey et al. 2013). New pharmacological therapies may improve the clinical situation of patients who are highly sensitive to low gluten exposure, but how these drugs will influence the risk of RCD and EATL remains to be seen.

There is no standard approach to the management of patients diagnosed with RCD. The management of type I RCD includes strict adherence to a GFD and aggressive nutritional support, if needed (Rubio-Tapia et al. 2013). There is not sufficient clinical data available to justify recommending any pharmacological therapy (Woodward 2013). The situation is even more obscure in RCD II, which is usually resistant to any known therapy and the progression to EATL cannot be prevented (Tack et al. 2010a). It has been suggested that a regular clinical follow-up could comprise a PET scan and enteroscopy biannually in RCD II and annually in RCD I, but there is no scientific evidence that this intensive surveillance protocol improves the prognosis of these patients (Malamut et al. 2012). Continual monitoring of both the immunophenotype and clonality of IELs is important (Liu et al. 2010), and flow cytometry with a 100% negative predictive value and sensitivity may improve the risk assessment of EATL (Verbeek et al. 2008b). At the moment, EATL is frequently found in a disseminated stage, and the poor clinical condition of patients prevents treatment with aggressive chemotherapy (Daum et al. 2003). Earlier diagnosis of EATL and a better maintained nutritional status would improve the prognosis of these patients (Daum et al. 2003, Malamut et al. 2013). Due to the difficulty of diagnostic and therapeutic regimens, these patients should be referred to regional centres with expertise in the management of RCD (Daum et al. 2005, Woodward 2013).

On the other hand, over half of the patients diagnosed with RCD have type I disease, and do not progress to overt lymphoma (Tables 4 and 12). Repeated endoscopies and PET scans in addition to conventional CT scans are burdensome to the patients and expensive to the health care system. The available therapies can control malabsorption and improve bone mineral density, but currently there are no means to prevent progression to EATL, and ultimately improve the prognosis of these patients.

9. Malignant diseases in coeliac disease and dermatitis herpetiformis

9.1 Overall risk of malignant diseases in coeliac disease and in dermatitis herpetiformis

The risk of malignant diseases in coeliac disease has been evaluated in numerous cohorts, including only clinically diagnosed, only screen-detected or both patient groups. There is a definite detection bias when asymptomatic patients remain undiagnosed and do not contribute with a dilution effect to the estimation of cancer risk (Catassi et al. 2002, Kane et al. 2011). In early studies including mainly coeliac patients suffering from classic symptoms, the overall risk of cancer was two-fold compared to that of general population (Holmes et al. 1989). In recent cohorts, also including patients diagnosed with atypical symptoms, the risk ratio (RR) has been only modestly elevated, 1.0-1.5 (Table 13). In screen-detected patients the RR has been even lower, 0.67-0.94 (Anderson et al. 2007, Lohi et al. 2009). In a recent meta-analysis combining both clinically diagnosed and screen-detected patients, the odds ratio (OR) was 1.07 (Tio et al. 2012). The RR for malignancies in dermatitis herpetiformis has been 1.0-1.3 (Table 16).

The increased risk of cancer in coeliac disease is claimed to result from chronic inflammation causing impaired immunosurveillance, nutritional deficiencies and increased permeability of the gut, enabling carcinogens to enter the immune system (Green et al. 2003). A GFD is thought to protect against future malignancies, at least if initiated early in life (Holmes et al. 1989, Silano et al. 2008), but there are contradictory reports, where no effect was seen (Mearin et al. 2006, Olen et al. 2011, Elfström et al. 2012). The cancer incidence usually declines after the first years from diagnosis and treatment of coeliac disease, and the high cancer incidence during the first years has been thought to reflect an ascertainment bias created by coincidentally diagnosed coeliac disease while investigating cancer-related symptoms or vice versa (Askling et al. 2002, Card et al. 2004, West et al. 2004, Goldacre et al. 2008, Tio et al. 2012).

Table 13. Risk of malignancy in clinically diagnosed coeliac disease patients

Study	Country	Follow-up (years)	Patients (n)	Malignancies (n)	SIR with 95% CI
Holmes et al. 1989	UK	1974-1985	210	39	2.0 (1.4-1.8)
Collin et al. 1994	Finland	1980-1990	335	10	1.5 (0.7-2.8)
Askling et al. 2002	Sweden	1964-1995	11019	249	1.3 (1.2-1.5)
Green et al. 2003	USA	1981-2000	381	43	1.5 (0.3-5.7)
Card et al. 2004	UK	1978-2001	869	31	1.0 (1.1-1.6)
West et al. 2004	UK	1987-2002	4732	134	1.3 (1.1-1.6)
Viljamaa et al. 2006	Finland	1960-2000	781	49	1.2 (0.9-1.5)
Silano et al. 2007	Italy	1982-2005	1968	55	1.3 (1.0-1.7)
Goldacre et al. 2008	UK	1963-1999	1997	91	1.2 (0.9-1.4)
Grainge et al. 2012	UK	1970-2004	435	69	1.4 (1.1-1.8)

SIR standardized incidence ratio, CI confidence interval, UK United Kingdom, USA United States of America

9.2 Risk of lymphoma

The risk of lymphoma has also been evaluated in both clinically diagnosed and screen-detected coeliac patients. In early studies, the risk of NHL was markedly increased, up to 43-fold (Holmes et al. 1989), but significantly lower in later studies, namely to 2.1-12-fold (Table 14). In screen-detected patients the RR for NHL has been 0.6-7.5 (Table 15). In the previously mentioned meta-analysis, again combining these two types of cohorts, the OR for NHL was 2.61 (Tio et al. 2012). Although the risk of NHL is greatest during the first years after diagnosis of coeliac disease, it has been reported to remain elevated for up to 15 years from the diagnosis (Grainge et al. 2012). This contradicts the supposedly beneficial effect of a GFD (Holmes et al. 1989, Anderson et al. 2009, Gao et al. 2009, Grainge et al. 2012). In DH, the RR for NHL has been 1.6-10.3, which is the same level as in the most recent studies on coeliac disease (Table 16).

Chronic antigen stimulation causing persistent activation of the immune system is considered to result in elevated risk of lymphoma (Green et al. 2003). Lymphomas occurring in coeliac patients are not exclusively EATLs, since B cell lymphomas have also been detected, and in DH patients B cell lymphomas even outnumber T cell lymphomas (Farre et al. 2004, Mearin et al. 2006, Hervonen et al. 2005, Smedby et al. 2005, Smedby et al. 2006, Elfström et al. 2011, Ansell et al. 2011). Increased risk of B cell lymphoma may be related to a concomitant autoimmune disease instead of coeliac disease (Smedby et al. 2005). The combination of coeliac disease with another autoimmune disease may render a patient extremely susceptible to lymphoma (Smedby et al. 2005, Smedby et al. 2006, Ansell et al. 2011).

Table 14. Risk of non-Hodgkin's lymphoma (NHL) in clinically diagnosed coeliac disease (CD) patients.

Study	CD (n)	NHL (n)	SIR (95% CI)
Holmes et al. 1989	210	9	43 (20-81)
Delco et al. 1999	458	13	4.5 (2.0-10)
Askling et al. 2002	11019	44	6.3 (4.2-13)
Green et al. 2003	381	9	9.1 (4.7-13)
Card et al. 2004	869	12	5.8 (1.6-15)
West et al. 2004	4732	23	4.8 (2.7-8.1)
Smedby et al. 2005	11650	56	6.6 (5.0-8.6)
Smedby et al. 2006	19	3055	2.1 (1.0-4.8)
Mearin et al. 2006	13	1446	3.3 (1.4-7.9)
Viljamaa et al. 2006	781	5	3.2 (1.0-7.5)
Silano et al. 2007	1968	20	4.7 (2.9-7.3)
Goldacre et al. 2008	1997	9	3.3 (1.5-6.3)
Gao et al. 2009	54	37869	5.4 (3.6-8.1)
Grainge et al. 2012	435	14	12 (6.6-20)

SIR standardized incidence ratio, CI confidence interval

Table 15. Risk of non-Hodgkin's lymphoma (NHL) in screen-detected coeliac disease (CD) patients

Study	CD (n)	NHL (n)	SIR (95% CI)
Catassi et al. 2002	6	653	3.1 (1.3-7.6)
Farre et al. 2004	2	298	0.6 (0.1-3.8)
Mearin et al. 2006	9	1446	1.3 (0.6-2.7)
Anderson et al. 2007	490	2	7.5 (0-18)
Lohi et al. 2009	73	2	6.4 (1.5-27)

SIR standardized incidence ratio, CI confidence interval

Table 16. Risk of any malignancy and non-Hodgkin's lymphoma (NHL) in dermatitis herpetiformis.

Study	Country	Patients (n)	Any malignancy; SIR (95% CI)	NHL; SIR (95% CI)
Sigurgeirsson et al. 1994	Sweden	976	males: 1.4 (1.1-1.7)	6.4 (2.7-12.5)
			females: 1.2 (0.8-1.7)	4.5 (0.9-13.2)
Collin et al. 1996	Finland	305	1.3 (0.7-2.1)	10.3 (2.8-26.3)
Askling et al. 2002	Sweden	1354	1.2 (1.0-1.4)	1.9 (0.7-4.0)
Viljamaa et al. 2006	Finland	366	1.0 (0.6-1.5)	6.0 (2.4-12.4)
Lewis et al. 2008	UK	846	1.0 (0.7-1.5)	1.6 (0.4-6.1)

SIR standardized incidence ratio, CI confidence interval, UK United Kingdom

9.3 Risk of gastrointestinal and other common malignancies

The risk of malignancies of the gastrointestinal tract has been found to be elevated in coeliac disease. The risk of oesophageal cancer has reportedly been elevated up to four-fold and the risk of stomach cancer up to three-fold (Table 18). The risk of small-intestinal carcinoma has been markedly increased, up to 34-fold (Green et al. 2003). Although the risk of colon and liver cancer in coeliac patients has been reported to be increased in some studies, there are also contradictory findings, and the issue remains debatable (Table 18).

The reported risk of lung cancer has been significantly decreased in only one study (West et al. 2004), whereas the risk of breast cancer has been decreased in all previous reports (Table 17).

Table 17. Risk of common cancers in coeliac disease.

Study	Patients	Breast		Lung		Prostate	
	(n)	SIR	95% CI	SIR	95% CI	SIR	95% CI
Askling et al. 2002	11019	0.3	0.1-0.5	1.0	0.5-1.7	0.7	0.4-1.2
Green et al. 2003	381	1.2	0.2-7.2	0.8	0.1-7.2		
Card et al. 2004	869	0.6	0.1-1.7	1.5	0.6-3.3		
West et al. 2004	4732	0.4	0.2-0.7	0.3	0.1-1.0	1.0	0.4-2.4
Viljamaa et al. 2006	781	0.9	0.4-1.7	0.6	0.1-2.1		
Silano et al. 2007	1968	0.2	0.0-0.6				
Goldacre et al. 2008	1997	0.5	0.2-1.0	1.1	0.6-1.8	0.7	0.2-1.7
Grainge et al. 2012	435	0.7	0.2-1.7	0.8	0.3-1.7		
Ludvigsson et al. 2012a	10995					0.9	0.8-1.1
Ludvigsson et al. 2012b	18365			1.1	0.9-1.3		
Ludvigsson et al. 2012c	17852	0.9	0.7-1.0				

SIR standardized incidence ratio, CI confidence interval

Table 18. Risk of gastrointestinal cancers in coeliac disease.

	Patients	Oesophagus		Stomach		Small intestine		Colon		Liver		Pancreas	
	(n)	SIR	95% CI	SIR	95% CI	SIR	95% CI	SIR	95% CI	SIR	95% CI	SIR	95% CI
Askling et al. 2002	11019	4.2	1.6-9.2	0.9	0.3-2.0	10	4.4-20	1.9	1.2-2.8	2.7	1.3-4.7	1.9	0.9-3.6
Green et al. 2003	381	12	6.5-21			34	24-42	0.8	0.1-7.2				
Viljamaa et al. 2006	781	0.0	0-11	1.2	0.2-4.5	0	0-80	1.1	0.3-2.8				
Silano et al. 2007	1968			3.0	1.3-4.9	25	8.5-51	1.1	0.7-1.6				
Goldacre et al. 2008	1997	2.6	0.8-6.1	1.8	0.8-3.6			1.2	0.6-2.2			0.6	0.1-2.1
Elfström et al. 2012	28882	1.2	0.6-2.7	1.1	0.7-1.8	2.2	1.2-4.1	1.1	0.9-1.4	1.8	1.2-2.6	1.0	1.0-2.0
Grainge et al. 2012	435	2.9	0.6-8.4			11	0.3-62	1.2	0.4-2.5				
Volta et. al 2014	1757							0.3	0.1-0.5				

SIR standardized incidence ratio, CI confidence interval

1. Aims of the dissertation

The aim of this dissertation was to describe various forms of coeliac disease that do not respond to a GFD. The dissertation comprises the spectrum of non-responsive coeliac disease from persistent villous atrophy and intraepithelial lymphocytosis on the small-intestinal mucosa to overt RCD, and finally describes the risk of malignancies in coeliac disease compared to population in general.

The specific aims were

1. To ascertain the frequency of persistent villous atrophy and intraepithelial lymphocytosis on the small-intestinal mucosa of coeliac disease patients with a long-term strict GFD, and to assess predisposing factors to and the clinical relevance of persistent intraepithelial lymphocytosis without villous atrophy (I).

2. To describe the prevalence and clinical characteristics of RCD in Finland, and to assess the predictive effect of clinical characteristics present at the primary coeliac disease diagnosis on subsequently developing RCD (II).

3. To establish the risk of malignant diseases, especially NHL, in a large, unselected cohort of coeliac disease patients (III).

2. Patients

2.1 Patients in Study I

The study cohort comprised of 177 volunteer adult coeliac disease patients, who had been on a strict GFD for at least two years. The diagnosis of coeliac disease had to be biopsy-proven. Patients with symptomatic RCD were excluded from this study, which concentrated on the mucosal healing of long-term treated coeliac disease patients. Newspaper advertisements and a newsletter from the Finnish Coeliac Society were used to achieve nationwide recruitment of patients.

The patients enrolled were divided into three subgroups according to the degree of mucosal recovery seen in the duodenal biopsies performed. The "normal" group comprised patients with completely healed mucosa. Patients in the "inflammation" group had persistent intraepithelial lymphocytosis, but normal villous architecture. The "atrophy" group comprised patients with persistent villous atrophy.

2.2 Patients in Study II

The Finnish guidelines for coeliac disease advocate control biopsies to be taken from all adult coeliac disease patients after one year on a GFD, and if there is evidence of persistent villous atrophy or ongoing symptoms, these patients are to be evaluated for complications. In the Finnish health care system, these evaluations are made exclusively in the central and university hospitals. For this study, specialists from all hospital districts in Finland were asked to submit clinical data on all their patients diagnosed with RCD since 2000. Colleagues from 11 out of 21 hospital districts responded to this survey, providing the data on altogether 44 RCD patients. According to the international diagnostic criteria of RCD (Biagi and Corazza 2001, Rubio-Tapia and Murray 2010, Ludvigsson et al. 2013b), patients with dietary non-adherence, other aetiologies of villous atrophy and intestinal lymphomas at diagnosis of RCD were excluded. All patients were evaluated by expert dieticians in the respective hospitals trained to evaluate the diet even for hidden gluten traces.

To assess the prevalence of RCD in coeliac disease, the number of adult coeliac disease patients living on the participating hospital districts was obtained from the

Social Insurance Institution of Finland (SII). The SII has maintained a register of all Finnish coeliac disease patients receiving compensation for the additional costs of their GFD since October 2002. A prerequisite for this payment has been that the diagnosis of coeliac disease is biopsy-proven. The quality of the register has been evaluated in earlier studies and 99% of the diagnoses of coeliac disease have been proven correct (Collin et al. 2007, Virta et al. 2009). The SII also provided the number of adult inhabitants living on the hospital districts in December 2012, when the point prevalence of RCD in the population in general was calculated.

Patients with uncomplicated coeliac disease served as controls while the clinical charasteristics present at the primary coeliac disease predicting the risk of future RCD were assessed. This cohort comprised of 886 biopsy-proven coeliac disease patients enrolled in an earlier study investigating different clinical, pathogenic and genetic aspects of coeliac disease (Kurppa et al. 2012). These patients were recruited in a nationwide search using newspaper advertisements and via local coeliac societies. Only patients diagnosed with coeliac disease in adulthood were included, since no childhood coeliac disease diagnoses were present among the RCD patients. This control group was diagnosed with coeliac disease during the same time period as the RCD cohort and came from the same geographical regions. All patients in the control group responded well to a GFD.

2.3 Patients in Study III

The abovementioned register of the SII was also used in Study III. For this study, all coeliac disease patients receiving the dietary reimbursement since October 2002 were included. In 2002-2003 most patients with an earlier coeliac disease diagnosis applied for this compensation, and at the end of 2003, there were 20,448 coeliac patients on the register. Since then, approximately 1,700 people have been added to the register annually, giving altogether 32,439 coeliac disease patients on the register at the end of the year 2011.

2.4 Ethical considerations

The protocols of Studies I and II were approved by the Ethics Committee of Tampere University Hospital. All subjects in Study I and subjects in the control group of Study II gave their written informed consent. The protocols of Studies II and III were approved by the National Institute for Health and Welfare.

3. Methods

3.1 Upper intestinal endoscopy and small-intestinal biopsies in Study I

Altogether six biopsies were taken from the distal duodenum in upper intestinal endoscopy. The Vh/CrD was determined and a ratio below 2.0 was considered compatible with villous atrophy and crypt hyperplasia (Marsh III). There is some variation in the literature regarding the cut-off values applied for intraepithelial lymphocytosis (Lee et al. 2003, Bardella et al. 2007, Lanzini et al. 2009, Aziz et al. 2010). As values between 25 to 29 IELs per 100 enterocytes represent a grey area, values over 30 IELs per 100 enterocytes represent a definite pathological intraepithelial lymphocytosis (Villanacci et al. 2011). In this study the density of IELs was measured and values over 30 IELs per 100 enterocytes were first regarded as intraepithelial lymphocytosis, but a separate analysis was carried out applying the lower cut-off value (25 IELs per 100 enterocytes) as well. In endoscopy, biopsies were also taken from the stomach for routine histological assessment and *H. pylori* staining.

3.2 Clinical and dietary evaluation in Study I

Patients were interviewed on their symptoms leading to the diagnosis of coeliac disease, duration of GFD, family history of coeliac disease and other diagnosed autoimmune diseases. Current use of NSAIDs and proton-pump inhibitors (PPIs) were recorded. A trained dietician evaluated the strictness of the diet and the consumption of fibre, wheat starch and oats by interviewing all patients and also by assessing 4-day food diaries self-administered by patients. Body mass indices (BMI) were computed as weight in kilograms per height in metres^2.

Current gastrointestinal symptoms were evaluated by a validated Gastrointestinal Symptom Rating Scale (GSRS) questionnaire, which is widely used in coeliac disease and comprises 15 items with 5 subdimensions: diarrhoea, indigestion, constipation, abdominal pain and reflux (Svedlund et al. 1988, Midhagen and Hallert 2003, Viljamaa et al. 2005b, Kurppa et al. 2010). Each item is

graded from one to seven and a higher score indicates more gastrointestinal symptoms.

Quality of life was evaluated by a validated Psychological General Well-Being (PGWB) questionnaire, which is also widely used in coeliac disease and contains 22 items with 6 subdimensions: anxiety, depression, well-being, self-control, general health and vitality (Dimenäs et al. 1996, Viljamaa et al. 2005b, Roos et al. 2006, Nachman et al. 2009, Kurppa et al. 2010, Ukkola et al. 2011). Total score ranges from 22 to 132 and higher a score indicates better psychological well-being.

Serum IgA-class EMAs were determined by an indirect immunofluorescence method and a serum dilution over 1:5 was considered positive. TG2-ab titers were measured by ELISA (Celikey, Pharmacia Diagnostics, Germany) and values over 5.0 were considered positive (Sulkanen et al. 1998). Blood haemoglobin (Hb), red blood cell mean corpuscular volume (MCV), serum iron and erythrocyte folic acid levels were measured by standard laboratory methods. HLA DQ2 and DQ8 allele genotyping was performed by DELFIA® Coeliac Disease Hybridization Assay (Perkin-Elmer Life and Analytical Sciences, Wallac Oy, Finland) or the SSP DQB1 low resolution kit (Olerup SS AB, Sweden). Some of the samples were typed by HLA tagging SNPs (Koskinen et al. 2009).

3.3 Collection of data on RCD patients in Study II

For the enrolled RCD patients gender, age at primary diagnosis of coeliac disease, symptoms leading to the diagnosis, coeliac serology (EMA and TG2-ab positivity), presence of HLA DQ2 or DQ8 haplotypes, serum Hb levels and histological findings in the first diagnostic small-intestinal biopsy were recorded. Concomitant autoimmune diseases were obtained from medical records. Trained dieticians interviewed all the enrolled RCD patients in the respective hospitals and previous dietary transgressions were recorded; although all the included patients had adhered to a strict diet for at least 12 months before the diagnosis of RCD was made. Patient's age and symptoms at the time when RCD was first diagnosed were also recorded, likewise serum Hb and albumin levels, coeliac antibody positivity and BMI. Clinical manifestations of RCD, including ulcerative jejunitis, hyposplenism and mesenterial lymph node cavitations were listed if found. Treatment of RCD was documented: duration of the medication as well as clinical and histological response. EATLs evolving and other diagnosed malignancies as well as dates and causes of death were recorded.

For differential diagnosis between RCD type I and type II, histological and immunogenetical reports were obtained from the corresponding departments in the participating hospitals. Patients presenting with over 50% abnormal IELs,

determined by CD3- and CD8-immunostaining were considered to have type II RCD and the differential diagnosis was confirmed by gamma chain T cell monoclonal rearrangement analysis (Biagi and Corazza 2001, Rubio-Tapia and Murray 2010).

3.4 Collection of data on control subjects in Study II

Age, presenting symptoms, coeliac serology titers and duodenal mucosal histology at diagnosis of coeliac disease were collected from medical records and in structured interviews. The presence of other autoimmune diseases and adherence to GFD were listed.

3.5 Collection of data for Study III

The coeliac cohort was collated with the Finnish Cancer Registry that covers over 98% of diagnosed malignancies in Finland, and includes of both clinical and histopathological data (Teppo et al 1994). Follow-up began in October 2002, when the SII register was founded and the endpoints were 31st December 2011, or at death, yielding a total of 232,505 person years. Cancers occurring before entry to the SII register were excluded.

Time of the coeliac disease diagnosis was not recorded in the SII Register for 20,448 patients added to the register soon after it was founded in 2002-2003. For the 11,991 patients added to the register in 2004 or later, the exact month of the diagnosis was known. A subgroup analysis was carried out with these cases, stratifying the follow-up to under 2 years, 2-4.9 years and over 5 years.

3.6 Statistical analysis

In Study I, the quantitative data were expressed as medians and ranges or means and 95% CIs. Statistical differences were evaluated using Mann-Whitney test. Fisher´s and Kruskal-Wallis tests were used in cross-tabulations. P values <0.05 were considered statistically significant. Statistical testing was performed using SPSS 17.0.

In Study II, results were summarized using means and percentages. Fisher´s exact test was used for categorical variables and t-test for continuous variables to

compare patients with uncomplicated disease to RCD patients. SPSS version 19.0 was used for statistical analysis.

In Study III, the expected numbers of malignant diseases were calculated by multiplying the person-years of follow-up by the incidence rate of each malignant disease in the respective sex, age and calendar period in the Finnish population. SIR was defined as the ratio of observed-to-expected cancers. 95% CIs were assessed by assuming that the observed numbers followed a Poisson distribution.

4. Results

4.1 Mucosal recovery and factors associated with persistent intraepithelial lymphocytosis in long-term treated coeliac disease in Study I

Seven (4%) out of the 177 enrolled coeliac patients had persistent villous atrophy and in view of this small number, were not included in the statistical analysis, but were presented for comparison. Of the patients 96 (54%) had normal villous structure, but persistent intraepithelial lymphocytosis while 74 (42%) had completely normal duodenal mucosa.

The duration of GFD was longer in patients with completely recovered small-intestinal mucosa than in those with persistent intraepithelial lymphocytosis (10 vs 9 years respectively, p=0.014). Besides this, only the consumption of oats was more frequent in patients with persistent mucosal inflammation compared to those with normal mucosa (84% vs 64%, p=0.012). The use of products containing wheat starch and fibre was similar between the respective study groups (Table 19). Age and gender distributions; family history and clinical presentation of coeliac disease were also similar between the study groups (Table 19). The prevalence of other autoimmune diseases and *H. pylori* gastritis, and the use of medications were also comparable between the groups (Table 19).

Table 19. Clinical data on patients in Study I (n=177) categorized according to small-intestinal mucosal histology.

	Villous atrophy (n=7)	Inflammation (n=96)	Normal (n=74)	Normal vs inflammation (P value)
Female, n (%)	4 (57)	74 (77)	51 (69)	0.293
Age, median (range); years	52 (38-72)	55 (23-81)	57 (21-75)	0.712
Duration of GFD, median (range); years	7 (3-19)	9 (2-41)	10 (3-34)	0.014
Clinical presentation, n (%)				0.717
Gastrointestinal symptoms*	7 (100)	78 (81)	59 (80)	
Extraintestinal symptoms †	0 (0)	17 (18)	13 (17)	
Screen-detected	0 (0)	1 (1)	2 (3)	
Family history of coeliac disease, n (%)	3 (43)	34 (35)	30 (41)	0.526
Other autoimmune disease, n (%)	0 (0)	17 (18)	15 (20)	0.696
Using NSAID, n (%)	2 (29)	18 (21)	10 (17)	0.670
Using PPI, n (%)	2 (29)	10 (12)	7 (12)	1.000
H. pylori gastritis, n (%)	0 (0)	4 (5)	1 (2)	0.649
Consuming wheat starch, n (%)	6 (100)	87 (93)	62 (89)	0.421
Consuming oats, n (%)	4 (67)	79 (84)	46 (64)	0.012
Fibre, median (range); g/day	17 (8-27)	18 (9-41)	18 (8-51)	0.502

GFD gluten-free diet, NSAID non-steroidal anti-inflammatory drug, PPI proton-pump inhibitor

*Abdominal pain, diarrhoea, weight loss

†Anaemia, dermatitis herpetiformis, osteoporosis, neurologic manifestations

4.2 Clinical outcome of persistent intraepithelial lymphocytosis in Study I

Patients with persistent intraepithelial lymphocytosis had a significantly lower Vh/CrD than those with completely healed mucosa, the figures being still within the normal range (> 2.0) in both study groups (Table 20). There were no differences in BMI, malabsorption parameters, current gastrointestinal symptoms and psychological well-being (measured by GSRS and PGWB respectively) between the study groups (Table 20). Two malignancies occurred in both the inflammation group (one uterine and one prostate cancer) and in the normal group (one breast cancer and one lymphoma). There were no malignancies in the atrophy group.

The data were stratified according to the duration of GFD in the following time strata: 2-5 years, 5-10 years, 10-15 years, 15-20 years and over 20 years. In these time strata; respectively 85%, 63%, 51%, 42% and 48% had persisting intraepithelial lymphocytosis. Vh/CrD, malabsorption parameters, GSRS and PGWB did not differ between these stratified groups (data not shown).

Table 20. Clinical outcome of the patients in Study I categorized according to mucosal recovery.

	Villous atrophy (n=7)	Inflammation (n=96)	Normal (n=74)	P value*
Vh/CrD, median (range); ratio	1.3 (0.4-1.8)	2.9 (2.1-4.3)	3.2 (2.1-4.5)	0.042
BMI, median (range); kg/m^2	24 (21-29)	25 (17-36)	26 (19-38)	0.213
Hb, median (range); g/dl	140 (128-161)	139 (112-181)	144 (110-166)	0.295
MCV, median (range);fl	90 (86-96)	90 (83-100)	90 (86-96)	0.692
Serum iron, median (range); g/dl	19 (15-27)	19 (6-37)	18 (9-34)	0.809
Erythrocyte folic acid, median (range); nmol/l	486 (243-1849)	505 (179-2005)	583 (218-2832)	0.374
GSRS, mean (95% CI)	1.73 (1.39-2.07)	1.80 (1.24-2.37)	1.83 (1.34-2.31)	0.875
PGWB, mean (95% CI)	113 (111-115)	105 (91-120)	106 (96-117)	0.674

* Normal vs inflammation

Vh/CrD villous height/crypt depth ratio, BMI body mass index, Hb haemoglobin, MCV mean corpuscular volume, GSRS Gastrointestinal Symptom Rating Scale, PGWB Psychological General Well-Being

4.3 Lower cut-off value for increased lymphocyte count in Study I

When a lower cut-off value of 25 IELs per 100 enterocytes was applied, no factors contributing to the intraepithelial lymphocytosis were found. The association between persisting intraepithelial lymphocytosis and consumption of oats was no longer present (p=0.120), neither was there a lower Vh/CrD ratio in the inflammation group (p=0.575). There were no differences between the

inflammation and normal group in malabsorption parameters, GSRS and PGWB (data not shown).

Using this lower cut-off value for intraepithelial lymphocytosis and time stratification by the duration of GFD; after 2-5 years on the GFD 89% had persistent intraepithelial lymphocytosis, after 5-10 years 75%, after 10-15 years 64%, after 15-20 years 67% and after 20 years 67%.

4.4 Prevalence of RCD in coeliac disease and in general population

In December 2012, 38 RCD patients were resident in the participating hospital districts as well as 12,243 adult coeliac disease patients and 1,730,555 adult inhabitants, giving a point prevalence of 0.31% of RCD in diagnosed coeliac disease and 0.002% in general population. The prevalence of diagnosed coeliac disease was 0.7% in these hospital districts at the same time-point.

4.5. Clinical characteristics of RCD patients

The primary diagnosis of coeliac disease was made in RCD patients at a mean age of 53 years, and 59% were females. Total or subtotal villous atrophy was found in 58% in the diagnostic small-intestinal biopsy and the remainder had partial or unknown atrophy (Table 22). Negative coeliac serology at diagnosis was found in 30%. Symptoms leading to the diagnosis were predominantly classical and none of the RCD patients were detected by screening. Three RCD patients presented with dermatitis herpetiformis.

The mean age at diagnosis of RCD was 63 years: 61% had total or subtotal villous atrophy, and 27% had positive coeliac serology despite a strict GFD at diagnosis of RCD. Of the 44 enrolled RCD patients, 30 (68%) had type I and 10 (23%) had type II disease; in 4 (9%) the type remained undetermined. Approximately 40% of the patients suffered from diarrhoea, abdominal pain and weight loss (Table 21), while 37% were anaemic and 34% had hypoalbuminaemia. Seven patients had ulcerative jejunitis and two hyposplenism with mesenterial lymph node cavitations. Four had microscopic colitis. Twelve patients had concomitant autoimmune diseases: autoimmune thyreoiditis, diabetes mellitus type I, polymyalgia rheumatica, psoriasis, IgA deficiency, rheumatoid arthritis, glomerulonephritis, autoimmune pancytopenia, colitis ulcerosa, autoimmune haemolytic anaemia and sarcoidosis. Nine patients had had dietary lapses in the

past, but at diagnosis of RCD they had all been on a strict diet for a minimum of 12 months.

Nine RCD I and three RCD II patients were treated with corticosteroids and all nine RCD I patients also received azathioprine. One RCD II patient was treated with cladribine. The majority, 25 (75%) of the RCD patients, had no other treatment than a GFD.

The RCD patients were followed-up for a mean 45 months (range 0-168) after diagnosis. During that time four progressed to overt lymphoma and two of them died. Apart from these two, three RCD patients died of malabsorption and one of neutropenic sepsis. Four patients were diagnosed with other malignancies: one adenocarcinoma of the stomach, one peripheric T cell lymphoma, one prostate carcinoma and one melanoma.

Table 21. Characteristics of patients with refractory coeliac disease (RCD) (n=44) in Study II at diagnosis of RCD.

	RCD all (n=44)	RCD I (n=30)	RCD II (n=10)	RCD undetermined (n=4)
Female, n (%)	26 (59)	18 (60)	5 (50)	3 (75)
Age at diagnosis of RCD, median (IQR); years	64 (53-73)	63 (49-70)	66 (64-70)	73 (63-76)
Histology at diagnosis of RCD, n (%)				
Total villous atrophy	27 (61)	17 (57)	9 (90)	1 (25)
Partial villous atrophy	17 (39)	13 (43)	1 (10)	3 (75)
Seropositivity in RCD, n (%)†	11 (27)	8 (27)	2 (20)	1 (100)
Diarrhoea in RCD, n (%)	17 (39)	8 (27)	7 (70)	2 (50)
Weight loss in RCD, n (%)	17 (39)	10 (33)	6 (60)	1 (25)
Abdominal pain in RCD, n (%)	18 (42)	10 (33)	6 (60)	2 (50)
Hb, mean (range); g/dl	129 (87-164)	129 (108-160)	127 (87-164)	128 (121-133)
Albumin, mean (range); g/dl	34 (13-47)	36 (16-47)	28 (13-43)	36 (34-38)
BMI, mean (range); kg/m^2	23 (17-28)	23 (20-28)	23 (19-25)	21 (17-28)
Progressed to EATL, n (%)	4 (9)	1 (3)	3 (20)	0 (0)
Diagnosed with another malignancy, n (%)	4 (9)	2 (7)	2 (20)	0 (0)
Died, n (%)	6 (14)	3 (10)	3 (30)	0 (0)

IQR interquartile range, Hb haemoglobin, BMI body mass index, EATL enteropathy-associated T cell lymphoma

† Denominator varies depending on the available data

4.6. Comparison of RCD and uncomplicated coeliac disease

RCD patients were older at primary coeliac disease diagnosis than patients with uncomplicated disease, 53 vs 43 years respectively ($p<0.001$). Seronegativity at diagnosis was significantly more common in RCD patients than in controls, but family history of coeliac disease was more common in controls (Table 22). Haemoglobin levels or the degree of mucosal damage did not differ significantly between groups. This notwithstanding, patients with RCD had experienced significantly more often weight loss or diarrhoea before the diagnosis. Dietary transgressions were significantly more common among patients with subsequent RCD. About 30% of patients in both cohorts were diagnosed with other autoimmune diseases, but malignant diseases were significantly more common among the RCD patients.

Table 22. Comparison of patients with refractory coeliac disease (RCD) and uncomplicated disease at primary diagnosis of coeliac disease.

	RCD (n=44)	Uncomplicated disease (n=866)	P value
Female, n (%)	26 (59)	672 (76)	0.012
Age at diagnosis, median (IQR); years	56 (41-63)	44 (33-53)	<0.001
Histology, n (%)			0.293
Total villous atrophy	25 (58)	436 (49)	
Partial villous atrophy	13 (30)	243 (27)	
Unknown atrophy	5 (12)	175 (20)	
Normal	0 (0)	32 (4)	
Seronegativity, n (%)	8 (30)	23 (5)	<0.001
Weight loss, n (%)	13 (36)	138 (16)	0.001
Diarrhoea, n (%)	20 (54)	337 (38)	0.050
Blood Hb, mean (range); g/dl	123 (87-164)	127 (61-173)	0.258
Family history of coeliac disease, n (%)	12 (28)	578 (66)	<0.001
Duration of GFD, mean (range); years	13 (0-44)	10 (0-37)	0.017
GFD, n (%)			<0.001
Strict	35 (80)	852 (96)	
Lapses	9 (20)	34 (4)	
Other autoimmune diseases, n (%)	13 (30)	252 (29)	0.911
Malignant diseases, n (%)	7 (16)	43 (5)	0.002

IQR interquartile range, Hb haemoglobin, GFD gluten-free diet

4.7 Risk of malignant diseases in coeliac disease

There were 32,439 patients in the whole cohort, of whom 11,281 were males and 21,158 females, yielding a total of 80,675 and 151,830 person-years respectively. On being added to the register 5,218 patients were 15-29 years of age (28,921 person years), 18,134 were 30-59 years (120,930 person-years) and 9,087 were over 60 years (82,654 person-years).

Altogether 1,626 cancers were detected in these patients, while 1,735 were expected, giving an SIR of 0.94 (95% CI 0.89-0.98, p<0.01) for any malignancy. In females the SIR was lower (0.89, 95% CI 0.83-0.94, p<0.001) and in males equal to

that of general population (1.00, 95% CI 0.93-1.07). NHLs occurred in 129 coeliac patients, while 66 were expected, giving a increased SIR of 1.94 (95% CI 1.62-2.29, p<0.001).

Regarding gastrointestinal malignancies, 27 small-intestinal cancers were detected in coeliac patients while only 6 were expected, giving an increased SIR of 4.29 (95% CI 2.83-6.24, p<0.001). The SIR for colon cancer was also slightly increased (1.35, 95% CI 1.13-1.58, p<0.01). For pancreatic cancer the SIR was decreased (0.73, 95% CI 0.53-0.97, p<0.05).

Breast cancers were detected in 239 female coeliac disease patients, while 340 were expected, giving a decreased SIR of 0.70 (95% CI 0.62-0.79, p<0.001). The SIR for lung cancer was decreased (0.60, 95% CI 0.48-0.74, p<0.001). SIRs for renal (0.72, 95% CI 0.51-0.99, p<0.05) and bladder cancer (0.53, 95% CI 0.35-0.77, p<0.001) were also decreased in coeliac disease. The SIR for basal cell carcinoma of the skin was slightly increased (1.13, 95% CI 1.03-1.22, p<0.05).

4.8 Time-stratified analysis in Study III

In the 11,991 patients diagnosed with coeliac disease in the period 2004-2011, follow-up of 0-1.9 years yielded 21,508 person years, 2-4.9 years 21,556 person-years and over 5 years 7,794 person-years.

For any cancer the SIR was equal to that of general population in the first five years from diagnosis of coeliac disease, but increased slightly after that (1.31, 95% CI 1.04-1.63, p<0.05). This was largely attributable to the increased risk of colon cancer after the first five years from diagnosis of coeliac disease (SIR 3.12, 95% CI 1.56-5.58, p<0.01). The SIR for NHL, on the other hand, was only increased during the first two years from diagnosis of coeliac disease (SIR 2.56, 95% CI 1.37-4.38, p<0.01), but not in the long-run. The SIR for skin basal cell cancer was only increased in the first two years from diagnosis of coeliac disease (1.37, 95% CI 1.00-1.83, p<0.05).

5. Discussion

5.1 Persistent villous atrophy and intraepithelial lymphocytosis

This dissertation found mucosal recovery in 96% of long-term treated coeliac patients with only 4% evincing persistent villous atrophy. Compared to earlier studies, where 24-78% of coeliac patients reportedly had persistent villous atrophy (Table 3), this is an exceptionally good result. Variation on the reported mucosal recovery figures is partially explained by the differences in the duration of GFD; at shortest it was only one year (Sharkey et al. 2013), while in the present study it was mean 11 years. Nevertheless, no marked improvement in the villous structure was observed after two first years on a GFD in a recent study (Lebwohl et al. 2014), implying that the length of GFD alone did not explain good mucosal recovery in the present study. In children, mucosal recovery is usually faster and more complete than in adults (Wahab et al. 2002, Lanzini et al. 2009). The mean age of patients in this study was 56 years, which indicates that young age did not contribute to the excellent mucosal recovery, either. A plausible explanation for the high mucosal recovery rate in this study would be that only patients maintaining a strict GFD were included. Although current scientific data on the influence of gluten ingestion on complications of coeliac disease is inconsistent (Elli et al. 2014), dietary non-compliance is the most common reason for poorly controlled disease (Ciacci et al. 2002, Lanzini et al. 2009, Rubio-Tapia et al. 2010b, Sharkey et al. 2013, Lebwohl et al. 2014). Of note, about 90% of Finnish coeliac disease patients maintain a strict diet, the labelling of gluten-free products is strictly controlled by the legislation, availability of gluten-free products and public awareness of the disease are good (Kurppa et al. 2012). This all indicates that although only patients maintaining a strict diet were included in this study, they were not strikingly different from the general coeliac population of Finland.

Although persistent villous atrophy was uncommon in this study, persistent intraepithelial lymphocytosis was not, and was found in 54% of coeliac patients maintaining a strict GFD, which was confirmed by detailed dietary assessments and negative coeliac serology. Consumption of oats was the only factor found to contribute to the persistent inflammation, although most of the subjects with completely healed mucosa were also consuming oats and the association was no longer present when a lower cut-off value (<25 IELs per 100 enterocytes) was applied. Nevertheless, Peräaho et al. (2004b) found the same association between consumption of oats and persistent inflammation of the small-intestinal mucosa in

an earlier study. Oat varieties are known to differ in their immunological safety for coeliac disease patients (Comino et al. 2011). In the present study, patients were consuming a wide variety of commercially available oat products, meaning that there was a possibility of gluten contamination, but gluten contamination may also be present in products labelled as naturally gluten-free (Collin et al. 2004b). A wide variation in sensitivity to gluten traces has also been reported in earlier studies (Catassi et al. 1993, Lähdeaho et al. 2011). Based on the current scientific evidence, pure oats should be permissible for coeliac patients, verified by numerous studies on the safety of oats (Janatuinen et al. 1995, Janatuinen et al. 2002, Störsrud et al. 2003b, Högberg et al. 2004, Peräaho et al. 2004b, Holm et al. 2006, Srinivasan et al. 2006, Sey et al. 2011, Cooper et al. 2013, Kaukinen et al. 2013).

The duration of GFD was one year longer in the normal group than in the inflammation group (10 vs. 9 years), and this small difference was statistically significant. However, as mucosal recovery usually occurs much faster (Wahab et al. 2002, Hutchinson et al. 2010, Rubio-Tapia et al. 2010b, Lebwohl et al. 2014), this can hardly be clinically relevant. Furthermore, in time-stratified analysis according to the duration of GFD; the Vh/CrD ratio, density of IELs, laboratory parameters, BMI, GSRS and PGWB did not differ significantly between the groups.

In this study, 56% of patients with recovered small-intestinal mucosal villous structure still presented with intraepithelial lymphocytosis despite a strict GFD. This may suggest that even in well-treated coeliac disease, intraepithelial lymphocytosis may not disappear even over time. While $\alpha\beta$+ IELs often decrease within months in response to gluten withdrawal, the increase of $\gamma\delta$+ IELs has been considered highly sensitive and specific for uncomplicated coeliac disease, regardless of dietary treatment and mucosal morphology (Kutlu et al. 1993, Järvinen et al. 2003). Autoimmune diseases, drugs and *H. pylori* gastritis, which are the most common aetiologies of intraepithelial lymphocytosis in general, were not found to contribute to the condition in coeliac patients. The ultimate reason for persistent inflammation of the small-intestinal mucosa in coeliac patients remained obscure, and further studies are needed to reveal it.

Persistent inflammation of the small-intestinal mucosa was associated with a lower Vh/CrD ratio, but again, this difference was no longer present when a lower cut-off value for intraepithelial lymphocytosis was applied. Patients with persistent intraepithelial lymphocytosis had no more gastrointestinal symptoms or impaired quality of life than patients with completely healed mucosa, nor did they have any signs of malabsorption or increased prevalence of malignancies. Although persistent villous atrophy may predispose coeliac patients to complications (Kaukinen et al. 2007b, Lebwohl et al. 2013b), persistent intraepithelial lymphocytosis did not seem to affect the good clinical outcome of patients with normalized villous structure.

The main strength of this study was a long follow-up period, which ranged up to 40 years in the patients enrolled. The study was not restricted to a referral centre with nationwide recruitment of patients, and thus the results can be generalized, at least in Finland. The lower rates of mucosal recovery in other countries may raise a suspicion of a selection bias in our cohort, as only patients who were in good health would have volunteered for such a study with an upper endoscopy in the study protocol. However, the results of this study are in line with earlier studies from Finland, demonstrating good mucosal recovery in the country, possible due to generally good compliance with a GFD (Collin et al. 2004a, Kaukinen et al. 2007b, Kurppa et al. 2012).

The limitations of the study are related to the cross-sectional design leading to a non-uniform protocol of control biopsies and no data on the clinical outcome of the patients after the last control biopsies in individual patients. Another limitation of the study is that the phenotype of persisting IELs was not investigated; this could have described the nature of persistent intraepithelial lymphocytosis more accurately, and should be done in further research.

5.2 Refractory coeliac disease

RCD was found to be very rare in Finland, with a prevalence of only 0.002% in general population. The prevalence of RCD among coeliac disease patients was 0.31%, which is much lower than the previously reported 1.5-10% (Table 5). As demonstrated in Study I and in earlier studies from Finland (Collin et al. 2004a, Kaukinen et al. 2007b), persistent villous atrophy is rare here, and the low prevalence of RCD is also in line with earlier findings. Actually, exactly the same prevalence of RCD in coeliac disease (0.3%) was found in a smaller study from Finland involving only one tertiary centre (Kaukinen et al. 2007b). The good outcome of coeliac disease in Finland could be explained at least in part by the efficient case-finding policy adopted here, which facilitates early diagnosis and treatment of the disease (Collin et al. 2007). Also contributing to this is the excellent compliance with GFD in Finland (Kurppa 2012). Moreover, the Finnish guidelines advocate control biopsies to be taken from all coeliac patients diagnosed in adulthood after one year on a GFD, enabling detection of patients with poor mucosal recovery despite a good clinical response, and prompt clinical assessments for the non-responsiveness (Sharkey et al. 2013).

The RCD patients in this cohort experienced a fairly benign course of the disease, as most had RCD type I with no severe symptoms or signs of malabsorption and only 9% progressed to overt lymphoma, compared to 12-67% in earlier studies (Table 12). Supporting this, most of our patients did well on a

strict GFD only, and received no medication for the disease, although the limited proof of efficacy in any available therapy so far may have contributed to the low use of medication in RCD II.

Older age and severe symptoms at diagnosis of coeliac disease as well as male gender were found to be risk factors for evolving RCD. Although 59% of patients with RCD were female, the proportion of females was 76% in uncomplicated coeliac disease, and this difference was statistically significant. Males with RCD are also at greater risk of progressing to EATL (Al-toma et al. 2007a). The ultimate reason for this is unknown. In a recent Italian study (Biagi et al. 2014a) patients over 48 years of age at diagnosis of coeliac disease were, as in the present study, found to be at risk for complications, including RCD. In the present study baseline seronegativity was also associated with ensuing RCD, accounting in older age and probably longer diagnostic delay in RCD patients, as it has been shown that in longstanding coeliac disease antibodies with increasing avidity for intestinal TG2 are produced and there is less spillover of the antibodies into serum (Salmi et al. 2006). Family history of coeliac disease was found to prevent RCD, possibly indicating an earlier diagnosis of coeliac disease in the family context, where the awareness of the disease is higher. All these factors together indicate that a long diagnostic delay in symptomatic coeliac disease may increase the risk of later developing RCD. This study was not designed to evaluate the role of dietary transgressions in acquiring RCD, but patients with RCD were found to have had more dietary lapses in the past than patients with uncomplicated disease, although at the time of diagnosis of RCD, they had all been on a strict diet for at least one year.

The main strength of this study was that it was not limited to a tertiary centre, but instead covered 39 % of the total Finnish adult population and thus was able to estimate the prevalence of RCD in general population. The coeliac background population was also large (12,243 adult patients), and well-representative of clinically diagnosed coeliac disease. With a large control group including patients with uncomplicated disease, new data on the risk factors for RCD were presented. This study attempted to present at least some tools for identifying the at-risk patients for intensive surveillance (Biagi et al. 2014b), also taking into consideration the increasing prevalence of coeliac disease.

As to the limitations of this study, this was a retrospective multi-centre study, and the patients with RCD were not investigated under a uniform protocol. Some coeliac patients may be extremely sensitive to gluten traces, which are not revealed by detailed dietary assessments and these patients may have been falsely diagnosed with RCD type I. However, this is a universal problem and widely addressed in the literature (Dewar et al. 2012, Hollon et al. 2013, Woodward 2013). It may be that a recently proposed method for the assessment of gliadin 33-mer equivalent epitopes

in patients´ faeces will help in future to better identify these patients with involuntary infringements (Comino et al. 2012). The differential diagnosis between RCD and other causes of persistent villous atrophy was made in the participating hospitals according to international guidelines (Biagi and Corazza 2001, Rubio-Tapia and Murray 2010, Rubio-Tapia et al. 2013). Given the low prevalence of RCD found in this study, it is highly unlikely that patients with other aetiologies of persistent villous atrophy would have been included.

Flow cytometry is not available in Finland for RCD purposes, which means that there may have been some inaccuracy in the differential diagnosis between types I and II. The type of RCD was determined by independent pathology departments in the participating hospitals, mainly according to immunohistochemical staining, but all biopsy samples with over 50% of abnormal IELs also contained TCR γ-chain rearrangements, making the diagnosis of RCD II more reliable (Rubio-Tapia and Murray 2010, Woodward 2013). Not relying solely on monoclonality in immunogenetic analysis on the other hand, implies that the number of patients with RCD II was not overestimated, either (Woodward 2013). DQ2-homozygoticity has been acknowledged as a risk factor for RCD (Al-toma et al. 2006a, Malamut et al. 2009), but in this cohort it could not be assessed since HLA-typing was available in only a few patients.

5.3 Malignancies in coeliac disease

The risk of cancer overall in clinically diagnosed coeliac disease patients has been reported in most studies to be increased (Table 13), but in this study the risk was decreased, mainly due to a decreased risk of breast and lung cancer. Contributing to the low cancer incidence in coeliac disease, were the decreased numbers of pancreatic, renal and bladder cancers. Neverthless, in the subgroup analysis the SIR slightly increased five years after diagnosis of coeliac disease. This indicates that no ascertaiment bias occurred in the study, but may also argue against the protective value of a GFD.

The reduced risk of female breast cancer shown in this study is in line with many previous studies (Table 17). It is highly speculative if the reduced reproductive period with late menarche and early menopause found in coeliac women could account for this (Santonicola et al. 2011, Collaborative group on hormonal factors in breast cancer 2012). The risk of lung cancer has similarly been reportedly decreased in many previous studies (Table 17), although statistically significantly in only one (West et al. 2004). Coeliac patients are reported to smoke less than individuals in general (Snook et al. 1996, Austin et al. 2002, Hervonen et

al. 2012), which may contribute to the lower risk of lung cancer; and also to the lower risk of renal and bladder cancers.

Although this study confirmed an increased risk of NHL in coeliac patients, the SIR of 1.94 was lower than in any of the earlier studies (Table 14). There is some heterogeneity in the earlier studies, which is largely explained by the method used to identify patients with coeliac disease: only serology; first serology, then biopsy; biopsy only or medical records (Kane et al. 2011). Different study designs and control populations may also account for this heterogeneity. In contrast, the effect of an ascertainment bias on the risk of NHL was suggested to be minimal in a recent meta-analysis (Kane et al. 2011), where removal of NHLs diagnosed close to the diagnosis of coeliac disease gave a similar risk estimate to that overall. In this study the SIR for NHL was increased only in the first two years after diagnosis of coeliac disease, indicating that some ascertainment bias could have occurred.

The risk of small-intestinal cancer was increased in this study, as in many earlier studies (Table 18). This probably results from a long-standing inflammation in the small-intestinal mucosa, although small-intestinal adenocarcinomas may also arise from pre-existing adenomas (Green et al. 2003, Rambertap et al. 2003). Although the relative risk was high, small-intestinal cancer was found in only 0.08% of coeliac patients in this study, meaning that the absolute risk was minimal.

A modestly increased risk of colon cancer was found in this study, which concurs with some of the earliers studies (Table 18), although in only one was the increase statistically significant, and this study included only hospitalized patients (Askling et al. 2002). A GFD contains less fibre than the normal gluten-containing diet (Wild et al. 2010) and alters the intestinal microbiota (Pozo-Rubio et al. 2012), which may affect the risk of colon cancer in coeliac patients. The risk of rectal cancer was not increased in this study. This may support the role of the diet, since although dietary fibre protects against colon cancer, the beneficial effect is lost in the rectum due to the short retention time (Hansen et al. 2011).

The slightly increased risk of skin basal cell carcinoma found in this study has not been addressed in any earlier report. This finding may well be coincidental, as the increased risk was present only during the first two years after diagnosis of coeliac disease. Possibly the regular visits of coeliac patients to the health care system, and especially in the case of DH to the dermatology units, may have facilitated the diagnosis, but this is highly speculative.

The main strength of this study was the large, unselected cohort of coeliac patients covering the whole spectrum of clinically diagnosed coeliac disease, also including patients with atypical presentation. With a large cohort, narrow confidence intervals were obtained, even in less common malignancies, such as small-intestinal cancer. The cohort was also very up-to-date, as the follow-up started in 2002 and ended in 2011.

The main limitation of the study was that patients with coeliac disease and DH could not be analysed separately, since patients with DH could have been labelled as having coeliac disease in the SII register. Nevertheless, as most patients with DH do have small-intestinal mucosal lesions compatible with coeliac disease (Reunala et al. 1984), the disease entity is by and large the same, and this also applies to the risk of malignancies (Viljamaa et al. 2006). Compliance with a GFD and other lifestyle factors possibly affecting the cancer risk could not be estimated, either, as they were not obtainable from the registers used in this study. On the other hand, in a Finnish study with screen-detected patients BMI, smoking habits, educational status, alcohol consumption, physical activity or number of births did not change the risk level in multivariate adjustment (Lohi et al. 2009). Finally, the precise time of the diagnosis of coeliac disease was unknown in patients entering the register in 2002-2003, making a time-stratified analysis of the follow-up impossible in the whole cohort, although a subgroup analysis was carried out to compensate for this.

The increased risk of NHL in coeliac disease has long been acknowledged, but the association has not been considered adequate to justify serological mass screening aiming at early diagnosis of coeliac disease (UEGW 2001, Catassi et al. 2002, Lohi et al. 2009). Instead, it has been demonstrated that the health care system is able to identify the majority of coeliac patients by active case-finding (Collin et al. 2007). Furthermore, some patients may develop NHL without a preceding history of coeliac disease and these patients often present with an advanced disease causing small-intestinal perforation or obstruction (Al-toma et al. 2007a, van de Water et al. 2010). Small-intestinal cancers are rare and diagnosis can be difficult, but in at-risk patients, especially those presenting with iron-deficiency anaemia, the diagnostic yield of capsule endoscopy followed by double-balloon enteroscopy if needed, is good (Rambertap et al. 2003, Tomba et al. 2014). In two large series including coeliac disease patients who underwent colonoscopy, the incidence of colorectal adenomas and carcinomas was equal to that in controls (Lebwohl et al. 2010, Pereyra et al. 2013). This challenges the rationale of surveillance colonoscopies in coeliac patients; although an increased risk of colon cancer was found in this study. Whether the reduced intake of fibre in GFD really contributes to the increased risk of colon cancer needs to be assessed in future research. The risk of basal cell carcinoma of the skin should also be assessed in different cohorts before any recommendations can be made on the need for special attention to protecting the skin against sunlight in coeliac patients.

6. Summary and conclusions

Although mucosal non-recovery is reportedly common in coeliac disease (Ciacci et al. 2002, Wahab et al. 2002, Lee et al. 2003, Tursi et al. 2006, Bardella et al. 2007, Lanzini et al. 2009, Hutchinson et al. 2010, Sharkey et al. 2013, Lebwohl et al. 2014), in this study persistent villous atrophy was rare, although persistent intraepithelial lymphocytosis was common, even in long-term treated patients on a strict GFD. Consumption of oats may have contributed to this, but on the other hand, there is convincing data on the safety of oats in a GFD (Janatuinen et al. 1995, Janatuinen et al. 2002, Strösrud et al. 2003b, Högberg et al. 2004, Peräaho et al. 2004b, Holm et al. 2006, Srinivasan et al. 2006, Sey et al. 2011, Cooper et al. 2013, Kaukinen et al. 2013). Furthermore, 70% of Finnish coeliac patients include oats in their diet (Peräaho et al. 2004a), and despite this, mucosal recovery is excellent here and the incidence of RCD and malignancies is low, as demonstrated in this dissertation. Furthermore, in this study the clinical outcome of patients with persistent intraepithelial lymphocytosis did not differ from those with completely healed mucosa, implying a non-significant nature of this whole finding. According to current scientific evidence, our national guidelines allowing pure oats in a GFD should not be changed.

RCD was found to be extremely rare in this study, which may suggest that active case-finding and early initiation of GFD could contribute to a lower incidence and also less dismal course of RCD. Apart from active case-finding, a rational follow-up in coeliac disease may also prevent complications (Haines et al. 2008, Sharkey et al. 2013). Although evidence on the influence of persistent villous atrophy on mortality in coeliac disease is somewhat contradictory (Rubio-Tapia et al. 2010b, Lebwohl et al. 2013a), the increased risk of osteoporosis, RCD and NHL has not been disputed (Wahab et al. 2002, Kaukinen et al. 2007b, Lebwohl et al. 2013b). Clinical and serological response to a GFD does not guarantee mucosal recovery, which means that a control biopsy is warranted (Kaukinen et al. 2002a, Midhagen et al. 2004, Rubio-Tapia and Murray 2010, Sharkey et al. 2013, Woodward 2013). Non-invasive markers of mucosal recovery are being developed, and the measurement of DP-AGA may turn out to be useful in monitoring mucosal recovery on a GFD (Spatola et al. 2014).

Older age, severe symptoms and seronegativity at diagnosis of coeliac disease may indicate a long diagnostic delay, and are risk factors for future RCD, thus patients with these characteristics should be followed up carefully.

The risk of malignant diseases in clinically diagnosed coeliac disease was found to be even lower than in general population. This resulted mainly from the decreased incidence of breast and lung cancer in coeliac patients. The risks of NHL and small-bowel cancer were increased, but to a much lesser extent than previously

reported, and the absolute risk of these malignancies was low. This notwithstanding, the cancer incidence increased after five years from diagnosis of coeliac disease and this was mainly attributable to an increased risk of colon carcinoma. As an increased risk of colon cancer in coeliac disease has currently been verified in only one study (Askling et al. 2002) besides this, further studies are needed before colonoscopic surveillance can be recommended for all coeliac disease patients.

To conclude, this dissertation demonstrated that persistent villous atrophy, refractory coeliac disease and malignancies are rare in Finland. Active case-finding and appointed duodenal control biopsies, as advocated in the national guidelines, may have contributed to this.

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Tuire Ilus

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Persistent Duodenal Intraepithelial Lymphocytosis Despite a Long-Term Strict Gluten-Free Diet in Celiac Disease

Ilus Tuire, MD[1], Lähdeaho Marja-Leena, MD, PhD[2], Salmi Teea, MD, PhD[3], Haimila Katri, PhD[4], Partanen Jukka, PhD[4], Saavalainen Päivi, PhD[5], Huhtala Heini, MSc[6], Mäki Markku, MD, PhD[2], Collin Pekka, MD, PhD[1] and Kaukinen Katri, MD, PhD[1]

OBJECTIVES: **In celiac disease, complete histological normalization of the small-intestinal mucosa occurs in only 8–20% of adult patients after commencing a gluten-free diet. Intraepithelial lymphocytosis may persist for years while villous morphology normalizes. Factors contributing to this and the clinical relevance of persistent intraepithelial lymphocytosis were here investigated.**

METHODS: **Altogether 177 adult celiac disease patients adhering to a long-term strict gluten-free diet were enrolled. Co-morbidities, ongoing medications, and consumption of oats and wheat-starch were recorded. Small-bowel morphology and intraepithelial lymphocyte count as well as laboratory parameters of malabsorption were evaluated. Gastrointestinal symptoms and psychological well-being were measured by structured questionnaires.**

RESULTS: **In all, 170 (96%) out of the 177 patients evinced normal villous architecture and 7 (4%) villous atrophy. Among patients with normal villous structure, 96 (56%) had persistent intraepithelial lymphocytosis and 74 (44%) completely normal small-intestinal mucosa. Consumption of oats was the only factor contributing to the persistent intraepithelial lymphocytosis. Co-morbidities, *Helicobacter pylori* gastritis, drugs, or wheat-starch in the diet had no effect. The clinical outcome of the patients with persistent intraepithelial lymphocytosis was good, since no signs of malabsorption, excess malignancies, increase in gastrointestinal symptoms, or impaired quality of life were associated with it when compared to subjects with completely normal mucosa. The only outcome found in this study was a significantly lower, although normal villous height–crypt depth ratio among the patients with persistent intraepithelial lymphocytosis as compared to those with completely normal mucosa.**

CONCLUSIONS: **Despite excellent villous recovery in this study, persistent intraepithelial lymphocytosis was still common among celiac disease patients on a long-term strict gluten-free diet. Consumption of oats was associated with persistent duodenal lymphocytosis and this calls for further investigations. The prognosis of patients with persistent intraepithelial lymphocytosis seems to be good while adhering to a gluten-free diet for a mean of 11 years.**

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INTRODUCTION

In celiac disease, the ingestion of dietary gluten induces villous atrophy with crypt hyperplasia and intraepithelial lymphocytosis in genetically predisposed persons (1). While elimination of gluten from the diet improves the villous architecture and reduces the number of intraepithelial lymphocytes (IELs), previous studies have shown that complete histological normalization occurs in only 8–20% of adults even when they maintain a strict gluten-free diet (2–8). The most common cause of persisting villous atrophy is gluten exposure, advertent or inadvertent (9,10). However, some patients may develop refractory celiac disease, a rare complication defined by persistent symptoms and signs of malabsorption and

[1]Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, School of Medicine, University of Tampere, Tampere, Finland; [2]Pediatric Research Center, University of Tampere, Tampere University Hospital, Tampere, Finland; [3]Department of Dermatology, Tampere University Hospital, Tampere, Finland; [4]Finnish Red Cross Blood Service, Helsinki, Finland; [5]Research Programs Unit, Molecular Medicine, Haartman Institute, Department of Medical Genetics, University of Helsinki, Helsinki, Finland; [6]School of Health Sciences, University of Tampere, Tampere, Finland. **Correspondence:** Kaukinen Katri, MD, PhD, School of Medicine, University of Tampere, FIN-33014, Finland. E-mail: katri.kaukinen@uta.fi
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villous atrophy despite strict adherence to a gluten-free diet (11). There is also some concern that persistent villous atrophy without symptoms in long-term treated celiac disease is not a harmless condition, but carries a risk of complications: malignancies and osteoporosis (12). Supporting this, Rubio-Tapia *et al.* (13) have reported a trend towards an association between persistent mucosal damage and increased all-cause mortality in celiac patients on a gluten-free diet.

While a strict gluten-free diet induces normalization of the small-intestinal villous structure, intraepithelial lymphocytosis may persist for years (4,14,15). Small-bowel mucosal lymphocytosis is an early finding in the recurrence of mucosal damage when a celiac disease patient is again exposed to gluten (16–18). However, mucosal lymphocytosis as such is an unspecific finding, and apart from celiac disease, it may be caused by drugs, especially NSAIDs (non-steroidal anti-inflammatory drugs) and PPIs (proton-pump inhibitors). Furthermore, *Helicobacter pylori* gastritis and viral gastroenteritis are often associated with small-bowel mucosal intraepithelial lymphocytosis. Other common etiologies include food protein intolerance, primary immunodeficiency diseases, Crohn's disease, microscopic colitis, rheumatoid arthritis, and other autoimmune diseases (17,19,20). A possible association between duodenal lymphocytosis and symptoms of irritable bowel syndrome may also exist (20).

The poor prognosis of symptomatic refractory celiac disease is well addressed in the current literature (10,11). Yet, little information is available regarding the precipitating factors and the clinical significance of persisting intraepithelial lymphocytosis with recovered villous architecture in celiac disease patients on a strict gluten-free diet. Here, we carried out a cross-sectional study on long-term treated, voluntary celiac disease patients aiming to establish the prevalence and clinical relevance of persisting small-bowel intraepithelial lymphocytosis in the presence of normal villous architecture. A further objective was to identify factors that distinguish the subjects with normal IEL counts from those with persisting intraepithelial lymphocytosis.

METHODS

Patients and study design

The study was carried out at the Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Finland. Altogether 177 volunteer long-term treated celiac disease adult patients adhering to a strict gluten-free diet for at least 2 years were invited to participate in a health survey. The diagnosis of their disease had to be biopsy-proven (21) and patients with symptomatic refractory celiac disease with signs of malabsorption were excluded. The volunteered patients of the study were recruited by a nationwide search using newspaper advertisements and the Finnish Celiac Society newsletter. The study protocol included upper intestinal endoscopy with small-bowel biopsies and clinical and dietary evaluations. Serum and whole blood samples were analyzed for malabsorption parameters, celiac serology, and human leukocyte antigen (HLA)-typing.

Long-term treated celiac disease patients were divided into three groups based on follow-up small-bowel mucosal biopsy findings: (i) normal villous morphology and no inflammation (normal group), (ii) normal villous morphology but intraepithelial lymphocytosis (inflammation group), and (iii) villous atrophy with crypt hyperplasia and inflammation (atrophy group). The main objective was to evaluate differences between normal and inflammation groups; data on the atrophy group are presented for comparison only.

Upper gastrointestinal endoscopy and small-bowel mucosal biopsies

Altogether six small-bowel biopsy specimens were taken from the distal part of the duodenum upon esophagogastroduodenoscopy. The biopsies were processed as previously described (22), and evaluated by the same investigator without prior knowledge of disease history or clinical findings. The villous height–crypt depth ratio (Vh/CrD) was determined from well-oriented small-bowel mucosal biopsy samples from multiple sites. A ratio below 2.0 was considered abnormal and compatible with villous atrophy and crypt hyperplasia (Marsh III). The density of IELs was measured. According to literature values between 25 and 29 IELs per 100 enterocytes have been regarded as increased (6,7,20), but values over 30 IELs per 100 enterocytes represent pathological intraepithelial lymphocytosis in duodenum (3,23). In this paper, small-bowel mucosal intraepithelial lymphocytosis was taken to be present when over 30 IELs per 100 enterocytes were counted. The data with cutoff value 25 IELs/100 enterocytes are also presented, for comparison.

Upon endoscopy, mucosal biopsy samples were also taken from the stomach (corpus and antrum) for routine histological assessment and *H. pylori* staining.

Clinical and dietary evaluation

Patients were interviewed on past medical history, signs and symptoms leading to the diagnosis of celiac disease, duration of gluten-free diet, and family history of celiac disease. The current use of NSAIDs or PPIs was also recorded. A detailed dietary analysis and history of occasional or regular consumption of gluten-containing products, oats, gluten-free wheat-starch and fiber were assessed by means of an interview by a trained dietitian and by a 4-day record of food intake. The medical history of the study subjects was reviewed from patient files. Body mass index was computed as weight in kilograms/height in meters^2 (normal range: 18–25 kg/m^2).

Current gastrointestinal symptoms were evaluated by the self-administered GSRS (Gastrointestinal Symptom Rating Scale) questionnaire, which is well validated also for celiac disease (24–26). The questionnaire comprises altogether 15 items within five subdimensions: diarrhea, indigestion syndrome, constipation, abdominal pain, and gastro-esophageal reflux. Each item is graded from one to seven; a higher score indicates more gastrointestinal symptoms.

Quality of life was evaluated by the structured PGWB (Psychological General Well-Being) questionnaire. This measures

self-perceived health-related well-being and distress, and has previously been validated and is widely applied in celiac disease research (27–29). The questionnaire contains altogether 22 items, which can be divided into six subdimensions: anxiety, depressed mood, positive well-being, self-control, general health, and vitality. Total scores may range from 22 to 132, higher scores indicating better psychological well-being.

Laboratory parameters and HLA-typing

Serum IgA-class EmA (endomysial antibodies) were determined by an indirect immunofluorescence method, a serum dilution of 1:⩾5 being considered positive (30). In addition, serum tissue TGA (transglutaminase antibody) titers were measured by enzyme-linked immunosorbent assay (Celikey, Pharmacia Diagnostics, GmbH, Freiburg, Germany) according to manufacturer's instructions; values of 5.0 unit values (U) or greater were considered positive. None of the study subjects suffered from selective IgA deficiency. The following laboratory values were measured by standard laboratory methods: blood hemoglobin level (reference values: men, 13.4 – 16.7 g/dl; women, 11.7–15.5 g/dl), red blood cell mean corpuscular volume (reference values: 82–98 fl), serum iron level (reference values, 50–190 g/dl), red blood cell folic acid level (reference values, 200–700 nmol/l).

HLA DQ2 and DQ8 allele genotyping was performed by DELFIA® Celiac Disease Hybridization Assay (Perkin-Elmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) or the SSP DQB1 low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) according to the manufacturers' instructions. In part of the samples typing was based on HLA tagging SNPs genotyped according to Koskinen *et al.* (31).

Statistics

Quantitative data were expressed as medians and ranges or means and 95% confidence intervals. Statistical differences were evaluated using Mann–Whitney test. Fisher's and Kruskal–Wallis tests were used in cross-tabulations. P values <0.05 were considered statistically significant. Statistical testing was performed using SPSS 17.0 (SPSS, Chicago, IL).

Ethical considerations

The study protocol was approved by the Ethical Committee of Tampere University Hospital. All subjects gave their written informed consent.

RESULTS

Background data

Small-bowel villous morphology was normal in altogether 170 (96%) out of the 177 treated celiac disease patients, and villous atrophy with crypt hyperplasia was present in seven (4%). All seven patients with persistent villous atrophy were in a good condition, asymptomatic and had no signs of malabsortion and thus they did not fulfill the criteria of refractory celiac disease. Of those with normal villous structure, 74 (56%) also had normal IEL counts, while 96 (44%) evinced persisting intraepithelial lymphocytosis (**Table 1**). All patients were strictly adherent to a gluten-free diet according to detailed dietary assessments and negative celiac serology. The duration of the gluten-free diet was 9 years in the inflammation and 10 years in the normal group, this 1-year difference being statistically significant. Besides this, only the consumption of oats was statistically significantly more frequent in those with mucosal inflammation compared to those with completely normal mucosa (**Table 1**). Subjects in the atrophy group were consuming oats less frequently than those in the inflammation group, although statistical comparisons were not made. The use of wheat-starch-containing products and fiber were similar in the respective study groups, as were age and gender distributions, family history, and clinical presentation of celiac disease at diagnosis. The prevalence of other autoimmune diseases as well as *H. pylori* gastritis and the use of medications were comparable between the groups (**Table 1**). None of the patients was on immunosuppressive medication. All 87 tested patients had alleles encoding the DQ2- or DQ8-molecyles substantiating the diagnosis of celiac disease.

Clinical outcome

Patients with persisting intraepithelial lymphocytosis on a long-term gluten-free diet had significantly lower Vh/CrD than those with completely normal mucosa, the Vh/CrD still being within the normal range (>2.0) in both study groups (**Table 2**). There were no differences in body mass index or malabsorption parameters between the groups. Current gastrointestinal symptoms and psychological well-being were also equal as measured by GSRS and PGWB total and subdimensional scores (**Table 3**). There were two malignancies in the inflammation group (one uterine and one prostate cancer) and similarly two in the normal group (one breast cancer and one lymphoma). No malignancies were recorded in the atrophy group. The data were divided in the following groups according to the duration of gluten-free diet: 2–5 years, 5–10 years, 10–15 years, 15–20 years, over 20 years. In each time strata, 85%, 63%, 51%, 42%, and 48% had persisting intraepithelial lymphocytosis, respectively. Small-bowel mucosal villous morphology, inflammation, malabsorption data, symptoms, and quality of life were not significantly different between the groups (P values for Vh/CrD 0.533, density of IELs 0.069, blood hemoglobin 0.638, serum iron 0.565, erythrocyte-folic acid 0.132, body mass index 0.526, total GSRS 0.562, and total PGWB 0.746, respectively; data not shown).

Lower cutoff value for increased lymphocytes

When lower cutoff value 25 IELs per 100 enterocytes was applied, no factors contributing to the intraepithelial lymphocytosis were found. The association between lymphocytosis and consumption of oats was not statistically significant ($P=0.210$). No signs of malabsorption, excess malignancies, increase in gastrointestinal symptoms, or impaired quality of life were associated with lymphocytosis when compared to subjects with completely normal mucosa (data not shown). Small-bowel mucosal Vh/CrD did not differ between the groups ($P=0.575$). By using lower cutoff value for IELs, it was found that after 2–5 years on a gluten-free diet 89%

Table 1. Clinical data on 177 long-term treated celiac disease patients divided according to small-bowel biopsy findings at the time of follow-up

	Villous morphology normal			
	Normal (*n*=74)	Inflammation (*n*=96)	Villous atrophy (*n*=7)	Normal vs. inflammation (*P* value)
Female, *n* (%)[a]	51 (69)	74 (77)	4 (57)	0.293
Age, median (range); years	57 (21–75)	55 (23–81)	52 (38–72)	0.712
Duration of GFD, median (range); years	10 (3–34)	9 (2–41)	7 (3–19)	0.014
Clinical presentation at diagnosis, n (%)				0.717
Gastrointestinal symptoms	59 (80)	78 (81)	7 (100)	
Extraintestinal symptoms	13 (17)	17 (18)	0 (0)	
Screen-detected	2 (3)	1 (1)	0 (0)	
Family history of celiac disease, *n* (%)	30 (41)	34 (35)	3 (43)	0.526
Autoimmune thyroid disease, *n* (%)	12 (16)	14 (15)	0 (0)	0.831
Any autoimmune disease, *n* (%)	15 (20)	17 (18)	0 (0)	0.696
Using NSAID, *n* (%)	10 (17)	18 (21)	2 (29)	0.670
Using PPI, *n* (%)	7 (12)	10 (12)	2 (29)	1.000
H. pylori gastritis, *n* (%)	1 (2)	4 (5)	0 (0)	0.649
Consuming wheat starch, *n* (%)	62 (89)	87 (93)	6 (100)	0.421
Consuming oats, *n* (%)	46 (64)	79 (84)	4 (67)	0.012
Dietary fiber, median (range); g/day	18 (8–51)	18 (9–41)	17 (8–27)	0.502
Celiac-type HLA DQ2 and/or DQ8, *n* (%)	41 (100)	44 (100)	3 (100)	

GFD, gluten-free diet; HLA, human leukocyte antigen; NSAID, non-steroidal anti-inflammatory drugs; PPI, proton-pump inhibitors.
[a]Denominator varies depending on the available data.

Table 2. Small-bowel mucosal villous height–crypt depth ratios (Vh/CrD), current body mass index (BMI), laboratory parameters in 177 long-term treated celiac disease patients divided according to follow-up small-bowel biopsy findings

	Villous morphology normal			
	Normal (*n*=74)	Inflammation (*n*=96)	Villous atrophy (*n*=7)	Normal vs. inflammation (*P* value)
Small-bowel Vh/CrD, median, range; ratio	3.2 (2.1–4.5)	2.9 (2.1–4.3)	1.3 (0.4–1.8)	0.042
BMI, median (range); kgm^2	26 (19–38)	25 (17–36)	24 (21–29)	0.213
Blood hemoglobin, median (range); g/l	14.4 (11.0–16.6)	13.9 (11.2–18.1)	14.0 (12.8–16.1)	0.295
Blood MCV, median (range); fl	90 (86–96)	90 (83–100)	91 (87–96)	0.692
Serum iron, median (range); g/dl	18 (9–34)	19 (6–37)	19 (15–27)	0.809
Erythrocyte folic acid, median (range); nmol/l	583 (218–2832)	505 (179–2005)	486 (243–1849)	0.374

MCV, red blood cell mean corpuscular volume.

of the treated celiac disease patients had persisting intraepithelial lymphocytosis, after 5–10 years 75%, after 10–15 years 64%, after 15–20 years 67%, and after >20 years 67%.

DISCUSSION

This study revealed intestinal mucosal recovery in 96% of celiac disease patients on a long-term strict gluten-free diet, this being an excellent achievement when compared with previously reported results (**Table 4**). Nevertheless, persistent intraepithelial lymphocytosis proved here again to be a much more common finding in long-term treated celiac disease patients (2–8) than in the general population, in 42% and 4% (32), respectively. Intraepithelial lymphocytosis has been postulated to be a sensitive marker of ongoing gluten-ingestion in celiac disease patients (3,7). However, all patients in the current study were on a strict gluten-free diet, confirmed by detailed dietary assessments and negative celiac serology.

Table 3. Current gastrointestinal symptoms measured by Gastrointestinal Symptom Rating Scale (GSRS) and quality of life measured by Psychological General Well-Being (PGWB) questionnaires in 177 long-term treated celiac disease patients divided according to follow-up small-bowel biopsy findings

	Villous morphology normal		
	Normal (*n*=74)	Inflammation (*n*=96)	Villous atrophy (*n*=7)
GSRS, mean (95% CI)			
Total score	1.83 (1.34–2.31)	1.80 (1.24–2.37)	1.73 (1.39–2.07)
Diarrhea	1.57 (0.83–2.31)	1.48 (0.71–2.26)	1.10 (0.93–1.26)
Indigestion	2.25 (1.47–3.02)	2.18 (1.40–2.97)	2.04 (1.43–2.64)
Constipation	1.76 (0.73–2.79)	1.94 (0.69–3.19)	1.43 (0.57–2.28)
Abdominal pain	1.78 (1.21–2.36)	1.70 (1.10–2.30)	1.57 (1.00–2.14)
Reflux	1.55 (0.78–2.32)	1.48 (0.69–2.27)	2.71 (1.33–4.09)
PGWB, mean (95% CI)			
Total score	106.4 (95.9–116.8)	105.3 (90.9–119.7)	112.7 (110.6–114.8)
Anxiety	24.6 (22.2–27.1)	23.9 (19.8–28.0)	26.3 (24.2–28.4)
Depression	16.6 (15.0–18.2)	16.6 (14.8–18.5)	17.7 (17.1–18.2)
Well-being	17.3 (14.7–19.8)	17.3 (14.6–20.0)	18.0 (18.0–18.0)
Self-control	16.2 (14.6–17.8)	15.2 (12.4–17.9)	16.3 (15.8–16.9)
General health	13.7 (11.2–16.2)	14.3 (11.5–17.0)	13.7 (11.2–16.2)
Vitality	18.1 (15.3–20.9)	18.0 (14.9–21.1)	20.7 (20.1–21.2)

Data are given as mean values and 95% confidence intervals (CIs). Differences between the groups were not statistically significant.

Consumption of oats was the only factor to emerge as contributing to the persistent inflammation of the small-intestinal mucosa, although most of the subjects with completely healed mucosa were also consuming oats. In a previous study, Peräaho *et al.* (33) found similarly in a different patient series that the consumption of oats was associated with intraepithelial mucosal inflammation. Our observations conflict somewhat with earlier studies showing oats to be safe in the gluten-free diet, also when used long-term and in large quantities (34–36). Furthermore, celiac disease patients in Finland have favored oats in their diets for years (37) and we have still achieved excellent mucosal recovery figures compared with countries where oats are not allowed for celiac disease patients (**Table 4**). The risk of gluten contamination of course remains, albeit not only in oats but also in products labeled as naturally gluten-free (38). Comino *et al.* (39) have shown that oat varieties differ in their immunological safety for celiac disease patients. In this present study, the patients were using a wide range of commercially available products, and it was impossible to establish which varieties were used by individual patients. Neither were we able to estimate the quantities of oats consumed. Wide variation in sensitivity to gluten traces between celiac patients has also been disclosed in earlier studies (18,40). Nevertheless, it must be noted that when lower cutoff value 25 IELs per 100 enterocytes was used for defining intraepithelial lymphocytosis, no association between oat consumption and lymphocytosis was found. Currently, gluten-free products containing pure oats have been permissible for celiac disease patients in some countries like Finland, the United Kingdom and Canada. Based on the current scientific evidence, the current treatment guidelines seem to be valid; however, studies on long-term safety of oats are still warranted.

Here, the duration of the gluten-free diet was a median 1-year longer in the normal group compared with the inflammation group. This small difference (9 vs. 10 years) was statistically significant, but it is difficult to see how it could be clinically relevant. Earlier reports have shown the mucosal recovery time to be much shorter, a few years at most (4,8,13). Interestingly, the proportion of patients having increased small-bowel mucosal lymphosytosis first seemed to decrease on a gluten-free diet. However, after 10 years approximately half of the patients evinced constantly persistent mucosal lymphocytosis despite a strict diet, suggesting that in treated celiac disease, IELs do not necessarily disappear over time. As mentioned above, we found that other common causes of intraepithelial lymphocytosis; autoimmune diseases, drugs, and *H. pylori* gastritis (14,17,19,20) did not contribute to the persistent inflammation of the small-bowel mucosa in celiac disease patients.

The clinical outcome of persistent intraepithelial lymphocytosis in celiac disease would appear to be harmless, since we found no signs of malabsorption. Nor were any excess malignancies associated with it, although the number of subjects was too small to draw firm conclusions. Neither did the patients with persistent mucosal inflammation report any more gastrointestinal symptoms nor impaired quality of life than those with completely normal mucosa. These findings were constant even if lower cutoff value for small-bowel mucosal intraepithelial lymphocytosis was applied. Regarding the low incidence of refractory sprue and intestinal lymphomas in our celiac disease patients in general (12,41), we do not expect a poor prognosis for these patients even in the long run. The only outcome of persistent intraepithelial lymphocytosis found in this study was the significantly lower, although normal Vh/CrD ratio in the inflammation group compared with the normal group. However, the Vh/CrD ratio of 2.9–3.2 found in long-term treated celiac disease patients in this study is excellent compared with previous studies, where ratios of 2.0–2.2 (13,40) have been reported.

The strengths of this study include a long follow-up period; some of the patients had been on a gluten-free diet over 40 years.

Table 4. Earlier studies on recovery rates (%) of small-bowel mucosa in patients on a long-term gluten-free diet

	No. of patients	Country	Duration of gluten-free diet, mean (range); years	Normal mucosa (Marsh 0)	Intraepithelial lymphocytosis (Marsh I)	Mucosal damage (Marsh II–III)
Lanzini *et al.* (7)	465	Italy	1.3 (1–9)	8	65	27
Tursi *et al.* (5)	42	Italy	2	60	16	24
Bardella *et al.* (6)	114	Italy	2 (1–23)	18	20	62
Hutchinson *et al.* (8)	284	UK	3 (1–8)	39	17	44
Wahab *et al.* (4)	158	Netherlands	5	41	24	35
Ciacci *et al.* (2)	390	Italy	7 (2–22)	44	9	47
Lee *et al.* (3)	39	USA	9 (1–45)	21		79
Current study	177	Finland	11 (2–41)	42	54	4

In our country, celiac disease diagnostics and follow-up in adults takes place oftentimes in primary healthcare and not in university hospital referral centers. In the current study, the enrollment of the patients was not restricted to one referral center. As the recruitment of patients was nationwide, the cohort can be considered representative for adult celiac disease as a whole (42). Higher rates of villous recovery than that reported in other countries (**Table 4**) may raise the suspicion of the selection bias among the volunteered patients. However, these figures are in line with our previous data (12). In addition, it would be unlikely that we would have missed a significant cohort of patients too sick to volunteer for biopsy due to malignancy or patients who had died during the first 5 years after diagnosis, as it has been earlier shown that in our country the mortality and malignancy figures in celiac disease are comparable to those in population in general (41). Potential limitations of this study are those related to a retrospective design, for instance follow-up biopsies were not taken at uniform intervals, some data might have been missing and the observation for morbidity/mortality after the last small-bowel biopsy was lacking. Furthermore, the results of the study might not be generalizable to all populations. As to limitations, we did not investigate the homozygosity of HLA DQ2 or the phenotype of the intestinal lymphocytes in this study. γδ+IELs remain elevated in the small-bowel mucosa of celiac disease patients for a long time after commencing a gluten-free diet and they seem to be regulatory in nature (43,44). By contrast, elevated αβ+IELs correlate with mucosal damage in active celiac disease and probably also with gluten-ingestion. Investigating the phenotype of the persistent IELs offers an interesting target for further studies.

We conclude that persistent small-intestinal mucosal intraepithelial lymphocytosis is a common finding in long-term treated celiac disease patients, but the clinical outcome of these patients does not differ from those with completely normal mucosa. Oats might contribute to the duodenal lymphocytosis, whereas drugs, *H. pylori* gastritis or other autoimmune diseases did not. Further studies are needed concentrating on the possible harmful effect of oats on the small-intestinal mucosa in the long term, and the phenotype of persistent IELs.

CONFLICT OF INTEREST

Guarantor of the article: Kaukinen Katri, MD, PhD.

Specific author contributions: Contributed to study design, data collection, interpretation of the results, and editing of the manuscript: Ilus Tuire, Collin Pekka, Kaukinen Katri, Salmi Teea, Mäki Markku, and Lähdeaho Marja-Leena; contributed to data analysis: Huhtala Heini; performed DNA analyses and interpreted the results: Saavalainen Päivi, Haimila Katri and Partanen Jukka. All authors read and approved the final article.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Complete histological normalization of the small-bowel mucosa occurs in only 8–20% of adult celiac disease patients after commencement of a gluten-free diet.
- ✓ Intraepithelial lymphocytosis may persist for years despite improved villous architecture in celiac disease patients on a gluten-free diet.
- ✓ Intraepithelial lymphocytosis as such is an unspecific finding and can be associated with drugs, other autoimmune diseases, and infections such as *H. pylori* gastritis.

WHAT IS NEW HERE

- ✓ The prognosis of patients with persistent intraepithelial lymphocytosis was good, since no signs of malabsorption, increased prevalence of malignancies, gastrointestinal symptoms, or impaired quality of life were associated with it.
- ✓ Consumption of oats might be associated with persistent intraepithelial lymphocytosis in celiac disease patients adhering to an otherwise strict gluten-free diet.
- ✓ Other autoimmune diseases, *H. pylori* gastritis and drugs were not associated with duodenal lymphocytosis in celiac disease.

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