On the work-up of (Refractory) Coeliac Disease

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"You will be more disappointed
by the things you did not do
than by the ones you did do.
So throw off the bowlines,
sail away from the safe harbour.
Catch the trade winds in your sails.
Explore. Dream. Discover."

Mark Twain

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SCOPE OF THE THESIS

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Coeliac disease (CD) is a major health care issue world wide, affecting genetically susceptible individuals of all ages. Currently, the diagnostic work-up and follow-up comprises serological analysis of CD associated antibodies as well as histopathological evaluation of duodenal biopsies obtained by a gastro-duodenal endoscopy. In a substantial number of CD patients diagnosed at an adult age, mucosal recovery is slow and incomplete despite adherence to a gluten-free diet (GFD) with subsequent normalised serology and disappearance of symptoms. Moreover, in particular the adult-onset coeliac patients are prone for developing complications, including refractory coeliac disease (RCD) and/or enteropathy associated T-cell lymphoma (EATL). In contrast to the slow responders, RCD is characterised by a primary or secondary lack of clinical and histological response upon strict adherence to a GFD for more than 12 months with normalised CD associated antibodies. This syndrome can be subdivided into type I and type II, with immunophenotypically normal and aberrant (cytoplasmicCD3+, surfaceCD3-, CD4-, CD7+, CD8-, TCR-, CD103+) intraepithelial T-cells (IELs) in the small intestinal mucosa, respectively. Aberrant IELs are considered to be a premalignant cell population from which aggressive EATL can evolve, which is observed in approximately half of the RCD II patients. The origin of these cells, however, is not fully clarified so far. Furthermore, easy applicable serological tests to identify and monitor patients that suffer from complicated forms of CD are lacking. Although the prognosis of RCD I is much more favourable than that of RCD II, as reflected in 5-year survival rates of around 90% and 44-58%, respectively, treatment is of utmost importance to prevent complications of longstanding malabsorption. Apart from a GFD and nutritional support, immunosuppressive therapy is the mainstay of treatment, yet there is no standardised treatment for both types of RCD. EATL is one of the main causes of death in patients with long-lasting untreated CD or RCD, due to its aggressive nature and unresponsiveness to currently available therapies. Within the spectrum of CD, this thesis aims to evaluate new diagnostic aspects, gain more insight in the underlying pathophysiological differences and explore novel therapeutic options.

Part I, the introduction, **chapter 1**, provides an overview of clinical aspects and treatment options of uncomplicated as well as complicated forms of CD.

Part II includes two studies investigating novel serum parameters beyond standard antibody testing in the spectrum of CD. **Chapter 2** describes the severity and course of enterocyte destruction in uncomplicated adult coeliac patients using serum intestinal fatty acid binding protein (I-FABP) levels at time of diagnosis and upon a GFD. **Chapter 3** sets out to investigate the potential use of novel serum parameters in the spectrum of CD, in order to discriminate between complicated and uncomplicated CD. Furthermore, part II includes two studies in which aberrant IELs and EATL are further investigated on the cellular and molecular level. **Chapter 4** investigates the origin of aberrant IELs by molecular analysis of

the T-cell receptor (TCR) rearrangements and by flow cytometric analysis of small intestinal biopsies. In addition, **chapter 5** reveals an extended phenotypic and genomic analysis of an exceptional case of EATL primarily presenting as ascites.

Part III evaluates new therapeutic alternatives for uncomplicated and complicated CD. **Chapter 6** assesses the safety and efficacy of *Aspergillus niger*-derived prolyl-specific endoprotease (AN-PEP) to mitigate the immunogenic effects of gluten in uncomplicated coeliac patients in a randomised double-blind placebo-controlled pilot study. **Chapter 7** evaluates safety and efficacy of the non-conventional thiopurine derivative tioguanine in RCD I. **Chapter 8** and **9** describe a relatively large cohort of RCD II patients treated with cladribine and in case of unresponsiveness autologous haematopoietic stem-cell transplantation (au-SCT) after conditioning with high dose chemotherapy, respectively. Finally, **chapter 10** reports two cases diagnosed with EATL that were treated with allogenic stem-cell transplantation (allo-SCT).

Part IV, the **general discussion**, summarizes the main findings of this thesis, points out future challenges in the work-up of (R)CD and provides recommendations for further research.

PART I

GENERAL INTRODUCTION

CHAPTER 1

General introduction

The spectrum of coeliac disease: epidemiology, clinical aspects and treatment

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ABSTRACT

Coeliac disease is a gluten-sensitive enteropathy that affects people of all ages worldwide. This disease has emerged a major health-care problem, as advances in diagnostic and screening methods have revealed its global prevalence. Environmental factors such as gluten introduction at childhood, infectious agents and socioeconomic features, as well as the presence of HLA-DQ2 and/or HLA-DQ8 haplotypes or genetic variations in several non-HLA genes contribute to the development of coeliac disease. Growing insight into the variable clinical and histopathological presentation features of this disease has opened new perspectives for future research. A strict life-long gluten-free diet is the only safe and efficient available treatment, yet it results in a social burden. Alternative treatment modalities focus on modification of dietary components, enzymatic degradation of gluten, inhibition of intestinal permeability and modulation of the immune response. A small group of patients with coeliac disease (2–5%), however, fails to improve clinically and histologically upon elimination of dietary gluten. This complication is referred to as refractory coeliac disease, and imposes a serious risk of developing a virtually lethal enteropathy associated T-cell lymphoma.

INTRODUCTION

Coeliac disease (CD) is the most common food intolerance in the Western population, and currently represents a major health-care issue. CD has an ancient history, first described in the 1st and 2nd century AD. In 1887, Samuel Gee described typical symptoms of CD in children, including irritability, chronic diarrhoea and failure to thrive, and cure by means of a diet was suggested for the first time.[1] Since then, insight into CD has undergone a revolutionary development regarding epidemiology, diagnostics and treatment.

Coeliac disease is a chronic, small intestinal enteropathy, which is triggered by gluten proteins from wheat, barley and rye. It is characterised by an autoimmune response in genetically susceptible individuals resulting in small intestinal mucosal injury. As a consequence, malabsorption develops which results in malnutrion-related problems including anaemia, vitamin deficiencies, osteoporosis and neurological disorders. Withdrawal of dietary gluten usually leads to prompt healing of the damaged small intestinal mucosa and improvement of nutrient absorption. A gluten-free diet (GFD) is sufficient to treat the overwhelming majority of patients with CD and clinical improvement is usually evident within a few weeks.[2]

A small percentage (2–5%) of patients with adult-onset CD, especially those diagnosed above the age of 50, does not respond to a GFD and is seen as suffering from refractory coeliac disease (RCD). The occurrence of an enteropathy associated T-cell lymphoma (EATL) is the major complication associated with RCD and is the main cause of death in this patient group.[3, 4] Early identification of patients with RCD enables early intervention, which results in reduction in morbidity and mortality.[4, 5]

This review gives an overview of the latest trends in epidemiology, clinical presentation, diagnosis, treatment and complications with respect to the spectrum of CD.

EPIDEMIOLOGY

Global population trends

Until the 1970s the estimated global prevalence of CD in the general population was 0.03%.[6] The currently estimated prevalence is 1%, with a statistical range of probability of 0.5–1.26% in the general population in Europe and the USA.[7] Even taking into account that the actual occurrence rate of CD has been underestimated for many decades, the prevalence of this disease is increasing. Advances in diagnostic methods and improvement in screening have played a part in this increase observed, but environmental factors have also been important.

Trends in diagnosis and screening

The introduction of gastro-intestinal endoscopic techniques in the 1970s, which provided the opportunity to take routine biopsy samples, opened new horizons in CD case-finding and diagnosis. In addition, identification of human leukocyte antigen (HLA) molecules typically

associated with CD, HLA-DQ2 and HLA-DQ8, in the late 1980s and early 1990s, respectively,[8] and development of highly sensitive and specific serologic tests have also been important. Furthermore, the implementation since the late 1980s of screening programs for detecting CD has contributed to a more realistic estimate of the actual disease prevalence.[9] The recognition that atypical, minor or extra-intestinal complaints can be associated with CD in patients of all ages and the detection of a range of histological abnormalities in the small intestine of patients with the disease have also contributed to improved diagnosis.[10-12]

Despite the advances in screening for CD, it remains underdiagnosed.[13] In the general population, the ratio between patients with CD who received an accurate diagnosis and those who were never diagnosed as having the disease was reported to range from 1:5.5[13] up to 1:10[14]. Since the 1980s, a trend towards earlier diagnosis of CD has been observed.[15] Unawareness of CD by physicians probably still underlies misdiagnosis and diagnostic delay.

Environmental risk factors

A Finnish population-based study has shown that the almost doubled prevalence of CD observed from 1980 (1.03%) to 2001 (1.99%) could not be ascribed only to screening and improved diagnostics, but was rather most probably attributable to environmental changes.[6, 16]

Infant feeding

The role of infant feeding on the development of CD has been intensely debated since the late 1980s, which has resulted in recommendation by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) committee.[17] This committee currently recommends that small amounts of gluten are gradually introduced between four and seven months of age during breastfeeding.[17] This recommendation is strongly supported by a meta-analysis[18] and, moreover, by lessons learned from the Swedish epidemic of CD (1984–1996), which arose as a consequence of changes in infant feeding[19]. In this birth cohort, gluten was mainly introduced abruptly after discontinuing breastfeeding at 6 months of age. At the same time, the gluten content of commercial infant food was increased. The prevalence of CD was almost threefold higher in this birth cohort compared with that in infants born after the epidemic, in whom gluten was introduced gradually while continuing breastfeeding.[19] Although dietary gluten exposure in children under the age of two seems more important with respect to CD risk when compared with exposure in older children[19], whether breastfeeding only delays clinical onset or whether it leads to permanent protection against CD remains to be elucidated. Interestingly, one study has suggested that breastfeeding during gluten introduction, which slightly delays onset of CD, also influences clinical appearance as well. Among children who develop CD during gluten introduction, those who were breastfed at time can present the typical (49%) or atypical (51%) form of disease, whereas the ones in which gluten introduction occurred after breastfeeding was stopped more frequently develop typical gastro-intestinal symptoms (90%).[20]

Infections

Infections after birth have been proposed to contribute to the development of CD. Whereas the role of infection with adenovirus type 12 in this process remains controversial, the association of hepatitis C infection and CD is well documented.[21] A prospective study showed that frequent rotavirus infections, the most common cause of childhood gastroenteritis, represent an independent risk factor for CD in genetically susceptible individuals.[22] Rotavirus infection changes the permeability of and the cytokine balance in the intestinal mucosa, potentially enhancing penetration of gluten peptides.[22] If this is the case, worldwide implementation of a rotavirus vaccine might diminish the occurrence of CD. The influence of infections with other common intestinal micro-organisms, including *Campylobacter jejuni*, *Giardia lamblia* and enteroviruses has not yet been clarified.[23]

Socioeconomic features

An epidemiological survey where comparisons were made between schoolchildren living in a prosperous area of Finland and children living in an adjacent poor region of Russia, whom in part shared genetic susceptibility and gluten intake, has suggested that worse socioeconomic conditions might protect against CD development.[24] Variation in gut flora, infections and differences in diet, which are factors involved in the maturation of immunoregulatory functions, may in turn precipitate CD development.[24]

GENETIC RISK FACTORS

HLA genes

CD is a multigenic disorder, in which the most dominant genetic risk factors are the genotypes encoding the HLA class II molecules HLA-DQ2 (encoded by *HLA-DQA1*0501* and *HLA-DQB1*02*) and HLA-DQ8 (encoded by *HLA-DQA1*0301* and *HLA-DQB1*0302*). About 90% of individuals with CD carry the DQ2 heterodimer encoded either in *cis* or *trans*, and practically all of the remaining patients express DQ8.[8] Deamidated gliadin peptides have a high binding affinity to HLA-DQ2 and HLA-DQ8 molecules, but not to other HLA class II molecules, which explains the immunogenicity of gluten in carriers of HLA-DQ2 and HLA-DQ8. A correlation has been found between homozygosity for the genes encoding HLA-DQ2 molecule and the development of serious complications of CD, in particular RCD and EATL, which implies a gene-dose effect.[25] These HLA-encoding genes are associated with approximately 40% of the heritable risk of developing CD.[26]

Non-HLA genes

Currently, several susceptibility loci not related to HLA have been identified by genome-wide association studies, each of which is estimated to be associated with only a small risk of developing CD. Most of these loci contain immune-related genes, in particular genes

implicated in the control of the adaptive immune response. The proteins encoded by these genes include an integrin (encoded by *ITGA4* at 2q31)[27], chemokines, cytokines and their receptors (*IL-2* and *IL-21* at 4q27, *IL-18RAP* at 2q11-2q12, *IL-12A* at 3q25-3q26, the *CCR1* and *CCR3* cluster locus at 3p21), and proteins involved in several signalling pathways (*RGS1* at 1q31, *SH2B3* at 12q24, *ATXN2* at 12q24, *TNFAIP3* at 6q23.3, *REL* at 2p16.1), regulating B-cell (*RGS1*) and T-cell activation (*TAGAP* at 6q25), and in maintaining cell adhesion and motility (*LPP* at 3q28)[28, 29]. However, association between the risk of CD development and *CCR3* and *IL-18RAP* could not be confirmed in other studies.[27, 30] The 4q27 region, which harbours the *IL-2* and *IL-21* genes, showed the strongest association.[27, 29] The latter association, however, accounted for less than 1% of the familial risk of CD and genetic variation in all currently known non-HLA genes together accounts for less than 10%.[27, 29] This indicates that many contributing polymorphisms in non-HLA genes still have to be discovered. An association between MYO9B polymorphisms and an increased risk for RCD and EATL has been found, however for uncomplicated CD this association remains controversial.[31] Further research is needed to determine the functions of the proteins that these genes encode and their involvement in the pathogenesis of CD.

POPULATION, GENDER AND AGE DISTRIBUTION

Variety in genetic factors including the frequency of non-HLA alleles and environmental factors including dietary habits underlie the variations in the frequency of CD observed in different world regions (Table 1). The HLA-DQ2 heterodimer is frequently found in white populations in Western Europe (20-30%), Northern and Western Africa, the Middle East and Central Asia, whereas HLA-DQ8 is more prevalent in Latin America and Northern Europe.[32] Gluten consumption is widespread in Northern Africa, South America and the northern wheat-eating parts of India. The Saharawi population of Arab-Berber origin living in Algeria has the highest prevalence of CD (5.6%) among all world populations.[33] High levels of consanguinity, high frequencies of HLA-DQ2 and gluten being used as staple food in this population may potentially explain this finding.[33] By contrast, CD seems to be rare in individuals of Japanese and Chinese ancestry, for whom the frequency of HLA-DQ2 is negligible.[32] The occurrence of CD may vary within individual countries, for instance in different parts of India.[34, 35] This variation is probably attributable to differences in dietary habits and to associations of specific genetic clusters and particular regions. Middle Eastern countries, including Iran, Turkey, Israel and Syria, seem to have similar frequency rates of CD to those of Western countries.[36]

As expected, in high-risk populations the prevalence of CD is much higher compared with that in the general population. A multicentre study conducted in the USA revealed that the prevalence of serotypes associated with risk of disease was 1:56 in individuals with clinical features of CD or CD associated extra-intestinal disorders.[37] Furthermore, the prevalence of

risk associated serotypes in first-degree and second-degree relatives of these patients was 1:22 and 1:39, respectively. In adults and children with symptoms that raise suspicion of CD, prevalence rates of 1:68 and 1:25, respectively, were observed.

Table 1 | Worldwide prevalence of coeliac disease for children and adults.

Country	Adults	Children
Europe		
Czech Republic	0.45% [84]	NA
Finland	0.55-2.00% [6, 14]	1.00% [42]
Germany	0.19% [85]	0.20% [85]
Great Britain	1.20% [86]	1.00% [87]
Italy	0.18% [88]	0.54-0.85% [89, 90]
Northern Ireland	0.82% [91]	NA
Russia	0.20% [24]	NA
Spain	0.26% [92]	NA
Sweden	0.46-0.53% [93]	1.30% [94]
The Netherlands	0.35% [95]	0.50% [96]
North and South America		
Argentina	0.60% [97]	NA
Brazil	0.15% [98]	NA
Mexico	2.60% [99]	NA
USA	0.40-0.95% [7, 37]	0.90-0.31% [7, 40]
Asia		
India	NA	1.00% [34, 100]
Iran	0.60% [101]	0.60% [102]
Israel	0.60% [103]	0.17% [103]
Kuwait	NA	0.02% [104]
Syria	1.60% [32]	NA
Turkey	1.30% [105]	0.90% [106]
Africa		
Algeria	NA	5.60% [33]
Tunisia	0.28% [107]	0.64% [108]
Oceania		
Australia	0.40% [109]	NA
New Zealand	1.20% [110]	NA

Abbreviation: NA, not available.

As with many other autoimmune diseases, CD is more common in women[38], with a female to male ratio of between 2:1 and 3:1. Some genetic loci are gender-influenced and immunoregulation is subject to hormones, which might explain these differences. By contrast, patients over the age of 60 who are diagnosed as having CD are more frequently male.[39]

CD can be diagnosed at any age, with a peak at early childhood and at the fourth and fifth decade of life for women and men, respectively. Currently, the reported global prevalence of CD in children ranges from 0.31% to 0.9%.[7, 40] The prevalence of CD in adults is approximately 1–2% in Europe[6] and 0.4–0.95% in the USA[7]. Whether diagnosing CD at advanced age is the result of diagnostic delay or of a true late onset of the disease is still debated. Whereas several studies reported a diagnostic delay in the elderly population[41], other reports suggest that CD may indeed develop later in life.[12, 16]

CLINICAL PRESENTATION

Coeliac disease has long been considered a paediatric syndrome, in which classical intestinal symptoms, including diarrhoea, steatorrhoea and weight loss, predominate. However, the disease has been increasingly diagnosed in older children and adults and has emerged to encompass a broad spectrum of clinical manifestations (Box 1), which are associated with a large variety of changes in the mucosa of the small intestine.[10, 11] About 50% of patients with CD present with atypical symptoms, such as anaemia, osteoporosis, dermatitis herpetiformis, neurological problems and dental enamel hypoplasia.[15, 42, 43] The variable clinical picture of CD is thought to have both genetic and immunological bases. Age of onset, extent of mucosal injury and dietary habits, but also gender[44], seem to affect the clinical manifestation of the disease.

The spectrum of CD currently encompasses four different types of which clinicians should be aware.[45] The classical form, which is mainly diagnosed between 6 and 18 months of age, is characterised by villous atrophy and typical symptoms of intestinal malabsorption. The atypical form is characterised by architectural abnormalities of the small intestinal mucosa and minor intestinal symptoms. Patients with this form present predominantly with various extra-intestinal signs and symptoms, such as osteoporosis, peripheral neuropathy, anaemia and infertility. The latent form is defined by presence of HLA-DQ2 and/or HLA-DQ8 molecules, normal architecture of the intestinal mucosa and possibly positive serology. Extra-intestinal signs and symptoms may or may not occur. In this form of disease the gluten-dependant changes appear later in life. The silent form is marked by small intestinal mucosal abnormalities and in most cases by positive CD associated serology, but is apparently asymptomatic.

Patients with the non-classical forms of the disease are usually detected by screening of high-risk populations or during upper-endoscopic analysis for other reasons. After starting a GFD, the majority of patients, irrespective of the clinical presentation, will notice improvement of their physical and psychological condition.[46] This improvement indicates that these asymptomatic, apparently healthy individuals are indeed affected by minor, unrecognised illness features such as lack of appetite, behavioural abnormalities and fatigue, which are most likely to be consequences of the presence of the disease for years.

The prevalence of several autoimmune diseases, predominantly organ-specific diseases, is higher in patients with CD than in the general population (Box 1).[47] First-degree relatives of and patients with Down, Turner or Williams syndrome are also at increased risk for the development of CD.[48]

Box 1 | Clinical presentation of coeliac disease.

Typical signs and symptoms	Atypical signs and symptoms	Some associated diseases
Abdominal distension	Alopecia areata	Addison disease
Abdominal pain	Anaemia (iron deficiency)	Atrophic gastritis
Anorexia	Aphthous stomatitis	Autoimmune hepatitis
Bulky, sticky and pale stools	Arthritis	Autoimmune pituitaritis
Diarrhoea	Behavioural changes	Autoimmune thyroiditis
Flatulence	Cerebellar ataxia	Behçet disease
Failure to thrive	Chronic fatigue	Dermatomyositis
Muscle wasting	Constipation	Inflammatory arthritis
Steatorrhoea	Dental enamel hypoplasia	Myasthenia gravis
Vomiting	Dermatitis herpetiformis	Primary biliary cirrhosis
Weight loss	Epilepsy	Primary sclerosing cholangitis
	Oesophageal reflux	Psoriasis
	Hepatic steatosis	Sjögren disease
	Infertility, recurrent abortions	Type 1 diabetes mellitus
	Isolated hypertransaminasemia	Vitiligo
	Late-onset puberty	
	Myelopathy	
	Obesity	
	Osteoporosis/osteopenia	
	Peripheral neuropathy	
	Recurrent abdominal pain	
	Short stature	

DIAGNOSIS

Given the broad clinical spectrum of CD, accurate histological and serological testing is essential for correct diagnosis. The diagnosis algorithm of CD currently follows the revised ESPGHAN criteria, published in 1990.[49] Briefly, a positive diagnosis is made when the following features are both present: typical small intestinal histopathological abnormalities defined as hyperplastic villous atrophy, and clinical remission on a strict GFD with relief of symptoms within weeks. In asymptomatic individuals a second biopsy is required to

verify mucosal recovery after withdrawal of dietary gluten. The presence of circulating CD associated antibodies at time of diagnosis and their normalisation after a GFD support a diagnosis of CD. As a consequence of the increased appreciation of the variable clinical and histological manifestations of the disease and improvement of serological and genetic tests, further revision of the ESPGHAN criteria that takes into account the results of multicentre studies has been repeatedly advocated.[10, 50]

Appropriate diagnosis of CD is extremely important to avoid life-long unnecessary commitment to a GFD in patients with other gastro-intestinal diseases and to enable rapid treatment of patients with CD, which decreases the risk of complications.

Histopathological analysis

Histopathological analysis of small intestinal biopsy samples of individuals with CD is characterised by typical architectural abnormalities. These are classified according to the modified Marsh classification[51]: normal mucosa (Marsh 0), intraepithelial lymphocytosis (Marsh I), intraepithelial lymphocytosis and crypt hyperplasia (Marsh II), and intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy (Marsh III). Mucosal villous atrophy has long been considered the hallmark of CD and remains the gold standard in CD diagnosis. False-positive and false-negative diagnosis, however, may occur as a consequence of interobserver variability, patchy mucosal damage, low-grade histopathological abnormalities and technical limitations.[52] For example, patients with low-grade histopathological abnormalities (Marsh I or Marsh II) can present with gluten-dependent symptoms or disorders before overt villous atrophy occurs. Furthermore, several patients with isolated intraepithelial lymphocytosis (Marsh I), who are not clinically suspected of having CD, develop CD during follow-up.[50] Although the mucosal changes in CD are thought to develop gradually, whether minor mucosal lesions in asymptomatic patients indicate CD in an early stage is not yet clear.[53] In case of strong clinical suspicion of CD, duodenal biopsy must be performed regardless of serological analysis[54]; in cases of low suspicion of disease or screening, duodenal biopsy only needs to be performed in seropositive patients (Figure 1).

Serological and genetic analysis

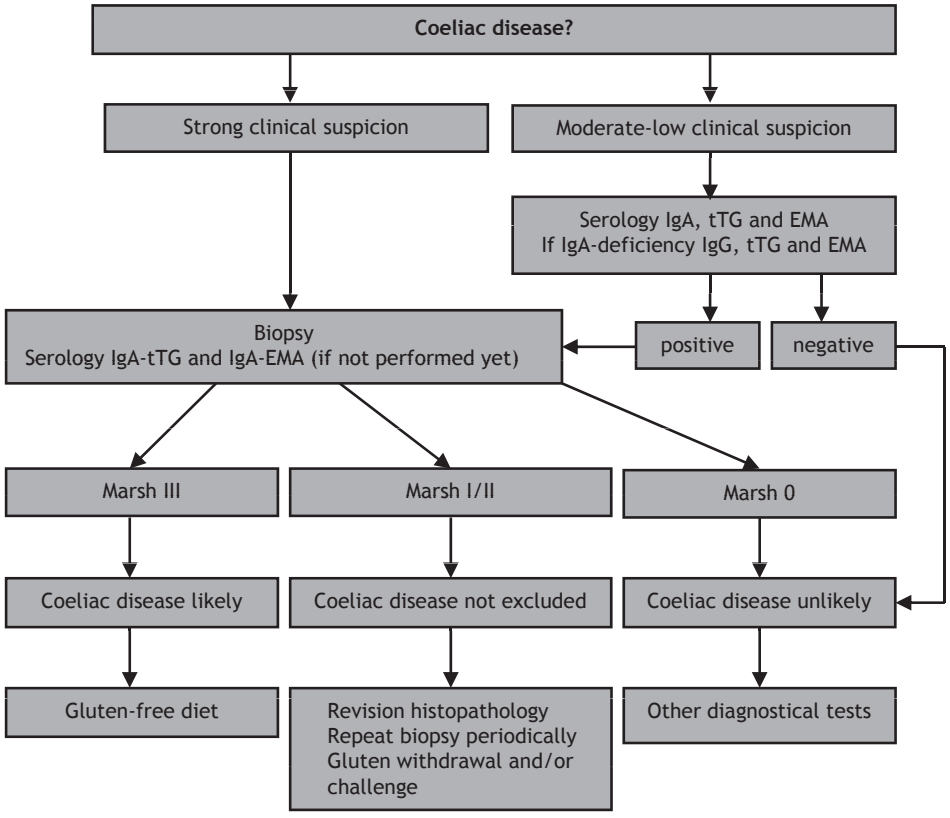
At present the most sensitive and specific serological tests for diagnosis of CD are assessments of the presence of IgA autoantibodies against the endomysium of connective tissue (IgA-EMA) and against tissue transglutaminase (IgA-tTG).[51] Tests for antibodies against deamidated gliadin peptides (which are part of gluten) have also become available and are promising diagnostic tools.[55] At diagnosis stage, at least anti-tTG antibodies should be measured and, if detected, the diagnosis of CD should be preferably verified with assessment of anti-EMA antibodies. Assessment of gliadin antibodies is a less specific and sensitive test than anti-tTG and anti-EMA testing, except in children younger than two years of age, in whom measurement of antibodies to gliadin is a more sensitive test.[56] An important pitfall in

serological testing for CD is the increased prevalence of IgA-deficiency observed in patients with the disease compared with that in healthy individuals.[57]

In order to avoid false-negative serological results in cases of IgA-deficiency, simultaneous monitoring of serum IgA levels is required. In case of IgA-deficiency, screening for IgG antibodies (either to tTG or to EMA) should be performed.[58]

Although HLA-DQ2 and/or HLA-DQ8 positivity is not an absolute requirement for diagnosis, as 40% of the healthy Western population also carry genotypes for these molecules[8], CD is highly unlikely in case both of them are absent. As a consequence of this high negative predictive value for developing CD, HLA-genotyping was proposed as a contributing element in diagnosis, in particular in the absence of villous atrophy.[59] Case-finding of patients with low-grade histopathological features of CD needs to be extended so that clinical studies that investigate the precise role of genotyping as a first-line examination in the diagnostic work-up can be started.

Figure 1 | Algorithm for diagnosis of uncomplicated coeliac disease.



Abbreviations: IgA-EMA, endomysial antibodies; IgA-tTG, tissue transglutaminase antibodies.

Furthermore, as negative serological testing does not exclude the development of CD later in life, HLA-genotyping has also been suggested as a powerful screening tool.[60] Nevertheless, this screening strategy does not seem cost-saving compared with first-line serological screening, although it might prevent unnecessary anti-tTG and anti-EMA diagnostic testing.[61] The Dutch Medical Coeliac Disease Society recommends serological testing in genetically susceptible patients for at least IgA antibodies against tTG every 2 and 5 years in adults and children, respectively.[62]

TREATMENT

The only currently available treatment for CD consists in dietary exclusion of grains containing gluten and supportive nutritional care in case of iron, calcium and vitamin deficiencies.[63] A life-long GFD is a well tolerated therapy that improves health and quality of life in the vast majority of patients with CD, even in those with minimal symptoms.[64] This treatment is, however, difficult to sustain, owing to small levels of gluten contamination in food products, high costs and restricted availability of gluten-free food alternatives and cultural practices leading to a substantial social burden.[65] Therefore, in the past decade researchers have become increasingly interested in therapeutic alternatives for continuous or intermittent use in patients with CD. Implementation of these is extremely challenging, as their potential adverse effects will always be difficult to accept as an alternative to a safe GFD.

Newly developed treatment modalities for CD are aimed at reducing the need for a strict GFD and are based on currently available insights into the pathogenesis of the disease (Table 2). These therapies focus on alteration of dietary food products, decrease of gluten exposure by rapid enzymatic degradation, inhibition of small intestinal permeability or modulation of the immune response.[66] Clinical trials for some of these therapies are still ongoing. For the time being, strict adherence to a GFD should be advised for all patients with CD, as it remains the only effective and safe therapy, which also seems to reduce the risk of complications.[67, 68]

COMPLICATIONS

The long-term consequences of CD are a matter of debate, particularly since the recognition of its broad clinical spectrum. The influence of non-compliance to a GFD and the substantial number of patients being undiagnosed are of greatest concern, as these factors could possibly contribute to the refractory form of CD and to the development of malignancies.

Table 2 | Treatment of uncomplicated coeliac disease.

Therapeutic aims and approaches	Therapies
Decrease gluten exposure	
Manipulation or selection of dietary components	Cereal modification[111, 112]
	Polymeric gliadin binders and neutralizers[113]
Enzymatic degradation of gluten	
	Aspergillus-niger derived prolyl endopeptidase[114]
	ALV003 enzyme cocktail[115, 116]
	Probiotics (VSL#3)[117]
Inhibit intestinal permeability	
Zonulin inhibition	Larazotide acetate[118]
Modulate the immune response	
Decrease adaptive immune activity	Blockers of tissue transglutaminase antibodies[119]
	Blockers of antigen presentation by HLA-DQ2 and HLA-DQ8[120]
	Gluten peptide vaccine[121]
	Infection with the hookworm *Necator americanus*[122]
Reduce inflammation	Interleukin-10[123]

Refractory coeliac disease

Some patients with adult-onset CD, especially among those diagnosed above the age of 50, show a lack of response to a GFD. They are diagnosed as having RCD when clinical and histological symptoms persist or recur after a former good response to a strict GFD and despite strict adherence to the diet for more than twelve months, unless earlier intervention is necessary.[4] The prevalence of RCD is currently unknown, but we believe that it might affect approximately 5% of patients with CD. According to the European Coeliac Disease working group, RCD can be subdivided into types I and II, with phenotypically normal and aberrant intraepithelial T-lymphocytes in the small intestinal mucosa, respectively.[69]

Intraepithelial T-lymphocytes (IELs) are considered aberrant when expressing cytoplasmic CD3, but lacking surface expression of the T-cell markers CD3, CD4, CD8[4] and the T-cell receptor[70]. To discriminate between RCD I and RCD II, a clinically validated cut-off value of 20% aberrant IELs, determined by analysis of small intestinal biopsy samples by flow cytometry, is used (Figure 2).[70] As flow cytometric analysis can not be performed in all medical centres, immunohistochemistry for CD3 and CD8 as a first-line screening, before performing flow cytometric analysis, in all patients diagnosed as having CD and who are above 50 years of age would also probably be helpful in identifying patients with RCD II, but proper studies are lacking.

Patients with RCD I have a less dismal prognosis compared with those diagnosed as having RCD II: the 5-year survival rates are 80-96% and 44-58%, respectively.[3, 71, 72] The reason for this difference is the higher risk of developing lymphoma in RCD II, as a consequence

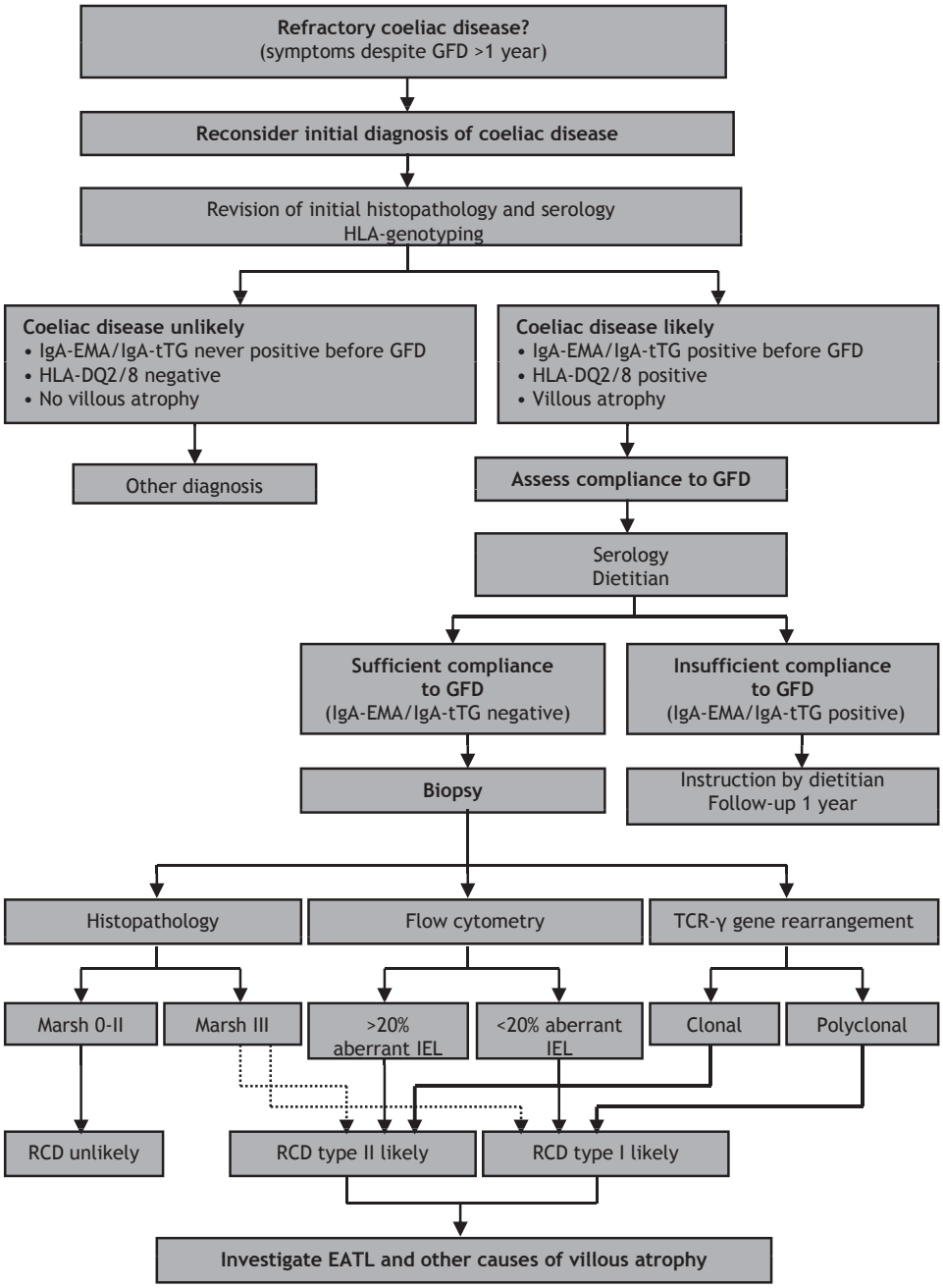
of clonal expansion and further transformation of aberrant IELs into EATL.[3, 4] EATL occurs in more than half of the RCD II patients within 4–6 years after RCD II diagnosis and is the main cause of death in this group of patients.[3, 71] Development of EATL was also observed in patients with RCD I in a single-centre study, albeit less frequently than in patients with RCD II (14%).[72] Furthermore, whereas RCD I can be treated effectively with prednisone with or without azathioprine in most cases[71, 73, 74], no standardised approach has yet been developed for RCD II, apart from nutritional support and strict adherence to a GFD.[72, 75] In the past decade several conventional and more experimental therapies have been evaluated to treat RCD II (Box 2), however this condition is usually resistant to any known therapy and transition into EATL could not be prevented successfully. Cladribine therapy[76] and autologous haematopoietic stem-cell transplantation[5] might be successful, but long-term results are awaited. Interleukin-15-blocking antibodies, which have been successfully used in the treatment of rheumatoid arthritis, might be a promising new therapeutic alternative, as this cytokine has a key role in the pathogenesis of RCD.[77]

Malignancies

CD is thought to be associated with an increased risk of malignancies, in particular lymphomas, but the risk currently estimated for this association, 1.3-fold greater than that of the general population[78], is much lower than that recorded in the 1970s and 1980s[79]. Earlier detection and treatment of CD in the past two decades may have contributed to this decline. Whether continued gluten exposure is associated with the higher incidence of malignancies in patients with CD is widely debated, as many patients are not recognised as having the disease and are, therefore, untreated. A Finnish cohort study showed no additional risk for malignancies among adult patients with CD who were diagnosed exclusively by serologic screening and followed for almost 20 years.[79]

The principal malignancy associated with CD is EATL, which has an annual incidence of only 0.5-1.0 cases per million people in Western countries.[80] Unexplained weight loss, abdominal pain, fever and night sweating should alarm physicians of the presence of an overt EATL. EATL can involve all areas of the small intestine, stomach and colon, being particularly frequent in the proximal jejunum. In some patients with CD, EATL may even occur outside the gastro-intestinal tract, for example in the lungs, ribs and spleen, without abdominal pain.[81] EATL is one of the main causes of death in patients with adult-onset CD, with 2-year and 5-year overall survival rates of 15–20% and 8–20%, respectively.[3, 82] This poor prognosis is mainly due to incomplete response to currently available therapies (Box 2), high rates of life-threatening complications such as perforation of the gut, and poor nutritional conditions.[75, 83]

Figure 2 | Algorithm for diagnosis of complicated coeliac disease.



Abbreviations: EATL, enteropathy associated T-cell lymphoma; IgA-EMA, endomysial antibodies; GFD, gluten-free diet; IEL, intra-epithelial lymphocyte; RCD, refractory coeliac disease; TCR-γ, T-cell receptor, γ-chain; IgA-tTG, tissue transglutaminase antibodies.

Box 2 | Treatment of complicated coeliac disease.

RCD I	RCD II	EATL
Azathioprine[74]	Alemtuzumab[126]	Allogenic stem-cell transplantation[133]
Azathioprine and prednisone[73]	Autologous stem-cell transplantation[5]	Autologous stem-cell transplantation[82, 134]
Budesonide[124]	Azathioprine and prednisone[73, 74]	CHOP[83]
Infliximab[125]	Budesonide[124]	Alemtuzumab and CHOP[135]
	Cladribine[76]	
	Cyclosporine[127,128]	
	Interleukin-10[129]	
	Pentostatine[130]	
	Mesenchymal stem-cell infusion[131]	
	Antibodies against interleukin-15[132]	

Abbreviations: CHOP, cyclophosphamide doxorubine vincristine prednisone; EATL, enteropathy associated T-cell lymphoma; RCD, refractory coeliac disease.

CONCLUSION

Many factors have contributed to the increased prevalence of CD, which has emerged as a common food intolerance worldwide that can be diagnosed at all ages. Growing insight into the clinical presentation of CD has resulted in novel diagnostic, prognostic and therapeutic dilemmas and highlights the importance of considering the current diagnostic criteria of CD and its complications, as well as the evaluation and development of new treatment modalities.

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PART II

DIAGNOSTIC ASPECTS

CHAPTER 2

Serum I-FABP as marker for enterocyte damage in coeliac disease and its relation to villous atrophy and circulating autoantibodies

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ABSTRACT

Objectives: Enterocyte damage is the hallmark of coeliac disease (CD) resulting in malabsorption. Little is known about the recovery phase of enterocyte damage and its clinical consequences. This study evaluates the severity of enterocyte destruction in adult CD patients and its time-related changes upon a gluten-free diet (GFD), using serum intestinal fatty acid binding protein (I-FABP) as marker. Furthermore, the correlation between enterocyte damage, CD autoantibodies and small intestinal mucosal abnormalities during the course of disease is studied.

Methods: Serum I-FABP levels were determined in 96 biopsy-proven adult CD patients and in 69 CD patients repeatedly upon a GFD. 141 individuals with normal anti-tissue transglutaminase antibody levels (IgA-tTG) served as controls. I-FABP levels were related to the degree of villous atrophy (Marsh grade) and IgA-tTG.

Results: I-FABP levels were significantly higher in untreated CD compared to controls, and correlated with Marsh grade and IgA-tTG. Upon a GFD serum levels decreased significantly, however, not within the range observed in controls, despite normalisation of IgA-tTG levels in most of the patients. High I-FABP levels not responding to treatment were found in patients with persistent histological abnormalities.

Conclusion: Enterocyte damage assessed by serum I-FABP correlates with the degree of villous atrophy and improves after initiation of a GFD. However, we now show that significant enterocyte damage persists upon a GFD despite absence of villous atrophy and low IgA-tTG levels in the majority of cases. Moreover, a lack of decrease in I-FABP levels during treatment is indicative of histological abnormalities and warrants further evaluation of patients.

INTRODUCTION

Coeliac disease (CD) is a common T-cell mediated enteropathy triggered by dietary gluten in genetically susceptible individuals of all ages worldwide.[1, 2] The disease is characterised by villous atrophy which is suggested to result from increased enterocyte apoptosis.[3, 4] However, a detailed understanding of the cellular behaviour of enterocytes in the intestine is lacking, particularly into the recovery of the small intestinal mucosa after initiation of a gluten-free diet (GFD). Nevertheless, mucosal damage in adult-onset CD is thought to recover slow and incomplete, and certain nutrient deficiencies are described to persist in a subgroup of patients despite treatment with a GFD.[5-8] As enterocytes are the anatomical and functional unit of the intestinal villi responsible for nutrient absorption and barrier function, insight in the course of enterocyte destruction in CD is of utmost importance in the pathogenesis of CD.

The limited knowledge of enterocyte damage in the course of CD is mainly caused by the absence of a reliable non-invasive tool for evaluation of intestinal damage. The CD associated antibodies anti-tissue transglutaminase (IgA-tTG), anti-endomysium (IgA-EMA) and anti-deamidated gliadin (IgA-DGA), which are used for monitoring disease activity, represent the immune response rather than intestinal damage or even repair.[9-13] Consequently, duodenal biopsies, taken through the invasive procedure of upper gastro-intestinal endoscopy, are required for the assessment of small intestinal disease activity, yet not providing information about damage at a cellular level.

Intestinal fatty acid binding protein (I-FABP), a small (15 kD) cytosolic protein abundantly present in enterocytes, has proven to be a sensitive marker for damage to the intestinal epithelium.[14-17] The expression of I-FABP is primarily limited to fully differentiated epithelial cells of the small intestine.[18, 19] Upon cellular damage of the enterocyte I-FABP is readily released into the systemic circulation. Our previous studies showed that in CD patients serum I-FABP levels represent small intestinal mucosal damage.[18, 20]

This study aims to evaluate the severity of enterocyte damage in untreated adult CD patients, and the time-related changes of enterocyte damage after initiation of a GFD by assessing serum I-FABP levels. In addition, the interplay between serum I-FABP levels as marker for enterocyte damage and CD autoantibodies as well as small intestinal villous atrophy was studied.

MATERIALS AND METHODS

Subjects

The study group included 96 adults with biopsy-proven CD evaluated at the Maastricht University Medical Centre (MUMC) or VU University Medical Centre Amsterdam (VUMC), The Netherlands, between 1996 and 2010. CD diagnosis was based on the combination of positive antibodies for IgA-tTG and/or IgA-EMA, and histological abnormalities grade III

according to the modified Marsh classification consisting of intra-epithelial lymphocytosis, crypt hyperplasia and some degree of villous atrophy.[21-23] All CD patients started a GFD. Patients with diagnosed and treated refractory coeliac disease (RCD), defined as persisting or recurring symptoms and mucosal villous atrophy despite strict adherence to a GFD for more than 12 months (assessed by a dietitian and negative IgA-tTG and IgA-EMA), and patients with enteropathy associated T-cell lymphoma (EATL) were excluded.[24, 25] Serum samples at time of diagnosis were available in 80 CD patients. Furthermore, serum samples of 69 CD patients were analysed at six months, one year, two years and/or three years after initiation of a GFD. Due to the retrospective study design, follow-up samples were not available of all patients at all moments of follow-up.

The control group consisted of 141 adults with a clinical suspicion of CD, yet with negative IgA-tTG and IgA-EMA levels included retrospectively at both academic centres. IgA-deficiency was not observed in the control group. Individuals recognised with autoimmune disorders were excluded from the control group.

All serum samples and biopsy specimens were obtained for diagnostic purposes and the procedures adhered to guidelines set by the institutional ethical committee.

Coeliac disease autoantibodies

In both CD patients and controls serum IgA-EMA was determined by means of a commercial indirect immunofluorescence test (Scimedx, Densvill, New Jersey, USA)[26] or by an in-house indirect immunofluorescence test according to Lerner using monkey oesophagus as substrate[27]. Serum IgA-tTG was determined by means of fluorescent-enzyme immuno-assay (FEIA, Thermo Fisher Scientific, Freiburg, Germany, cut-off value 10 U/ml) or with a standard enzyme-linked immunosorbent assay (ELISA) using recombinant human tTG (Diarect AG, Freiburg, Germany, cut-off value 6 U/ml). IgA-deficiency was excluded to avoid false-negative serology. Only patients evaluated with the ELISA technique for IgA-tTG were analysed to study the correlation with serum I-FABP levels.

Histology

Small intestinal biopsies of both untreated and treated patients were taken from the distal duodenum during endoscopy. Mucosal damage was graded according to the modified Marsh classification; Marsh 0 normal biopsy, Marsh I intraepithelial lymphocytosis (IEL), Marsh II crypt hyperplasia, Marsh IIIA partial villous atrophy, Marsh IIIB subtotal villous atrophy and Marsh IIIC total villous atrophy.[22, 23] The most severe grade of damage was reported. Small intestinal biopsies were not determined in the control group.

Intestinal fatty acid binding protein (I-FABP)

Serum of patients was stored at -20°C. Serum samples of the untreated patients were collected at a maximum of three months before CD was confirmed by small intestinal biopsy.

Serum I-FABP levels were determined in duplicate using a highly specific in-house ELISA that selectively detects human I-FABP (standard: 12.5-800 pg/ml). The optimal cut-off value for CD of the I-FABP ELISA was determined at 382 pg/ml. Storage time of the serum samples did not significantly influence levels of I-FABP.

Statistical analysis

Statistical analyses were performed using SPSS, version 19.0 for Windows (SPSS Inc, Chicago, Illinois, USA). For descriptive purposes, serum I-FABP levels (right-skewed distribution) are presented as median and interquartile range.

In order to find the cut-off point of serum I-FABP levels that most accurately discriminates patients with CD from patients without CD, a receiver operating characteristic (ROC) curve was drawn by plotting sensitivity against 1-specificity for all possible serum I-FABP thresholds. The overall accuracy of serum I-FABP for detecting CD was summarized using the area under the curve (AUC).

Log transformed (ln) baseline serum I-FABP levels were compared between CD patients and controls with a linear regression model adjusting for age. Patients with CD were stratified for Marsh grade, and compared with serum I-FABP levels also using the Kruskal Wallis test. Spearman's correlation coefficient test was used for identifying a correlation between serum I-FABP levels and both Marsh grades and IgA-tTG levels. A p-value below 0.05 was considered statistically significant.

Table 1 | Baseline characteristics of untreated coeliac disease patients and controls.

	coeliac disease n=80		control group n=141		p-value
Mean age in years (±SD)	47.1	(±17.0)	41.3	(±16.0)	0.015
Gender					
Male	31	(38.8%)	68	(48.2%)	0.174
Female	49	(61.3%)	73	(51.8%)	
IgA-EMA					
Positive	77	(96.3%)	-	-	
Negative	1	(1.3%)	141	(100%)	
ND	2	(2.5%)	-	-	
IgA-tTG					
Positive	71	(88.8%)	-	-	
Negative	1	(1.3%)	141	(100%)	
N.D.	8	(10.0%)	-	-	
Marsh grade					
IIIA	32	(40.0%)			
IIIB	23	(28.8%)		ND	
IIC	25	(31.3%)			

Abbreviation: ND, not determined.

RESULTS

In total, 96 adult biopsy-proven CD patients with elevated levels of IgA-tTG and/or IgA-EMA were included. 81 CD patients were analysed at time of diagnosis (Table 1). A total of 32 (40.0%) biopsies were classified as Marsh IIIA, 23 (28.8%) as Marsh IIIB and 25 (31.3%) as Marsh IIIC. Serum IgA-tTG and intestinal histology were available in 69 and 51 CD patients, respectively, during follow-up. The control group comprised 141 individuals with negative CD autoantibody levels.

Enterocyte damage assessed by serum I-FABP in untreated CD patients are associated with IgA-tTG levels and severity of villous atrophy

Serum I-FABP levels were significantly elevated in patients with untreated CD (median 691 pg/ml, interquartile range 447-1266 pg/ml) as compared to the levels in the control group (median 178 pg/ml, interquartile range 126-286 pg/ml; $p<0.001$; Figure 1A). With proper adjustment for age, the magnitude of the difference in serum I-FABP levels turned out to be much higher for older than younger subjects. Significant interaction with log transformed mean difference at the age of 30 and 60 years being 0.885 and 1.571, respectively (both $p<0.001$). The ROC curve for serum I-FABP yielded a high AUC of 0.88 (95% CI: 0.83–0.93; $p<0.001$) with an optimal cut-off value of 382 pg/ml and a sensitivity and specificity of 80% and 87.2%, respectively. The positive predictive value (PPV) and negative predictive value (NPV) were 78.0% and 88.5%, respectively. As depicted in Figure 1B, significantly elevated I-FABP levels account for each separate category of the Marsh classification; Marsh grade IIIA (median 530 pg/ml, interquartile range 384-836 pg/ml), Marsh IIIB (median 755 pg/ml, interquartile range 475-1248 pg/ml) as well as Marsh IIIC (median 860 pg/ml, interquartile range 556-1620 pg/ml). In addition, I-FABP levels correlated significantly with Marsh grade ($r=0.265$, $p<0.05$, $n=80$). Furthermore, serum levels of I-FABP correlated with IgA-tTG levels at time of diagnosis ($r=0.403$, $p<0.01$, $n=65$).

Incomplete normalisation of serum I-FABP levels in CD patients upon gluten elimination

I-FABP levels were investigated during dietary treatment in 69 patients. Six months after dietary exclusion of gluten an evident decline of I-FABP levels was shown ($p<0.01$; Figure 2). Serum I-FABP levels decreased further between six months and one year of adherence to a GFD, but did not reach significance ($p=0.071$, pair-wise comparison). Although I-FABP levels decreased during follow-up in CD, these levels seem to reach a plateau after 1-2 years of adherence to a GFD. The values did not decline towards the normal range observed in the control group, even despite the improvement of histological findings to Marsh 0 and or I ($p<0.05$) as shown in Figure 3. IgA-tTG levels normalised in 77% of the patients within two years of follow-up.

Six patients did not demonstrate a decrease in I-FABP levels upon a GFD for 1-2 years. Duodenal biopsies were available for five of these patients; with a mean diet time of 19

months (Table 2), showing crypt hyperplasia (n=4) and/or villous atrophy (n=1). In addition, IgA-tTG levels normalised in two patients with Marsh II. The positive and negative predictive values of serum I-FABP for intestinal damage in all CD patients on a GFD were 78,3% and 65,4%, respectively.

Figure 1 | Serum I-FABP levels in untreated coeliac disease patients compared to control subjects (A) and stratified for the degree of villous atrophy (B).

Serum I-FABP levels are expressed as medians with interquartile range.

Controls were defined as IgA-tTG negative, no biopsies were taken.

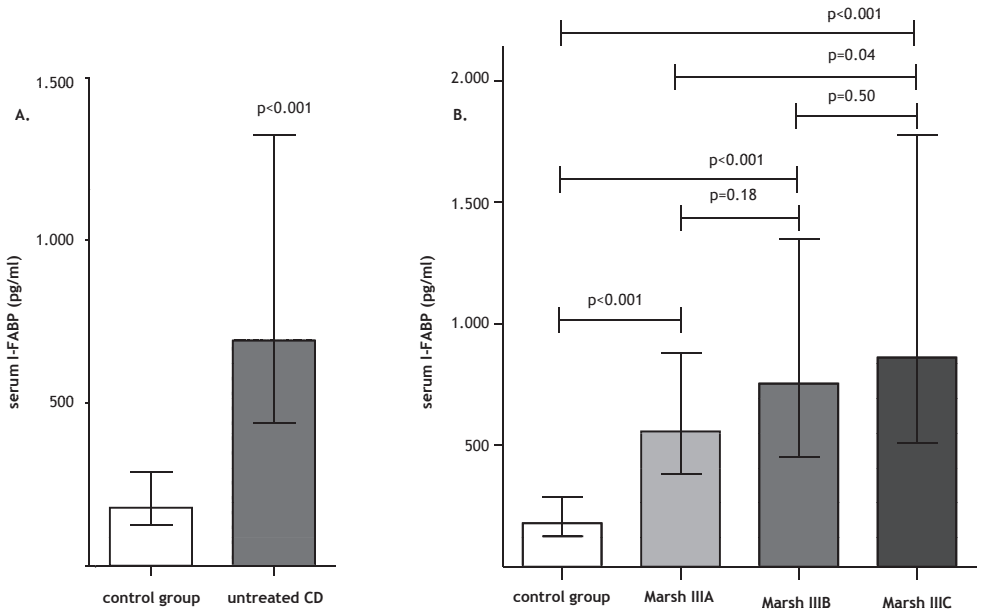


Figure 3 illustrates the significant correlation between serum I-FABP levels and villous atrophy in all CD patients upon a GFD (r=0.405, p<0.001, n=49). Otherwise, serum I-FABP levels did not correlate with IgA-tTG levels in CD patients upon a GFD (r=0.159, p=0.50).

Table 2 | Characteristics of coeliac disease patients with persisting elevated serum I-FABP levels upon a gluten-free diet for 1-2 years.

	Gender	Age (yr)	Duration of GFD (mo)	Marsh grade	IgA-EMA	IgA-tTG (U/mL)	I-FABP (pg/ml)
1	M	40	20	Marsh II	negative	negative (under detection limit)	587
2	F	68	23	Marsh IIIA	negative	positive (55 U/ml)	2992
3	M	64	16	Marsh II	borderline	borderline (8 U/ml)	632
4	M	63	18	Marsh II	positive	positive (> 80 U/ml)	581
5	M	56	24	ND	borderline	negative (under detection limit)	765
6	M	75	12	Marsh II	negative	negative (under detection limit)	853

Abbreviations: M, male; F, female; ND, not determined; GFD, gluten-free diet.

Figure 2 | Serum I-FABP levels and time-related changes after initiation of a gluten-free diet. Serum I-FABP levels were log transformed, the line displays estimated mean values during follow-up, according to the random intercept model.

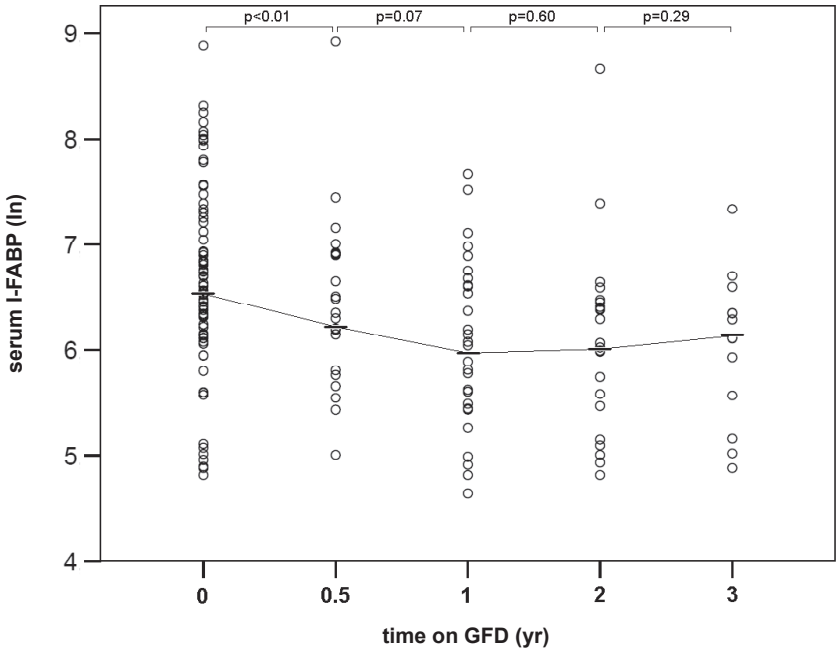
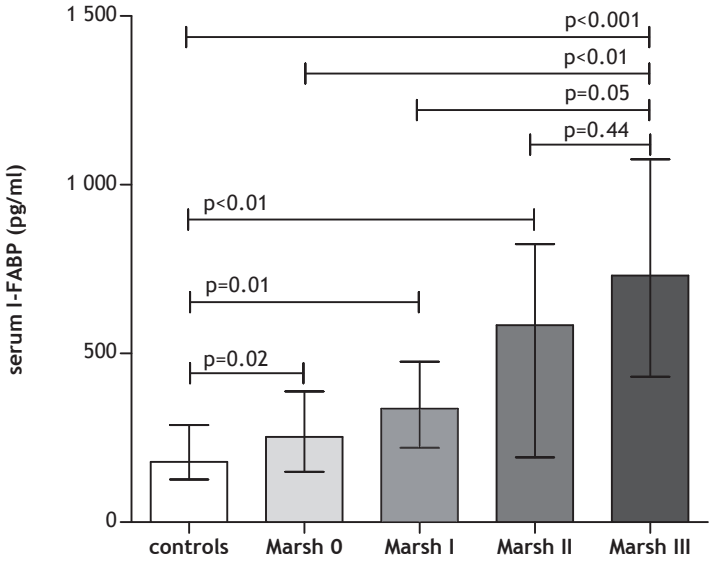


Figure 3 | Correlation between serum I-FABP levels and Marsh grade in coeliac disease patients on a gluten-free diet.

Serum I-FABP levels were expressed as medians with interquartile range.

Controls were defined as IgA-tTG negative, no biopsies were taken.



DISCUSSION

The findings of the present study, which is the first to directly evaluate enterocyte damage in adult CD patients, provide new information about two fundamental aspects of CD. First, serum I-FABP as disease independent marker for enterocyte damage is abundantly present in untreated CD and correlates with both the degree of villous atrophy and IgA-tTG levels. Second, significant enterocyte damage persists in treated CD patients after an initial decline of enterocyte damage in the first year after initiation of a GFD, despite absence of villous atrophy and low levels of IgA-tTG in the majority of cases.

Excessive enterocyte apoptosis has been proposed as one of the responsible mechanisms for the loss of absorptive villi in CD and correlates with the degree of villous atrophy.[3, 4, 28] The subsequent enhanced proliferative state does not compensate for the excessive loss of enterocytes, ultimately leading to villous atrophy.[28] After short-term adherence to a GFD apoptotic rate is suggested to fall to a normal level.[3] Besides apoptosis markers, different parameters have been investigated as evidence for intestinal epithelial damage in CD, including markers of intestinal paracellular permeability and absorptive surface area and function (plasma citrulline levels, C-sucrose breath test).[29, 30] However, these parameters do not represent enterocyte damage directly. As the enterocytes are the main target in CD

and the leading cause of the clinical features, direct insight in mucosal damage at a cellular level is mandatory.

This report showed significantly elevated levels of serum I-FABP at time of CD diagnosis as compared to control subjects. These levels correlate with the degree of villous atrophy and level of IgA-tTG. The latter implies an association between an activated immune response (IgA-tTG) and enterocyte damage (I-FABP). In agreement with previous studies on enterocyte apoptosis, this study revealed a correlation between serum I-FABP levels and villous atrophy in untreated CD patients, in line with the hypothesis that villous atrophy occurs as a consequence of enterocyte damage.[3, 4]

Furthermore, serum I-FABP levels decreased during the first year of follow-up after initiation of a GFD. However, I-FABP levels did not decline towards the normal range observed in the control group in a subgroup of patients despite treatment with a GFD up to three years, suggesting ongoing enterocyte damage. These findings are consistent with other reports showing slow and incomplete recovery of the small intestinal mucosa by histopathological assessment in adult-onset CD. Only 35-65% of these patients show histological remission within 2 years, despite apparent strict adherence to a GFD and remission of symptoms.[5-7, 31] Serum I-FABP levels correlate with villous atrophy in CD patients upon a GFD as well, yet persisting increased levels in a lower range were not related to villous atrophy. This discrepancy between persisting enterocyte destruction and absence of intestinal mucosal abnormalities is in line with recent studies. These reports show that morphometric indices including villous area and crypt length, improve upon a GFD in adult CD, however, rarely return to values observed in control subjects, even when the Marsh grade normalised.[32] Villous area was found to stabilise after one year of apparent adherence to a GFD and improved less than 50% after long-term treatment. In addition, electron microscopy revealed persisting enterocyte damage, despite a normalisation of the intestinal mucosa by routine histological assessment.[33, 34] Therefore, it is tempting to speculate that there are subtle alterations of the enterocyte integrity that persist for a long period after the start of a GFD in adult CD patients that can explain the significantly increased levels of I-FABP found in this study.

In contrast to the described results in adult CD patients, our previous study on serum I-FABP levels in children diagnosed with CD, showed a rapid decrease and normalisation of I-FABP levels after initiation of a GFD, which is faster than the decline of CD autoantibodies.[20] In line with these findings, mucosal recovery in children is thought to progress faster and more complete as compared to patients diagnosed at an adult age.[6, 35]

Although serum I-FABP levels in untreated CD patients correlated with IgA-tTG levels implying that the activated immune response is responsible for this enterocyte damage, our results showed that enterocyte damage assessed by serum I-FABP levels persists in CD patients upon a GFD despite low IgA-tTG levels. This suggests that enterocyte damage is independent of autoantibody production or at least from circulating autoantibodies. It has

been shown that serum autoantibody levels decline faster than the intensity of mucosal autoantibody deposits during dietary treatment and moreover the presence of deposits can persist for more than two years.[36]

The cause for the ongoing enterocyte damage in adult CD patients on a GFD remains to be revealed. Gluten ingestion might be one explanation. Despite the diet, all CD patients ingest at least minute amounts of gluten, and it is reported that up to 60% of CD patients are partially non-adherent.[10, 37-39] Dietary compliance is suggested to be related to the severity of symptoms of malabsorption before treatment. Individuals with adult-onset CD have mostly mild symptoms, whereas children often present with classic symptoms, which might contribute to age-dependent differences in mucosal recovery.[37, 39] In addition, prolonged gluten exposure in adult CD patients before final diagnosis may result in severe damage and consequently slow recovery of the mucosa, whereas early diagnosis in children might be related to mild intestinal damage and fast recovery.[7, 40] Furthermore, it has been suggested that the immune response to gliadin may persist even upon a GFD.[1, 7, 36, 41] However, in this study in most of the long-term treated patients circulating IgA-tTG autoantibodies were not detectable.

Currently, it is unknown whether the observed enterocyte damage in CD patients on a GFD influences nutrient absorption. Nevertheless, it is suggested that ongoing intestinal damage in CD patients is based on several mechanisms such as increased intestinal permeability and production of cytokines that may contribute to systemic symptoms, and associated with long-term complications of autoimmune and malignant disorders.[42, 43] For daily clinical practice, our findings suggest that serum I-FABP might be a potential marker for follow-up, but a prospective study is mandatory.

A limitation of this study is related to drop-out linked to the retrospective nature of the study design. It is possible that missing data biased our results; some patients may have undergone follow-up CD autoantibody tests and duodenal biopsy on account of their clinical deterioration. If so, drop-out was not at random (as assumed by the model), meaning that we could be missing complete histological responders potentially biasing our data. Otherwise, incompliant patients might have withdrawn from medical care. Our follow-up at three years on a GFD data included only 15% of the CD patients. Moreover, no intestinal biopsies were taken in the control group. Although the sensitivity of IgA-tTG tests is high, there might be hidden seronegative CD patients in this control group. As we expect that these patients could be responsible for the highest serum I-FABP levels in the control group, this will only emphasize the difference between enterocyte damage in CD patients (on a GFD) and controls. Although our data fit with the results of different studies reporting slow and incomplete recovery of the intestinal mucosa in adult-onset CD patients[5-7, 31, 32], a prospective study is indicated.

In conclusion, this study provides direct insight in damage to the small intestinal epithelium at enterocyte level during the course of CD in adult patients. We demonstrated

evident elevated levels of I-FABP associated with villous atrophy and IgA-tTG levels in untreated CD patients and improvement of this mucosal damage after initiation of a GFD in the majority of patients. Interestingly, significant enterocyte damage persists in CD patients despite apparent long-term adherence to a GFD. Serum I-FABP levels might be useful in daily practice in identifying non-compliance and unintentionally gluten intake, evaluating new therapeutic options, and might provide us with a minimal-invasive marker for detection of ongoing mucosal architectural abnormalities without the need of endoscopy.

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CHAPTER 3

Serum parameters in the spectrum of coeliac disease: beyond standard antibody testing

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ABSTRACT

Objectives: Invasive techniques are still required to distinguish between uncomplicated and complicated forms of coeliac disease (CD).

Methods: We set out to investigate the potential use of novel serum parameters, including IL-6, IL-8, IL-17, IL-22, sCD25, sCD27, granzyme-B, sMICA and sCTLA-4 in patients diagnosed with active CD (ACD), CD on a gluten-free diet (GFD), refractory coeliac disease (RCD) type I and II, and enteropathy associated T-cell lymphoma (EATL).

Results: In both active CD and RCD I-II elevated levels of the pro-inflammatory IL-8, IL-17 and sCD25 were observed. In addition, RCD II patients displayed higher serum levels of soluble granzyme-B and IL-6 in comparison to ACD patients. In contrast, no differences between RCD I and ACD or RCD II were observed. Furthermore, EATL patients displayed higher levels of IL-6 as compared to all other groups.

Conclusion: A series of novel serum parameters reveal distinctive immunological characteristics of RCD II and EATL in comparison to uncomplicated CD and RCD I.

INTRODUCTION

Coeliac disease (CD) is a chronic immune-mediated inflammation of the small intestine caused by a permanent state of intolerance to ingested gluten proteins affecting genetically susceptible individuals. Its hallmarks, lymphocytic infiltration of the lamina propria, expansion of the intraepithelial lymphocyte (IEL) population, hyperplasia of the crypts and atrophy of the villi are mediated by the interplay between an innate and adaptive immune response against gluten.[1] The complex of deamidated gliadin peptide interacting with the HLA-DQ2 and/or –DQ8 heterodimers on antigen presenting cells, is capable of activating the lamina propria T-helper lymphocytes, thereby initiating antibody production and a gluten-specific pro-inflammatory type 1 T-cell response (Th1).[1] Th1-cells play an important role in the pathogenesis of CD by secreting IL-2 that induces proliferation of T-lymphocytes and, in particular, by secreting the pro-inflammatory cytokine interferon-gamma (IFN-γ).[2] Furthermore, recent evidence has indicated that Th17-cells play a pathogenic role in CD.[3-5] In addition, IL-15 activates a cytotoxic response of intra-epithelial lymphocytes through IFN-y release and up regulation of NKG2D. In combination with epithelial stress, the NKG2D ligand MHC Class I chain-related A (MICA) is up regulated by enterocytes.[6, 7] The interaction of MICA and NKG2D induces enterocyte destruction by intraepithelial lymphocytes (IELs).

A gluten-free diet (GFD) results in mucosal recovery in the majority of patients, who are referred to as uncomplicated CD-patients. However, a small subset of adult-onset CD patients fails to regain intestinal homeostasis after elimination of dietary gluten, or symptoms recur after initial response.[8] After careful evaluation of dietary compliance and exclusion of other possible disease-entities causing villous atrophy, these patients are diagnosed to suffer from refractory coeliac disease (RCD).[9] RCD is considered a complicated form of CD, and is divided into type I (RCD I), when patients lack an aberrant IEL population, or type II (RCD II) in which a substantial (>20%) aberrant IEL population is found in the small intestinal mucosa.[10, 11] An aggressive type of lymphoma which carries a dismal prognosis, the enteropathy associated T-cell lymphoma (EATL), is thought to arise from this aberrant IEL population. An interesting observation is that both aberrant IELs as well as EATL cells display a cytotoxic phenotype and contain high levels of granzyme-B[12 13], which could therefore serve as a marker for complicated CD.

In consequence of a good response to immunosuppressive therapy, RCD I patients have a better prognosis than RCD II patients.[14-17] Therefore, early identification of CD patients developing RCD II and/or EATL enables early intervention, which will likely reduce morbidity and mortality.

Currently, antibodies against tissue transglutaminase (IgA-tTG), endomysium of connective tissue (IgA-EMA) and deamidated gliadin peptides (DGP) provide valuable and generally accepted serological parameters for the diagnosis and follow-up of uncomplicated CD. However, these antibodies are of no use in predicting and monitoring RCD I-II and EATL, implying that histological and flow cytometric analysis of duodenal biopsies are still required

to distinguish between the uncomplicated and complicated forms of CD. Additional serum markers could potentially provide us with a minimal-invasive, easy applicable test without the need to perform a gastro-duodenal endoscopy. In addition, immunological markers in the peripheral blood could provide more insight in the similarities and differences of the pathophysiology underlying the CD spectrum.

Therefore, in the present study we evaluated several immunological and biochemical parameters in sera from the five stages of CD, including ACD, CD on GFD, RCD I-II and EATL, for their ability to differentiate between complicated and uncomplicated forms, and secondly, to gain insight in the pathophysiological relations between these disease entities. For this purpose, we analysed serum levels of the inflammatory cytokines IL-6, IL-8, IL-17 and IL-22, the T-cell activation factors soluble (s)CD25 (IL-2R-alpha) and sCD27, the T-cell dysregulation factor sCTLA-4, shown previously to be up-regulated in different autoimmune diseases, the cytotoxic T-cell parameter granzyme-B, and sMICA, previously shown to be associated with the presence of epithelial stress and malignancies.

MATERIALS AND METHODS

A retrospective cohort study was conducted at a tertiary referral centre for CD in The Netherlands. Patients previously diagnosed with (complicated) CD in the VU medical centre were identified and included in our study when stored serum samples at time of diagnosis were available. Overall, 92 blood samples collected for diagnostic purposes between 1997 and 2010 were included. Serum levels of a substantial number of immunological markers were determined in the five different subsets of CD. In addition, results from several biochemical parameters of this cohort at time of diagnosis were collected from the electronic patient file in our centre.

Patients

CD diagnosis was based on the ESPGHAN guidelines.[18] All patients included in the active CD group had positive IgA-EMA and/or IgA-tTG, and histological abnormalities grade III according to the modified Marsh classification consisting of intra-epithelial lymphocytosis, crypt-hyperplasia and some degree of villous atrophy.[19] Furthermore, serum samples of these patients at time of an inactive phase of CD were collected. Remission of disease (GFD group) was defined by the disappearance of one or both CD antibodies upon a GFD, and if a gastro-duodenal endoscopy with subsequent collection of biopsies was performed during follow-up, normalisation of mucosal abnormalities (Marsh 0 or I) was required. Patients included in the RCD group had persisting or recurring symptoms and small intestinal villous atrophy, despite strict adherence to a GFD for at least one year (assessed by a dietitian and negative IgA-tTG / IgA-EMA). The clinically validated cut-off value of more than 20% of the IELs expressing an aberrant phenotype (surface CD3-, but cytoplasmic CD3+) as detected by

flow cytometric analysis was used to distinguish RCD type I and II.[11] All procedures were in accordance with the regulations of the medical ethics committee, and all patients declared their informed consent to store and use their blood samples collected for regular diagnostics for further research.

In total, 26 paired serum samples of CD patients at time of disease activity (ACD group) and after serological normalisation on a GFD (GFD group), and of an additional 40 patients with complicated CD at time of diagnosis were included. The latter group consisted of 12 RCD I, 16 RCD II and 12 EATL patients.

Serum parameters: enzyme linked immunosorbent assay (ELISA)

Levels of cytokines IL-6, IL-8, IL-17 and IL-22 were determined in serum using commercial ELISA kits (Pelikine-compact™ ,Sanquin, Amsterdam, The Netherlands), according to the manufacturer's instructions. Levels of soluble CD25 (sCD25), soluble CD27 (sCD27), soluble CTLA-4 (sCTLA-4), soluble MICA (sMICA) and granzyme-B were determined in serum using a specific commercial ELISA kit (Diaclone, Besancon, Cedex, France), according to the manufacturer's instructions.

Biochemical parameters

The concentration of C-reactive protein (CRP; g/L), erythrocyte sedimentation rate (ESR; mm per 1h), leukocyte count (WBC; 10E9/L), albumin (g/L) and haemoglobin (Hb; mmol/L) were extracted from the hospital patient file for all patients included at time of diagnosis.

Statistical analysis

Data were analysed and plotted using SPSS software (SPSS Inc. Chicago, Illinois, USA), using non-parametric tests as most variables examined in this study did not appear to be normally distributed. A Wilcoxon signed-rank test was used for pair wise comparison of the variability of immunological and biochemical parameters among the active CD and GFD group. The latter groups were individually compared to the complicated forms of CD by using the Kruskal-Wallis non-parametric test to identify possible serological and biochemical differences in the spectrum of CD. A receiver operating characteristic *(*ROC*)* curve was made of all significantly different parameters to represent the trade off between the false negative and false positive rates. As a considerable number of markers were determined, the level of significance was set at highly significant ($p<0.001$) and moderately significant ($0.001< p <0.05$).

RESULTS

Table 1 shows the characteristics of the five subsets of CD. Serum samples of 26 ACD patients at time of diagnosis and after remission of disease on a GFD were included. In all patients CD associated antibodies reverted to negative upon a GFD, and in 62% (16/26) a biopsy was taken which revealed mucosal healing (Marsh 0/I) in all those evaluated. Overall, 12 RCD I, 16 RCD II and 12 EATL patients were included. Significant differences were found for cytokine profiles between the five subsets of CD, as described in more detail below (Figure 1A-I).

ACD versus GFD

Serum levels of the inflammatory chemokine IL-8 (p=0.00) and the T-cell activation factor sCD25 were higher in active CD patients than in patients in remission on a GFD (p=0.00, highly significant) as well as the Th-17 lineage-defined cytokine IL-17 levels (p=0.011, moderately significant). Serum levels of IL-6, IL-22, sCD27, sMICA, granzyme-B and sCTLA-4 were not significantly elevated in the ACD group. In addition, levels of CRP, ESR, albumin and WBC were similar (data not shown).

Uncomplicated CD versus RCD I-II

The serum levels of IL-8, IL-17 and sCD25 in RCD type I and type II were comparable to those in the ACD group, however, significantly lower levels were observed in the GFD group. Moreover, in comparison to both ACD and GFD patients, RCD II patients showed increased levels of granzyme-B (p=0.020; p=0.016, both moderately significant) and IL-6 (p=0.018; p=0.024, both moderately significant), respectively. Furthermore, serum levels of soluble CTLA-4 were lower in RCD II patients than those in remission upon a GFD (p=0.003, moderately significant). Similar IL-22 serum levels were found in uncomplicated CD and RCD I-II.

Comparison of the inflammatory parameters CRP, ESR and WBC did not reveal significant differences between uncomplicated CD and RCD I-II. The concentration of albumin was lower in both RCD subsets when compared to ACD (p=0.003, moderately significant) and GFD (p=0.000, highly significant) (Figure 2).

The RCD complex

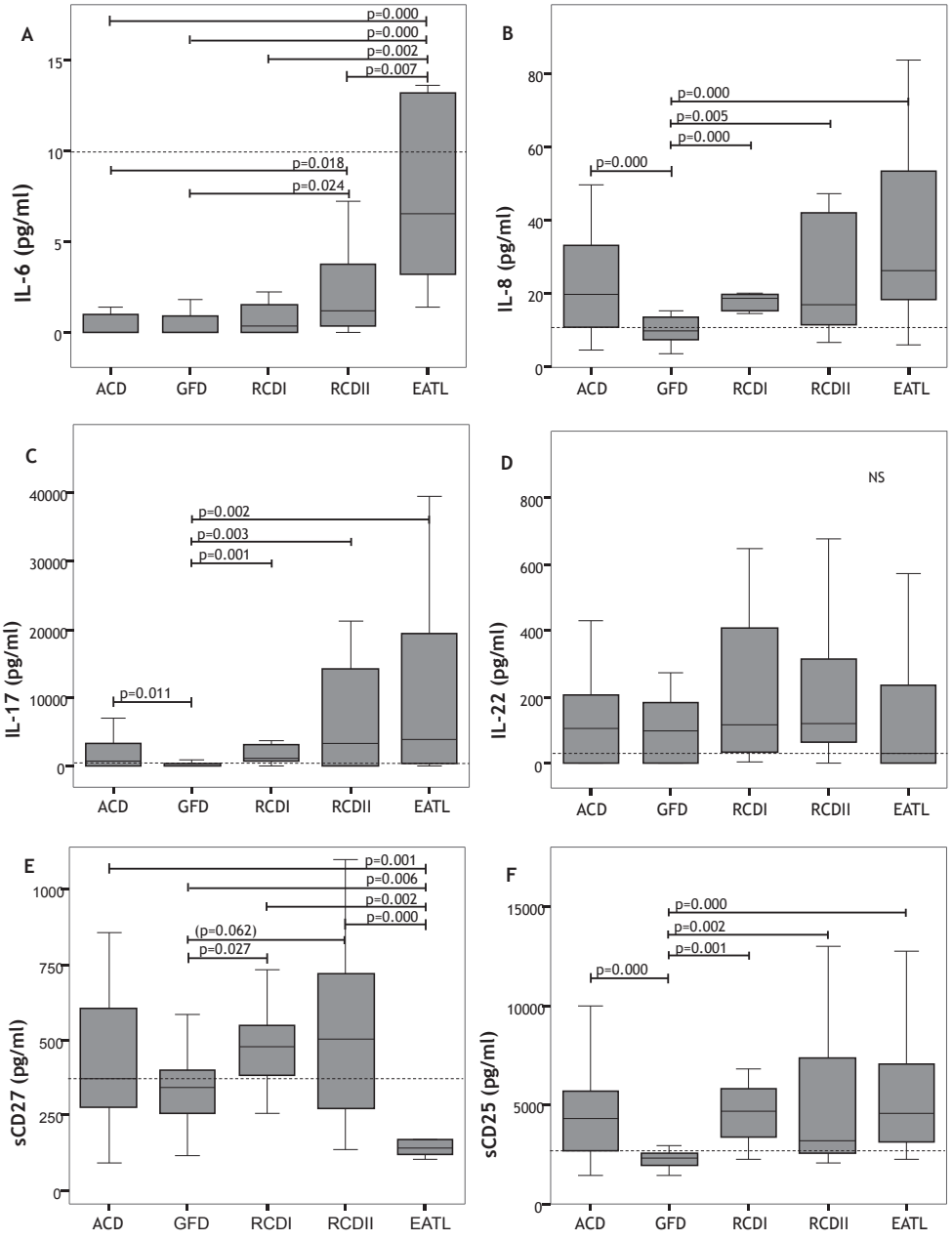
None of the markers tested was able to distinguish RCD II from RCD I. Moreover, no significant differences in levels of albumin or inflammatory parameters CRP, ESR and WBC were observed.

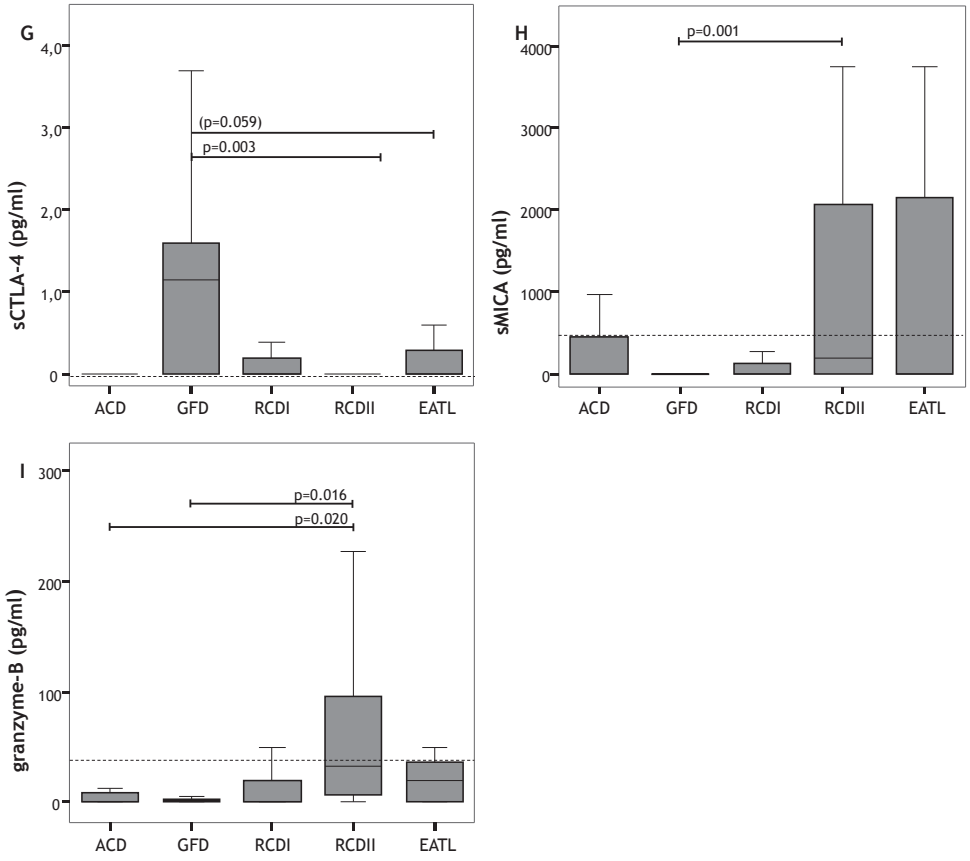
Table 1 | Characteristics of the different groups in the spectrum of coeliac disease.

	SPECTRUM OF CD				
	ACD	**GFD**	**RCD I**	**RCD II**	**EATL**
	n=26	**n=26**	**n=12**	**n=16**	**n=12**
Age at CD (yr), median (SD; range)	42.5 (16; 21-76)	45 (15; 21-77)	50.5 (16; 19-69)	61.5 (13; 27-74)	61.5 (7; 48-66)
Age at RCD (yr), median (SD; range)	-	-	59 (11; 35-74)	65 (10; 47-77)	64 (8; 51-72)
Age at EATL (yr), median (SD; range)	-	-	-	-	65 (6; 51-74)
HLA-staus					
DQ2 heterozygous	10	7	8	7	3
DQ8 heterozygous	9	13	-	-	3
DQ2/8 heterozygous	3	2	1	3	2
DQ2 homozygous	4	4	3	6	4
Marsh					
0	-	12	-	-	2
I	-	4	-	-	-
II	-	-	-	-	1
IIIA	11	-	8	9	5
IIIB	5	-	3	4	3
IIIC	10	-	1	3	1
ND	-	10	-	-	-
IgA-EMA					
Negative (-)	-	26	12	15	7
Dubious (+/-)	-	-	-	1	2
Weak positive (+)	3	-	-	-	-
Positive (++)	11	-	-	-	-
Strongly positive (+++)	11	-	-	-	2
ND	1	-	-	-	1
IgA-tTG					
Negative	-	7	1 2	14	6
Dubious	-	-	-	2	2
Weak positive	8	-	-	-	1
Positive	6	-	-	-	1
Strongly positive	9	-	-	-	1
ND	3	19	-	-	1
Aberrant IELs (%), median (SD; range)	-	-	4 (6; 2-19)	64 (23; 20-96)	7 (32; 1-87)
EATL type					
Primary	-	-	-	-	4
Secondary	-	-	-	-	8

Abrreviations: GFD, coeliac disease patients on a gluten-free diet; RCD I and II, refractory coeliac disease type I and II; EATL, enteropathy associated T-cell lymphoma; IgA-EMA; endomysial antibodies; IgA-tTG, tissue transglutaminase antibodies; IELs, intra-epithelial lymphocytes; ND, not determined.

Figure 1A-I | Concentration of serum parameters in the different subsets of the coeliac disease spectrum: IL-6 (A); IL-8 (B); IL-17 (C); IL-22 (D); sCD25 (E); sCD27 (F); sMICA (G); sCTLA-4 (H); granzyme-B (I).

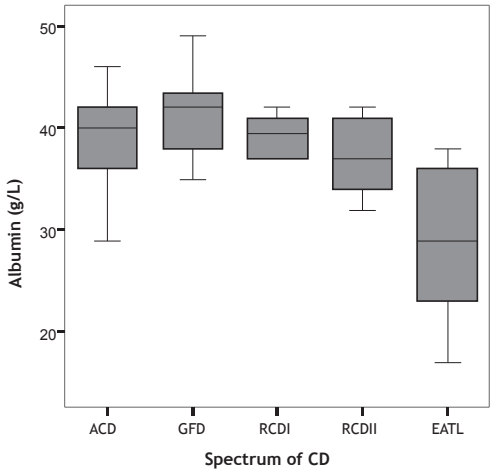




EATL versus ACD and RCD I-II

The highest serum levels of IL-6 were observed in the EATL group, which were higher (p=0.000, highly significant) as compared tot the active CD and GFD groups, as well as higher (p=0.002, p=0.007, moderately significant) than RCD I-II, respectively. Moreover, serum levels of IL-6 were clearly elevated in EATL over RCD II with an AUC of 0.82 (95% CI: 0.649-0.971). IL-6 levels in EATL patients tended to correlate (0.45, p=0.08) with CRP levels, but not with IL-17 levels. Furthermore, serum levels of sCD27 were decreased (moderately significant) in EATL patients as compared to all other groups, except RCD II in which (highly) significant (p=0.000) differences were found. Nevertheless, ROC analysis resulted in a very low AUC. In addition, similar levels of sMICA, granzyme-B and sCD25 were measured in EATL and RCD I-II. However, the serum albumin concentration in EATL patients was lower (moderately significant) than in ACD and both types of RCD (Figure 2).

Figure 2 | Concentration of albumin in the different subsets of the coeliac disease spectrum.



The concentration of albumin is significantly higher in both the ACD (p=0.003) and the GFD (p=0.000) subset compared to the complicated forms of CD. In EATL patients clearly lower albumin concentrations compared to both RCD I and II (p=0.003 and p=0.037, respectively) were observed.

DISCUSSION

Currently, physicians are unable to predict who will develop complicated CD. As invasive techniques are still required to differentiate between uncomplicated and complicated forms of CD, the purpose of the present analysis was to evaluate the ability of markers in peripheral blood to distinguish the various CD subsets. By doing so, it may also provide insight in the potential differences in underlying immunopathomechanisms of these disease subsets. The results could be biased by cytokine production from different cellular sources in the peripheral blood, and therefore may not exactly mimic the intestinal inflammation.[20] However, other investigators have previously shown that blood cytokine profiles do reflect intestinal mRNA expression in active CD patients.[2, 21-23] Care must be taken in case our findings are extrapolated to other age groups, as it can not be excluded that normal values vary over age.

ACD versus GFD

These results are in keeping with previous studies showing up regulation of IL-8[20, 24], a chemokine produced by macrophages, epithelial as well as endothelial cells that attracts leukocytes to a site of inflammation, as well as elevated levels of sCD25 that is cleaved off from the membranous CD25 (IL-2R-alpha) during T-cell activation.[25-27] Furthermore, the increased levels of the pro-inflammatory cytokine IL-17 observed in the current study, supports the view that Th17-cells that produce this particular cytokine are involved in several autoimmune diseases and are suggested to have a pathogenic role in CD.[3-5] In contrast to the (pro-) inflammatory environmental characteristics observed in this analysis and to previous

reports[20, 26, 28], the levels of IL-6, illustrative of an acute phase response and a potent inducer of the Th17 pathway[29], were low and similar in both uncomplicated CD subsets. A clear explanation for these findings is lacking, especially as strongly increased levels were found in RCD II and EATL patients. Another cytokine involved in gut inflammation is IL-22 that is produced by Th17 and/or Th22 cells, where it is currently believed to exert regulatory functions.[30, 31] Elevated levels of IL-22 have been found in the mucosa of Crohn's disease[32], but its role in CD is yet unclear[3, 33]. Nevertheless results failed to reveal a correlation between serum IL-22 levels and disease activity or disease entity.

sCD27 serum levels are increased in T-cell mediated diseases, including some auto-immune disorders.[34, 35] However, the sCD27 levels were normal in ACD. This in contrast to the previously reported increased production of another lymphocyte activation marker sCD25. This dissimilarity is remarkable as in SLE patients sCD25 and sCD27 levels are strongly correlated during the whole disease course.[36]

The RCD complex

Although, by general consent, RCD type I and II are considered two related disease entities within the spectrum of CD, it remains unclear whether both diseases share a similar pathogenesis responsible for the gluten-independent inflammation. In keeping with the current opinion, our results showed in both types of RCD similar inflammatory characteristics and T-cell activation based on serum levels of IL-8, IL-17, IL-22 and sCD25, respectively. On the other hand, a transition from RCD I to RCD II has only been reported sporadically.[16] Theoretically, granzyme-B is a suited parameter to differentiate between RCD type I and II, since aberrant IELs are clearly cytotoxic and express high amounts of intracellular granzyme-B.[12] Although increased levels of soluble granzyme-B were observed in RCD II patients, these were not significantly higher than in RCD I patients.

RCD I-II versus uncomplicated CD

Currently, it is unknown to what extent the gluten-independent inflammation as generally observed in RCD evolved from and/or differs from the gluten induced inflammation in active CD. In this study, the pro-inflammatory T-cell response, including IL-8, IL-17, sCD25, in both types of RCD and ACD patients shows resemblance, with exception of evidently increased IL-6 levels in RCD II over ACD. This finding suggests a higher inflammatory state in RCD II than in ACD, however, similar levels of the inflammatory parameters CRP, ESR and WBC were observed. In line with the lack of intestinal inflammation in patients adhering to a GFD, the pro-inflammatory response was significantly lower as compared to the complicated forms of CD. Interestingly, in comparison to ACD patients, RCD II patients displayed a distinctive cytotoxic T-cell activation profile based on elevated serum levels of granzyme-B. The levels of these parameters observed in RCD I patients did not differ from either ACD or RCD II patients.

Monitoring EATL development

RCD II patients carry a high risk to develop an EATL, yet, so far no serological parameters for EATL development have been identified, including efforts in the present analysis. Based on the fact that EATL cells contain large amounts of granzyme-B[13], these levels were measured in the peripheral blood, but EATL patients did not contain higher levels than ACD or RCD patients. Furthermore, elevated levels of sCD27[37] and sCD25[38] have been suggested to be associated with tumour burden in some lymphoid neoplasia. Neoplastic lymphoid cells in non-Hodgkin lymphoma express CD27 and are considered responsible for the increased sCD27 production.[37] The current analysis failed to show increased sCD27 levels in EATL patients and thereby suggests that EATL regards a distinct type of lymphoma and does not aid in the identification of this particular lymphoma. In line with a previous study showing elevated sCD25 levels in EATL[39], we found comparable levels of sCD25 in EATL, RCD and active CD. Another potential marker evaluated in this study is sMICA, that is cleaved from membranous MICA and has been shown to impair NKG2D mediated tumor surveillance in epithelial tumors[41], as well as it has shown potential as a prognostic parameter in haematopoietic malignancies since increased levels of sMICA were found in leukemia patients[41]. The only marker that distinguished EATL from all other groups was IL-6 and its levels correlated with CRP levels, indicating a more severe acute inflammatory response in EATL patients. In accordance with our data, an association with IL-6 levels and survival in Hodgkin lymphoma has been recognized almost twenty years ago.[42]

Clinical implication and potential application

Taken together, for daily clinical practice the results of this analysis suggest that apart from detection of CD associated antibodies and duodenal biopsies, other variables including IL-8, sCD25 and possibly IL-17, might be helpful in monitoring inflammatory disease status and differentiating between patients on a strict GFD and those diagnosed as having RCD. In case CD-specific antibodies remain mildly elevated, which is not rare in RCD patients, serum levels of granzyme B could possibly serve as an additional marker to distinguish active CD from RCD II, and will enable early intervention. However, the currently accepted diagnostic work-up of RCD remains required, pending prospective serological studies including larger series of patients.

Conclusion

In conclusion, both types of RCD are characterised by an ongoing and/or recurring inflammatory disease status showing great resemblance to that observed in ACD despite strict adherence to a GFD, yet differentiates itself by elevated serum IL-6 concentrations in RCD II. Furthermore, in addition to this increased pro-inflammatory profile, RCD II reveals a distinctive cytotoxic T-cell activation profile as compared to ACD based on elevated levels of granzyme B, whereas RCD I does not. Although no EATL-specific or -associated variables were found in this study, our ongoing efforts may identify relevant markers. Further research will also address the prospective and diagnostic value of the serum variables identified in this study in order to expand the clinical application of (R)CD serology beyond standard autoantibody testing.

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CHAPTER 4

Origin and immunophenotype of aberrant IEL in refractory coeliac disease II

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ABSTRACT

Objectives: Aberrant intraepithelial lymphocytes (IELs) are the hallmark of refractory coeliac disease (RCD) type II and considered a premalignant cell population from which aggressive enteropathy associated T-cell lymphoma (EATL) can evolve. The aim of this study was to gain further insight in the origin and characteristics of aberrant IELs by analysing T-cell receptor (TCR) rearrangements, and by immunophenotypic analysis of aberrant IELs.

Methods: Duodenal biopsies from 18 RCD II patients and three RCD II cell lines were analysed for the presence of TCR delta, gamma, and beta rearrangements. In addition, IELs isolated from biopsies derived from RCD II patients were phenotypically analysed.

Results: Aberrant IELs showed an up regulated expression of granzyme-B and a decreased expression of PCNA. TCR rearrangements in the aberrant IEL population in biopsies of RCD II patients were heterogenic, which is most likely due to a variation in maturity. Similarly, RCD II cell lines displayed a heterogenic TCR rearrangement pattern.

Conclusion: Aberrant IELs originate from deranged immature T-lymphocytes and display clear differentiation to a cytotoxic phenotype. Aberrant IELs displayed different stages of maturity between RCD II patients, of which only the patients harbouring the most mature aberrant IEL population developed an EATL.

INTRODUCTION

Coeliac disease (CD) is a common small intestinal enteropathy induced by dietary gluten proteins as well as other undefined environmental factors, affecting genetically predisposed individuals of all ages. A permanent state of intolerance to gluten-containing food leads to a chronic autoimmune mediated inflammatory response with subsequent remodelling of the proximal small bowel mucosa and nutrient malabsorption.[1] Withdrawal of dietary gluten usually leads to prompt healing of the damaged small intestinal mucosa and improvement of nutrient absorption. Although a substantial group of adult-onset CD patients lacks histological recovery after two years on a gluten-free diet (GFD) only a small subgroup of patients[2, 3], especially those diagnosed above the age of 50 years, develop primary or secondary resistance to a GFD with intestinal villous atrophy and persisting or recurring symptoms of malabsorption.[4] After evaluation of diet-compliance by a dietitian and exclusion of other underlying diseases known to cause villous atrophy, subjects are considered to suffer from refractory coeliac disease (RCD).[5] Based on the immunophenotype of intraepithelial lymphocytes (IELs), RCD can be subdivided into type I lacking a substantial aberrant IEL population (CD3-, CD45+, CD103+, CD7+, CD4-, CD8-, cytCD3+ cells) and type II in which an aberrant IEL population is present.[6] The distinction between RCD I and II is defined by a clinically validated cut-off of 20% aberrant IELs.[7]

As a consequence of a generally good response to immunosuppressive therapy, RCD I has a less dismal prognosis compared to patients suffering from RCD II, reflected in a 5-year survival rate of approximately 90% and 44-58%, respectively.[5, 8-10] More importantly approximately 40-50% of all RCD II patients develop an aggressive enteropathy associated T-cell lymphoma (EATL), which is considered to arise from the clonal expansion of the premalignant aberrant IEL population.[11-13] EATL is one of the main causes of death in RCD II patients, due to its aggressive nature and unresponsiveness to currently available therapies.[5, 8-10] Despite standardised treatment, one half of the RCD II patients develops an EATL whereas the other half does not, which could indicate that aberrant IEL populations between patients are heterogeneous and might be accompanied with a variable risk to develop an EATL. Even though RCD II and EATL patients are associated with HLA-DQ2 homozygosity[14], currently no histopathological or immunophenotypic features have been identified that have a prognostic value in the evolution of aberrant IELs into an EATL. Therefore it is of utmost importance to gain more insight in the origin and characteristics of aberrant IELs in RCD II, which will enable a better identification of high risk patients and the development of new therapeutic options.

Recently, elegant work addressing the expansion and function of aberrant IELs has been performed[15, 16], nevertheless, the exact role of aberrant IELs in the mucosa of the small intestine still remains unclear. Furthermore, the cells from which monoclonal aberrant IELs originate is currently under debate. Although aberrant IELs found in RCD II do not express the T-cell lineage-specific surface CD3-TCR complex, these cells do contain cytoplasmatic

CD3 antigen and display T-cell receptor (TCR) rearrangements, indicative of T-cell lineage commitment. It has been suggested that the TCR-CD3 complex is internalised due to overstimulation of IELs, implying that aberrant IELs originate from mature TCR+ IELs.[11] More specifically, it is hypothesised that these cells derive from gamma-delta T-lymphocytes based on the observed inversed correlation between aberrant IELs and gamma-delta cells in RCD II.[17, 18] Alternatively, a small, unique CD3-CD7+ population considered to be NK/T-cell precursors, which is found in the intestine of healthy individuals, is suggested to represent the physiological counterpart of aberrant IELs.[19-21] The presence of an immature lymphoid precursor population in the gut mucosa, which could hypothetically serve as origin for aberrant IELs, is emphasised by the ongoing extrathymic maturation of T-cells in the intestinal mucosa throughout life.[22]

Therefore, in this study we isolated lymphocytes from duodenal biopsies collected from RCD II patients and compared the expression of markers representing activation, proliferation, DNA-repair and lymphocyte development on aberrant lymphocytes to the expression on normal lymphocytes within the same patient. To elucidate the origin of aberrant IELs, we assessed these biopsies for the presence of TCR delta (TCR-δ), gamma (TCR-γ) and beta (TCR-ß) rearrangements. In addition, extensive analysis of TCR rearrangements in RCD II cell lines was performed.

MATERIALS AND METHODS

Patients

RCD II patients included in this study visited the out-patient department of gastroenterology at the VU University Medical Centre, Amsterdam, The Netherlands for diagnostic work-up or regular follow-up. The diagnosis of RCD II was based on persisting or recurring symptoms and small intestinal villous atrophy after a former good response despite strict adherence to a GFD for at least one year. Furthermore, the clinically validated cut-off value of 20% aberrant IELs as detected by flow cytometry was predominantly used to distinguish RCD type I and type II.[7] A lower percentage of aberrant T-cells was allowed in the presence of ulcerative jejunitis.

Small intestinal biopsies

During upper gastro-intestinal endoscopy multiple large spike forceps biopsies were taken from the second part of the duodenum. For TCR rearrangement analysis, 1-2 biopsy specimens were stored in liquid nitrogen until analysis. For flow cytometric evaluation, six biopsy specimens were collected from various locations in the duodenum, from which IELs were isolated and pooled before immediate analysis. All biopsy specimens were obtained for diagnostic purposes and the procedures were in accordance with the ethical guidelines of our institution.

Cell lines and cell culture

RCD cell lines P1, P2 and P3 were isolated from duodenal biopsies of RCD II patients as previously described.[23] In short: biopsy specimens were treated with 1mM dithiothreitol (Fluka, Buchs, Switzerland) and 0.75 mM ethylenediaminetetraacetic acid (EDTA, Merck, Darmstadt, Germany) after which the biopsy was cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Verviers, Belgium) supplemented with 10% normal human serum (NHS) and 10 ng/ml IL-15 (R&D systems Europe, Abingdon, UK). Released cells were propagated on IMDM containing 10% NHS and 10ng/ml IL-15 and restimulated approximately every four to five weeks with 1 µg/ml phytohemagglutinin, 10 ng/ml IL-15 and 1 x 10^6/ml irradiated allogenous peripheral blood mononuclear cells as feeder cells. A TCR-,$CD3^+$,$CD4^+$ T-cell clone isolated from a duodenal biopsy of a CD patient was maintained in IMDM containing 10% NHS supplemented with 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 (Proleukin, Chiron corporation, Emeryville, California, USA) and restimulated every two weeks.

Flow cytometric analysis

Multiparameter flow cytometric immunophenotyping was performed on IEL suspensions, isolated as previously described.[7] Briefly, biopsies were vigorously shaken at 37°C for 60 minutes in PBS supplemented with 1 mM DTT (Fluka, Buchs, Switzerland) and 1 mM EDTA (Merck, Darmstadt, Germany). The released IELs were washed twice with PBS supplemented with 0.1% BSA (Roche Diagnostics) and subsequently stained for 30 min on ice, with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC)-labeled monoclonal antibodies directed against CD3, CD4, CD5, CD7, CD8, CD16+56, CD19, CD25, CD30, CD34, CD45, CD45RA, granzyme-B, HLA-DR, Ki-67, NKG2D, PCNA (all from BD Biosciences, San Jose, California, USA), CD52 (Serotec, Düsseldorf, Germany), terminal deoxynucleotidyl transferase (TdT) and CD1a (Glostrup, Denmark), CD103 (IQ products, Groningen, The Netherlands), and IL-15R-alpha (eBioscience, Hatfield, United Kingdom). Cytoplasmic staining of CD3 was performed after cell permeabilisation by Cytofix/CytoPerm Plus (BD Biosciences), according to the manufacturer's instructions. Nuclear staining of Ki-67 and PCNA was performed after cell permeabilisation by eBioscience Cell Fixation/Permeabilisation (eBioscience), according to the manufacturer's instructions. Stained cells were washed with PBS containing 0.1% bovine serum albumin (BSA, Sigma) and analysed on a standard four-color flowcytometer (FACSCalibur, BD Biosciences). The data were analysed using Cellquest software (BD Biosciences). Care was taken to analyse only viable cellular events based on light scatter properties.

Both aberrant IELs (sCD3-, CD45+, cytCD3+) and normal IELs (sCD3+, CD45+, cytCD3+) were reported as percentage of the total number of CD45+ (lymphocytic) cells. The expression level of the aforementioned markers on aberrant lymphocytes was compared to that of IELs with a normal immunophenotype within each individual and reported in percentage and/or mean fluorescent intensity (MFI) index. For all markers corresponding isotype controls were used.

PCR-based analysis of TCR-δ, TCR-γ and TCR-β gene rearrangements

TCR-δ, TCR-γ and TCR-β gene rearrangements were assessed by multiplex PCR as previously described and resulting PCR products were further evaluated by GeneScan analysis.[24]

Southern blot

Southern blot analysis of TCR-β and TCR-δ genes was performed according to earlier published protocols, using TCR- δJ1, TCR-βJ1 and TCR-βJ2 probes.[25, 26]

Statistical analysis

Data were analysed in SPSS software (SPSS Inc. Chicago, Illinois, USA). Flow cytometric data were analysed with the non-parametric Mann-Whitney test to test for differences between aberrant lymphocytes and its normal counterpart. A p-value ≤0.05 was considered significant.

RESULTS

Flow cytometric analysis

An extensive panel of surface- and intracellularly expressed markers was evaluated by multiparameter flow cytometry. Overall, immunophenotyping of duodenal biopsies was performed during follow-up in 16 RCD II patients characterised by a median aberrant IEL percentage of 58.5% (range 21% - 97%; Table 1). All patients except one were treated with cladribine and three were subsequently treated with high dose chemotherapy followed by autologous haematopoietic stem-cell transplantation as previously described.[27, 28] In addition, six patients visited the department for follow-up more than once. In these patients, repetitive flow cytometric analysis showed almost identical results regarding the immunophenotype of aberrant and normal IELs (data not shown), indicating stability of the observed aberrant IEL phenotype. Table 2 shows an overview of all markers analysed and their respective expression by both aberrant and normal IELs.

All IELs showed strong expression of CD45 and low to intermediate forward and sideward scatter, indicating a relatively homogeneous population of lymphocytic cells (Figure 1A). In keeping with the generally accepted aberrant IEL immunophenotype found in RCD II patients, this population expressed cytoplasmic CD3 but lacked expression of surface CD3 (Figure 1B). In addition, these cells expressed CD7 (Figure 2A). Expression of the early T-cell development-associated markers terminal deoxynucleotide transferase (TdT), CD1a and CD34 was not detected (data not shown). Aberrant IELs did not show expression of CD5 (Figure 2B). Interestingly, 30% of the normal IEL population lacked CD5 expression, in contrast to the uniform expression of CD5 by peripheral T-cells.

Table 1 | Patient characteristics.

Patient	Analysis	Gender	Age RCD II	HLA-DQ	Dx_ Marsh	Dx_ aberrant IELs (%)	TCR_ aberrant IELs (%)	FACS_ Marsh	FACS_ aberrant IELs (%)	EATL develop-ment	Concurrent therapy	
											TCR	FACS
1	TCR	F	64	DQ2+/DQ8+ heterozygous	IIIB	50	27	ND	ND	No	No	ND
2	TCR	M	50	DQ2 homozygous	IIIA	67	9; UJ	ND	ND	No	No	ND
3	TCR + FACS	M	68	DQ2 homozygous	IIIB	87	88	0	91	No	No	2-CdA; au-SCT
4	TCR + FACS	M	67	DQ2 heterozygous	IIIA	61	61	0	58	No	No	2-CdA
5	TCR + FACS	F	66	DQ2 heterozygous	IIIC	53	53	0	45	No	No	2-CdA
6	TCR	F	62	DQ2 heterozygous	IIIB	50	50	ND	ND	No	2-CdA; au-SCT	ND
7	TCR + FACS	F	52	DQ2 homozygous	IIIB	87	87	IIIA	88	No	No	2-CdA
8	TCR + FACS	M	72	DQ2 homozygous	IIIA	59	59	0	80	No	No	2-CdA
9	TCR + FACS	F	67	DQ2 heterozygous	IIIC	55	71	IIIA	61	No	No	2-CdA
10	TCR	F	67	DQ2 heterozygous	IIIB	11	11; UJ	ND	ND	No	No	ND
11	TCR + FACS	M	62	DQ2 heterozygous	IIIA	75	75	IIIA	59	No	No	2-CdA
12	TCR	M	78	DQ2 homozygous	IIIA	74	74	ND	ND	No	No	ND
13	TCR + FACS	M	77	DQ2 heterozygous	IIIA	92	92	IIIA	97	No	No	2-CdA
14	TCR	M	57	DQ2 homozygous	IIIB	78	78	ND	ND	No	2-CdA	ND
15	TCR + FACS	F	70	DQ2 heterozygous	IIIA	37	37	IIIA	21	No	No	No
16	TCR	M	59	DQ2 homozygous	IIIA	95	95	ND	ND	Yes	No	ND
17	TCR	F	61	DQ2 heterozygous	IIIC	59	59	ND	ND	Yes	2-CdA	ND
18	TCR	F	64	DQ2 homozygous	IIIC	88	88	ND	ND	Yes	No	ND
19	FACS	F	64	DQ2 heterozygous	IIIB	21	ND	IIIA	21	No	ND	2-CdA
20	FACS	F	56	DQ2 heterozygous	IIIB	65	ND	0	76	No	ND	2-CdA; au-SCT
21	FACS	F	47	DQ2 heterozygous	IIIC	92	ND	0	65	No	ND	2-CdA; au-SCT
22	FACS	F	45	DQ2 homozygous	IIIA	25	ND	IIIA	50	No	ND	2-CdA
23	FACS	F	32	DQ2 homozygous	IIIA	42	ND	0	42	No	ND	2-CdA
24	FACS	M	50	DQ2 homozygous	IIIC	28	ND	0	40	No	ND	2-CdA
25	FACS	F	52	DQ2 heterozygous	IIIA	55	ND	0	53	No	ND	2-CdA

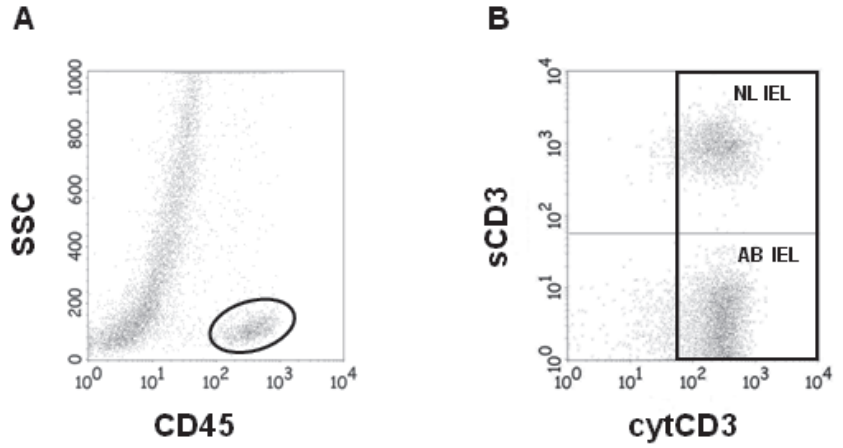
Abbreviations: F, female; M, male; TCR, T-cell receptor analysis; IELs, intra-epithelial lymphocytes; UJ, ulcerative jejunitis; EATL, enteropathy associated T-cell lymphoma; FACS, flow cytometric analysis; RCD II, refractory coeliac disease type II; DX, time of diagnosis; 2-CdA, cladribine; au-SCT, autologous stem-cell transplantation; ND, not determined.

Table 2 | Immunophenotype of aberrant and normal IELs determined by flow cytometric analysis.

		Aberrant T-cells (median; range)		Normal T-cells (median; range)		Mann-Whitney %	Mann-Whitney MFI-index
	n	%	MFI-index	%	MFI-index	p	p
CD45RA	10	55.5 (20-99)	4.5 (1.4-17.4)	45.0 (20-97)	4.2 (1.8-15.9)	NS	NS
IL-15R-alpha	14	3.2 (0.2-59.8)	1.0 (1-3.4)	9.6 (1.8-55.4)	1.3 (1-3.6)	0.056	NS
Ki-67	15	8.8 (3.4-44.7)	1.9 (1.3-5.6)	6.2 (2.8-23.2)	2.1 (1.1-6.1)	NS	NS
PCNA	11	17.6 (2-70)	1.9 (1-3.8)	49.9 (7.5-75.8)	2.8 (2-4.7)	0.047	0.007
NKG2D	8	34.0 (5-67.2)	2.4 (1.3-3.4)	29.2 (15-74.8)	2.6 (1.4-3.2)	NS	NS
HLA-DR	8	34.5 (7.2-82)	5.8 (2.4-16.6)	53.3 (15.5-76.5)	7.7 (5.2-30)	NS	NS
granzyme-B	8	78.7 (48.7-96)	8.2 (3.8-92.4)	38.7 (29-69.3)	6.1 (2.1-16.5)	0.03	NS

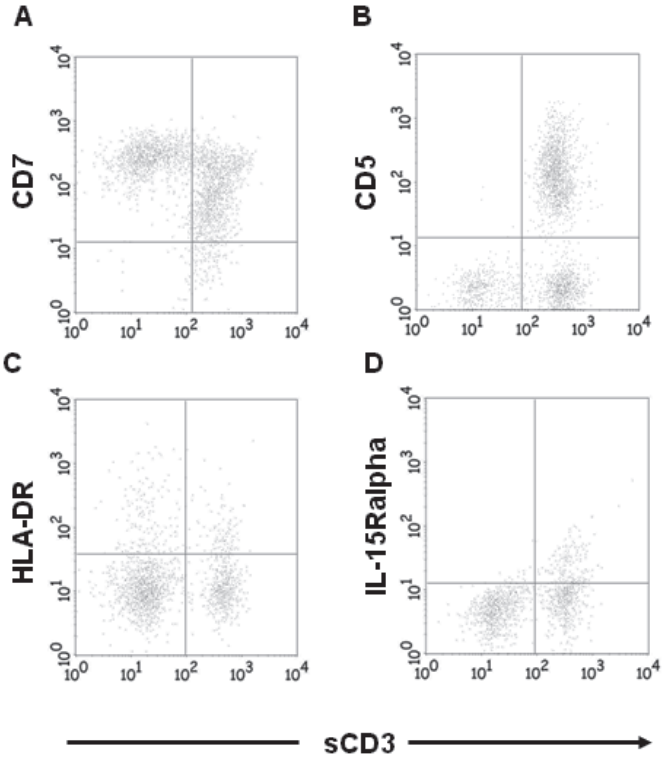
Abbreviation: NS, not significant.

Figure 1 | Gating strategy to identify aberrant and normal IELs. Cells with a strong CD45 expression and low to intermediate forward and sideward scatter were selected (A), after which IELs expressing intracellular CD3 expression were used for further studies (B).



Abbreviations: Ab IEL, aberrant IEL population (sCD3-, cytCD3+); NL IEL, normal IEL population (sCD3+, cytCD3+).

Figure 2 | Surface expression of CD7 (A), CD5 (B), HLA-DR (C) and IL-15R-alpha (D) on aberrant IELs and normal IELs derived from case 5, case 19, case 13 and case 5 (Table 1).

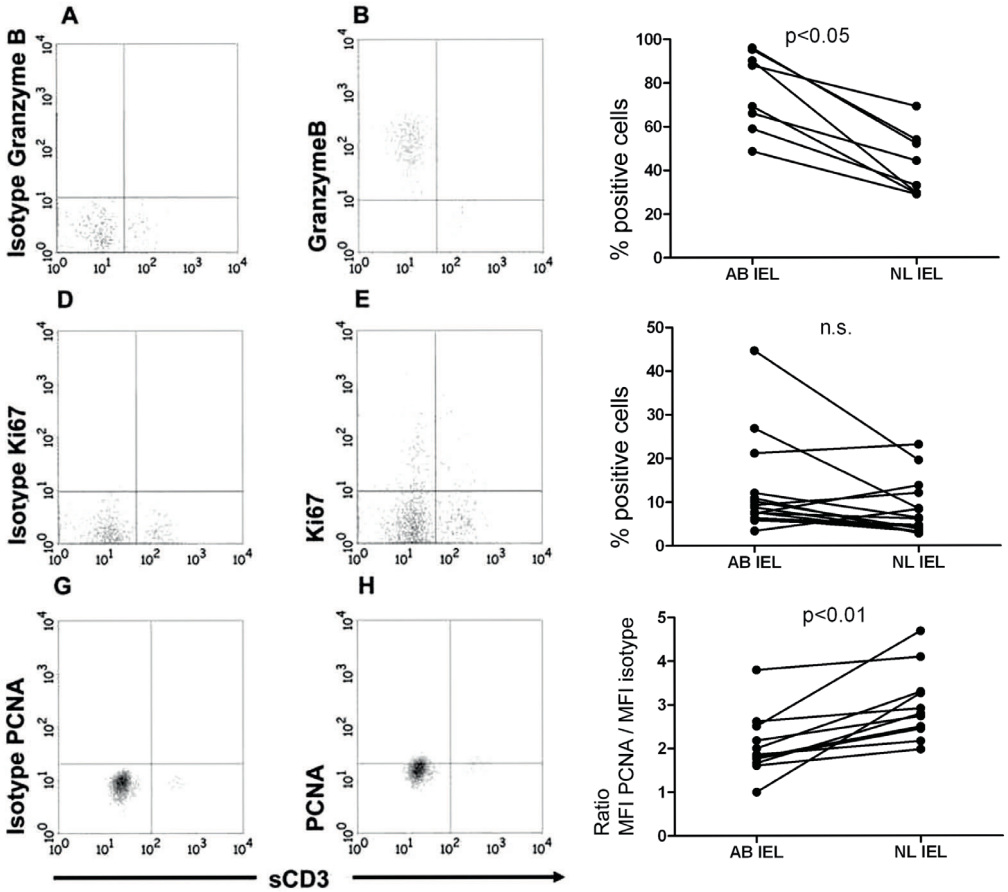


Significantly more aberrant lymphocytes than normal IELs expressed intracellular granzyme-B, illustrating a cytotoxic phenotype ($p<0.05$; Figure 3A-C; Table 2). No expression of CD30, or its ligand CD153 was observed on aberrant T-cells, thereby confirming the absence of an EATL.[29] However, we did find expression of CD45RA on both normal and aberrant IELs, which is known to be associated with cytotoxic activity in antigen experienced T-cells. Furthermore, natural killer (NK) associated receptor NKG2D was similarly expressed on aberrant IELs as on normal IELs (Table 2). Moreover, no differences in expression of the T-cell activation marker HLA-DR and IL-15R-alpha were found on both cell types (Figure 2C and D; Table 2).

Analysis of cell cycle activity of the intestinal IELs using nuclear staining of proliferating cell nuclear antigen (PCNA) revealed a significantly lower MFI expression in aberrant IELs compared to the immunophenotypically normal IELs ($p<0.01$; Figure 3G-I; Table 2). Even though only limited PCNA expression was observed in IELs, in all 11 patients tested PCNA levels were lower in the aberrant IEL population than in the normal IEL population. In

contrast, the expression of the proliferation marker Ki-67 was similar on both cell types (Figure 3D-F; Table 2). Both markers were tested simultaneously but no correlation between the two was observed.

Figure 3 | Intracellular expression of granzyme-B (B), Ki67(E) and PCNA (H) in aberrant IELs (Ab IEL) and normal IELs (NL IEL) derived from case 9, case 5 and case 13 (Table 1), with corresponding isotype controls (A, D and G). Depending on distribution, expression of granzyme-B (D) and Ki67 (F) was reported as percentage positive cells, while MFI was used to report PCNA expression which was correlated to the MFI of the isotype control (I). Aberrant populations revealed significant ($p<0.05$) higher granzyme-B expression (C) and significantly lower PCNA expression ($p<0.01$) as compared to normal IELs (I).



TCR rearrangement analysis

Detailed TCR rearrangement analysis was performed on duodenal biopsies of 18 well-defined RCD II patients: 15 at time of diagnosis, two following cladribine therapy and one after cladribine with subsequent autologous stem-cell transplantation (au-SCT). The patient characteristics are shown in Table 1.

Aberrant IEL populations in RCD II patients revealed a heterogeneous pattern of TCR rearrangements (Table 3). In one sample the DNA quality was probably inadequate (patient 1) as no amplification was found. The other cases could be subdivided into four groups. In the first group (patients 2-5) a polyclonal signal was detected. The second group (patients 6-8) revealed a monoclonal TCR-δ and/or TCR-γ gene rearrangement. The majority of the patients was present in the third group (patients 9-15) and all contained monoclonal TCR-δ and/or TCR-γ as well as incomplete TCR-ß gene rearrangements. The last group (patients 16-18) showed monoclonal TCR-δ and/or TCR-γ gene rearrangements, while a complete, monoclonal TCR-ß gene rearrangement was observed as well. Remarkably, all three patients in the last group, but none in the other groups, developed an EATL. No correlation between the above described groups and expression of any of the phenotypical markers was observed. Furthermore, in two patients (6 and 12) no clonal TCR-γ gene rearrangements were found, whereas the TCR-ß and TCR-δ gene rearrangements clearly revealed a monoclonal population.

Table 3 | Rearrangement analysis of the TCR-delta, gamma and beta chains in small intestinal biopsies.

Patient	TCR-delta	TCR- gamma	TCR-beta	Interpretation
1	No amplification			
2	NC			
3	NC			
4	NC			
5	NC			
6	C	NC	NC	
7	C	C	NC	
8	C	C	NC	
9	NC	C	NC	V-J beta loss
10	NC	C	NC	V-J beta loss
11	NC	C	NC	V-J beta loss
12	C	NC	C	V-J beta loss; TCR beta incomplete
13	C	C	C	V-J beta loss; TCR beta incomplete
14	C	C	C	V-J beta loss; TCR beta incomplete
15	NC	C	C	V-J beta loss; TCR beta incomplete
16	NC	C	C	Complete TCR beta
17	NC	C	C	Complete TCR beta
18	C	C	C	Complete TCR beta

Abbreviations: TCR, T-cell receptor; NC, no clonality; C, monoclonal.

In addition, the presence of TCR gene rearrangements in RCD cell line P1, P2 and P3 was assessed with multiplex PCR analysis and Southern blot analysis. PCR-based analysis indicated that in RCD cell line P1 TCR-δ gene rearrangements were absent while rearrangement of the TCR-γ gene and TCR-ß gene were, respectively, non-functional and incomplete (Table 4). Southern blot analysis of the TCR-δ gene and TCR-ß gene rearrangements confirmed these data as bi-allelic loss of the TCR-δ gene and incomplete rearrangement of the TCR-ß gene were found. Bi-allelic loss of the TCR-δ gene could point to rearrangement of both TCR-alpha (TCR-α) alleles in RCD cell line P1. RCD cell line P2 displayed incomplete TCR-δ gene rearrangement, non-functional TCR-γ gene re- arrangement and no rearrangement of the TCR-ß gene (Table 4) Southern blot analysis indicated that one allele of the TCR-δ gene was lost while rearrangement of the other was incomplete (data not shown). Furthermore, the TCR-ß gene was in germline configuration indicating that no rearrangements of the TCR-ß gene were made in RCD cell line P2 (data not shown). In contrast to RCD cell line P1 and P2, no TCR gene rearrangements were found with multiplex PCR for RCD cell line P3 (Table 4) and with Southern blot analysis of this cell line only germ line configuration of the TCR genes was found (data not shown). These data indicate that RCD cell lines can display a diverse phenotype regarding the TCR gene rearrangements, characterised by incomplete, non-functional or even total absence of TCR gene rearrangements (Table 4).

Table 4 | Rearrangement analysis of the TCR-delta, gamma, and beta chains in three RCD II cell lines.

Cell line	TCR-delta	TCR-beta	Interpretation
P1	NC	C	TCR-delta: D-J rearrangement
			TCR-gamma: non-functional V
P2	C	NC	TCR-delta: incomplete
			TCR-gamma: 1 out of frame, 1 non-functional V
P3	No rearrangements		
CD4+, TCR-alphabeta+	NC	C	Rearrangements fitting with TCR alpha-beta phenotype

Abbreviations: TCR, T-cell receptor; NC, no clonality; C, monoclonal.

DISCUSSION

Although it is generally accepted that EATL in RCD II patients originates from a clonal expansion of aberrant T-lymphocytic population located in the small intestine that tends to disseminate to the blood and the entire gastro-intestinal epithelium[30], it is unclear for what reason this has been observed in only half of the cases. Therefore, this study aimed to gain insight in the immunophenotypic characteristics and origin of aberrant IELs.

In this study, flow cytometric analysis revealed an increased granzyme-B expression on aberrant IELs over its normal counterpart, indicating a differentiation towards a

more cytotoxic phenotype, possibly in consequence of a continuous pro-inflammatory environment. In uncomplicated CD normal CD3+ IELs are thought to be reprogrammed into cytotoxic NK-like T-cells[31], and data is emerging that aberrant IELs also differentiated towards a cytotoxic NK-like phenotype with up regulation of KIRS, granzyme-H and NKG2D (Tjon et al. manuscript in preparation). Due to their cytotoxic phenotype aberrant IELs may be involved in the gluten-independent destruction of enterocytes in RCD II. However, since aberrant IELs lack a TCR, a TCR-independent mechanism is required in this process. Recently, it was shown that aberrant IELs, in a subset of RCD II patients, can acquire the ability to lyse epithelial cells via DNAM-1.[32] Nevertheless, a TCR independent mechanism is known to be involved in uncomplicated CD as well, since NKG2D expressed by cytotoxic T-lymphocytes is able to bind to its ligand MICA that is expressed on enterocytes, and subsequently induces apoptosis of these enterocytes.[33, 34] Therefore, NKG2D expression on aberrant IELs was evaluated, which revealed a similar NKG2D expression on aberrant IELs compared to normal IELs.

IL-15 has an anti-apoptotic effect on aberrant IELs and by doing so it plays an important role in the expansion of the aberrant IEL population.[15] Given the observation that the IL-15 threshold for IEL expansion in CD patients appears to be linked to an increased expression of IL-15R-alpha on these IELs, in combination with the report that a substantial number of EATL-cells expressed IL-15R-alpha, we compared IL-15R-alpha expression on aberrant and normal IELs, but did not find any difference in expression.[35, 36] It must be noted that although IL-15R-alpha has the highest affinity for IL-15, recently it became clear that IL-15R-alpha is not necessary for IL-15 induction since most IL-15 transduction is directed by the beta/gamma chain.[37]

In contrast to Malamut and colleagues[15] who observed low Ki-67 expression in aberrant IELs, our flow cytometric analysis showed a substantial percentage of the aberrant IELs expressing intracellular Ki-67, yet comparable to that in normal IELs. This suggests that aberrant IELs do proliferate to a similar extent as normal IELs, and together with the anti-apoptotic effect of IL-15 specifically on aberrant IELs, may provide the aberrant IEL population with a survival benefit with subsequent expansion in the intestinal mucosa. Furthermore, our results revealed a significant decrease of PCNA expression in aberrant IELs, with a concomitant non-different Ki-67 expression. Ki-67 and PCNA are both considered proliferation markers, whereas the latter has been implicated in the process of DNA-mismatch repair as well.[38] Therefore, the decreased PCNA expression found in aberrant IELs compared to its normal counterpart is unlikely the result of a lower proliferation and might rather indicate an impaired DNA-mismatch repair system in these cells. These findings are in line with previously reported chromosomal damage in aberrant IELs, and may facilitate further transformation of aberrant IELs into EATL.[39] Our findings might be influenced by previous treatment with cladribine, that was administered to all but one patient studied. Nonetheless, we recently reported that cladribine induces only a limited reduction of the

percentage of aberrant IEL in 40% of cases, while the majority still harbours a substantial aberrant IEL population after cladribine treatment.[28] This suggests that cladribine exerts a similar effect on aberrant and normal IELs, and therefore we feel that the comparison of the phenotype of both cell types provides useful information regarding their respective characteristics.

Currently it remains unclear whether this aberrant population originates from dedifferentiating mature IELs, or that it regards a monoclonal expansion of a unique, physiological subpopulation.[20, 21] In this study, a heterogeneous TCR gene rearrangement pattern in duodenal biopsies of a relatively large series of RCD II patients was observed, and confirmed in three RCD II cell lines. Based on TCR gene rearrangements four groups could be distinguished. First, a small group of RCD II patients showed no clonal TCR gene rearrangements indicating either a NK-cell origin, clonal T-cells in an early stage of their development prior to TCR rearrangements, or simply a polyclonal T-cell population. The second group contained clonal TCR-δ and TCR-γ gene rearrangements, but no TCR-ß gene rearrangements, suggesting either a gamma-delta T-cell origin or a deranged early developing T-cell. The latter results were in agreement with findings by Cerf-Bensussan and colleagues[18], who reported clonal TCRD-δ/TCR-γ gene rearrangements in several RCD II patients. The fourth group displayed complete, monoclonal TCR-ß gene rearrangements. It must be noted however, that mature alpha-beta T-cells not only contain a complete beta chain, but subsequently also rearrange an alpha chain. TCR-α gene rearrangements were not determined in this study due to lack of RNA, so from these results it cannot be concluded that these monoclonal populations are derived from fully mature T-cells. Strikingly, all three patients in the fourth group developed an EATL in a later stage, possibly indicating that secondary EATL evolve from T-cells that have reached a certain stage of maturity. These findings are in agreement with the current WHO-classification which classifies EATL as a mature T-cell neoplasm.[40] It could be hypothesised that these more mature cell populations are less responsive to currently available therapies.

Furthermore, the phenotype of RCD cell line P1 is closest to that of a mature T-cell as analysis of TCR gene rearrangements suggest functional rearrangement of the TCR-α gene but demonstrate incomplete rearrangement of the TCR-ß gene. This is also in line with the previous report describing that replacement of the TCR-ß chain restores TCR alpha-beta expression in this cell line.[23] RCD cell line P2 could represent an immature T-cell as TCR rearrangements were initiated in this cell line. The absence of TCR rearrangements in RCD cell line P3 could indicate that this cell line represents an even earlier stage in T-cell or NK cell development. Taken together, the TCR rearrangement analysis strongly suggests that the majority of the aberrant IEL populations in RCD II patients originates from a monoclonal expansion of immature T-cells, that deranged in their development before reaching full maturity. The latter finding is supported by the lack of expression of the early T-cell development-associated markers TdT, CD1-alpha and CD34 by aberrant IELs.

Furthermore, evolvement into an EATL was only observed in patients harbouring the most mature TCR rearrangement pattern. Although this patients series is small, this might be, at least in part, an explanation for the clinical observation that half of the RCD II patients eventually develops an EATL, but further research addressing the cause of this abnormal development is warranted.

For daily clinical practise it is relevant that two RCD II patients with a phenotypically aberrant IEL population revealed clonal TCR-ß or TCR-δ rearrangements however displayed polyclonal TCR-γ rearrangements. This can be explained by the fact that TCR-γ gene rearrangements are present in most, but not all alpha-beta T-cells.[24] Moreover, these results confirmed our previous findings that clonality analysis of only the TCR-γ gene rearrangement, as often used in the workup of RCD, misses at risk monoclonal populations, and that phenotypical identification of an aberrant population is a superior predictor of EATL development.[7] Therefore, TCR-ß gene rearrangement analysis in addition to phenotypical identification of aberrant IELs could be useful to identify an at risk aberrant IEL population. Further prospective studies are needed.

In conclusion, this study showed that aberrant IELs originate from deranged developing precursor T-lymphocytes and display clear differentiation to a cytotoxic phenotype. Aberrant IEL populations between RCD II patients displayed different stages of maturity, of which only the patients harbouring the most mature aberrant IEL populations developed an EATL.

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CHAPTER 5

Phenotypic and genomic analysis of an exceptional case of enteropathy associated T-cell lymphoma

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ABSTRACT

Approximately 2–5% of coeliac disease patients develops refractory coeliac disease (RCD), associated with the development of an aggressive enteropathy associated T-cell lymphoma (EATL). We report on immunophenotypic and genomic analysis of an exceptional case of type 1 EATL presenting as leukemic ascites. Our results, obtained by flow cytometry and karyotyping /comparative genomic hybridisation, indicate immunophenotypic and genomic aberrations previously reported for type 1 EATL. In addition, we found a substantial number of aberrations not reported so far and not included in the current WHO classification of lymphoid malignancies. The case described appears to represent an evolved form of type 1 EATL.

INTRODUCTION

Approximately half (30-50%) of all extra-nodal non-Hodgkin lymphomas arises from the gastro-intestinal tract[1] with more frequent localisation in the stomach compared to other intestinal sites.[2] Among them, the fast majority originate from B-cells[3, 4], whereas peripheral T-cell lymphomas (PTCL), although frequently manifested in the skin and upper airway, seldomly develop in the gastro-intestinal tract at initial presentation. Those PTCL that do primarily affect the digestive tract are most commonly located in the small intestine.[3] PTCL associated with coeliac disease (CD) are classified as (type 1) enteropathy associated T-cell lymphoma (EATL) according to the current WHO classification.[5] With an annual incidence rate of 0.5-1 per million people in the Western countries, this type of EATL accounts for less than 0.5% of all non-Hodgkin's lymphomas and covers approximately 35% of all small bowel lymphomas.[6]

EATL is a highly aggressive pleiomorphic form of high-grade T-cell lymphoma that can involve all areas of the small intestine, stomach and colon, with a particular preference for the proximal jejunum.[7, 8] Two thirds of EATL cases is initially limited to the gastro-intestinal tract and lymph nodes.[6] Dissemination is seen in many organs, including liver, spleen and skin.[6] Although ascites is commonly due to peritoneal metastasis, malignant lymphoma primarily presenting as ascites is very rare. Vakar-Lopez et al.[9] described in a review on initial presentation of lymphomas in ascites only two cases of T-cell origin neither of which represented EATL.

EATL shows a striking association with adult-onset CD, of which 2–5% develops refractory coeliac disease (RCD).[10, 11] In contrast to RCD type I, RCD type II is characterised by the presence of a population of aberrant intraepithelial T-lymphocytes (IELs) expressing cytoplasmic CD3 but lacking surface expression of CD3, CD4 and CD8.[12-14] About half of the RCD type II patients develops EATL, most probably originating from aberrant IELs upon clonal expansion and malignant transformation.[14, 15] EATL is more prevalent in males and has a peak incidence in the 7th decade in The Netherlands.[7] Despite its rare occurrence EATL is one of the main causes of death in patients with RCD II, with 1-, 2- and 5-year overall survival rates of respectively 31-39%, 15-20% and 8-20%.[15, 16] The poor prognosis is mainly due to incomplete response to currently available therapies, high rates of life-threatening complications such as perforation of the gut and poor nutritional conditions.[17, 18]

The immunophenotype of EATL plays an important role in its diagnostic work-up. EATL expresses CD3, CD7 and CD103, but lacks expression of CD4, CD5 and CD8 in the majority of cases. Furthermore, EATL usually exhibits a cytotoxic immunophenotype, characterised by expression of CD30 and the presence of cytotoxic granules recognised by the TIA-1 antibody.[2, 5, 19]

Current knowledge of EATL immunophenotype is almost exclusively based on immunohistochemistry. Although this technique has proven to be instrumental in the diagnostic work-up of EATL, it has limitations. In contrast to immunohistochemistry, flow

cytometric analysis allows simultaneous multiparameter immunophenotyping, analysis of expression levels and distinction between intra- and extracellular antigen expression. To the best of our knowledge, no extensive flow cytometric analysis of EATL cells primarily presenting as leukemic ascites has been performed to date. We report such analysis and show an immunophenotype that is in agreement with the known immunohistochemical features of EATL, expanded with antigens not previously analysed on EATL cells. Interestingly, we found several significant differences between the immunophenotype of EATL and aberrant intestinal T-cells associated with RCD type II. In addition, the leukemic presentation and resulting availability of single EATL cells provided the opportunity to perform both karyotype and array comparative genomic hybridisation (CGH) analysis in search of potential chromosomal abnormalities.

CASE HISTORY

A 62-year-old woman was diagnosed with CD by serology and small intestinal histology (Marsh IIIB) and subsequently treated with a gluten-free diet (GFD). Human Leukocyte Antigen typing showed a DQ2 heterozygous genotype. Seven months after diagnosis she was referred to our hospital with persisting diarrhoea, steatorrhoea, abdominal tenderness and distension, flatulence, regurgitation, night sweat, morning nausea and 10 kg of weight loss. Apart from CD, her medical history was unremarkable. Physical examination revealed a pale woman with a BMI of 23.5 kg/m2, no fever and she had a distended and slightly painful abdomen. Neither hepatomegaly nor lymphadenopathy was noted. Laboratory evaluation showed anaemia, elevated CRP, hypocalcaemia, hypomagnesaemia, hypoalbuminaemia and deficiency of zinc, folic acid, vitamin B12 and vitamin B6. Malnutrition was treated with total parenteral nutrition and all trace elements and vitamins were supplied. Nonetheless, the anaemia persisted and inflammatory markers, liver enzymes and lactate dehydrogenase further increased. Coeliac specific serology, including endomysial antibodies (IgA-EMA) and tissue transglutaminase antibodies (IgA-tTG), was still positive at the time of referral but substantially decreased in the following two months. A dietary inquiry performed by a skilled dietitian in CD showed strict adherence to the GFD.

At time of referral duodenal biopsies were classified as Marsh IIIB lesions based on intraepithelial lymphocytosis, crypt hyperplasia and subtotal villous atrophy, however without signs of malignancy. Immunohistochemical staining of the duodenal biopsies suggested the presence of an immunophenotypically aberrant IEL population, expressing CD3 but lacking expression of CD8. In order to confirm the presence as well as the precise quantity of aberrant IELs, intestinal lymphocytes were isolated from duodenal biopsies and analysed by flow cytometry as previously described.[13, 20] Within the IEL compartment, 66% of lymphoctes showed the aberrant immunophenotype CD3-, CD4-, CD8-, CD7+, cytoplasmicCD3+, indicating RCD type II associated aberrant IELs.[13] In addition, 22% of lymphocytes within the lamina propria compartment (LPL) showed a similar aberrant immunophenotype. Based

on the presence of >20% aberrant IELs, her clinical condition and persisting villous atrophy for which no other cause could be identified, she was diagnosed with RCD type II. Other investigations included abdominal ultrasound, CT- and PET-scan, showing slight dilatation of the pancreatic and common bile duct, a few slightly enlarged mesenteric lymph nodes with a maximum of 13 mm in diameter, hepatomegaly and steatosis hepatis. Cladribine, a purine analogue inducing T-cell depletion, was subsequently given intravenously for five days aiming to eradicate the aberrant intestinal T-cells.

After two months, no clinical and histological response was attained. Duodenal biopsies still showed subtotal villous atrophy in agreement with a Marsh IIIB lesion, however, no intraepithelial lymphocytosis was observed. In our experience the latter phenomenon is often observed upon cladribine treatment. In contrast to the persisting histological abnormalities, the relative percentage of aberrant IELs and LPLs decreased to 44% and 18%, respectively, after cladribine treatment. Subsequent CT-scan, video capsule endoscopy and MRI-enteroclysis showed abnormalities suspicious for lymphoma, including jejunal ulcers, multiple enlarged mesenterial lymph nodes, diffuse wall thickening of the jejunal-ileal border up to 12 mm, marked ascites and peritoneal lesions. Bone marrow analysis showed no abnormalities. Cytological analysis of the ascitic fluid showed many small to medium sized atypical lymphocytic cells most likely associated with lymphoma development. Importantly, an identical clonal TCR-γ gene rearrangement was found in the ascitic fluid derived cells as compared to duodenal biopsy derived lymphocytes, thereby supporting both the diagnosis EATL and the intestinal origin of this malignancy. Due to clinical deterioration no further treatment was possible and our patient deceased within three weeks after the diagnosis of this rare ascitic presentation of EATL.

MATERIALS AND METHODS

Cytological analysis

Ascitic fluid was obtained by peritoneal puncture and cells from this fluid were obtained by centrifugation at 530 g. Cells were either analysed directly or stored in liquid nitrogen. The ascites supernatant was stored at -80°C. Cytospins were generated and stained with May-Grünwald-Giemsa staining solution (Merck, Darmstadt, Germany) using standard procedures. Immunocytochemistry was performed with peroxidase labelled monoclonal antibodies directed against CD3, CD4, CD5, CD8, CD15, CD20, CD30, CD45 and CD79a (Dako, Glostrup, Denmark), using standard procedures.

Flow cytometric analysis

Multiparameter flow cytometric immunophenotyping was performed by staining cells for 30 minutes on ice with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC)-labelled monoclonal antibodies

directed against CD2, CD3, CD4, CD5, CD7, CD8, CD11a, CD14, CD16+56, CD18, CD19, CD25, CD30, CD45, CD45RA, CD45RO, CD54, CD69, CD95, CCR7, granzyme-B, HLA-DR, IL-2, IL-4, IL-10, IL-13, IFN-γ, Ki-67, NKG2D, PCNA, TCR-γδ (all from BD Biosciences, San Jose, California, USA), against CD52 (Serotec, Düsseldorf, Germany), against CD103 (IQ products, Groningen, The Netherlands) and against IL-15R-alpha (eBioscience, Hatfield, United Kingdom). Cytoplasmic staining of CD3, IL-2, IL-4, IL-10, IL-13 and IFN-γ was performed after cell permeabilisation by Cytofix/CytoPerm Plus (BD Biosciences), according to the manufacturer's instructions. Intracellular cytokine staining was proceeded by a four hour stimulation of the ascitic cells with 1 μg/ml PMA (Sigma, Zwijndrecht, The Netherlands) and 1 μM ionomycin (Sigma), in the presence of GolgiStop (BD Bioscience, according to instructions). Nuclear staining of Ki-67 and PCNA was performed after cell permeabilisation by eBioscience Cell Fixation/Permeabilisation (eBioscience), according to the manufacturer's instructions. Appropriate isotype controls were included. Stained cells were washed with PBS containing 0,1% bovine serum albumin (BSA, Sigma) and analysed on a standard four-colour flowcytometer (FACSCalibur, BD Biosciences). The data were analysed using Cell quest software (BD Biosciences). Care was taken to analyse only viable cellular events based on light scatter properties.

Enzyme linked immunosorbent assay (ELISA)

Levels of soluble CD25 (sCD25) were determined in the ascites supernatant using a commercial ELISA kit (Diaclone, Besancon, Cedex, France), according to the manufacturer's instructions. Levels of cytokines IL-1ß, IL-4, IL-6, IL-10, IL-13, TNF-α and IFN-γ were determined in the ascites supernatant using commercial ELISA kits (Pelikine-compact™ , Sanquin, Amsterdam, The Netherlands), according to the manufacturer's instructions.

Karyotype analysis

Metaphase chromosome preparations were obtained from EATL cells derived from ascites. The cells were cultured in RPMI 1640 with 10% fetal calf serum (both from Lonza, Verviers, Belgium) and incubated for 24 and 48 hours at 37ºC. The cultures were synchronized using FdU (0.03 μg/ml, Sigma) for 22 hours and Thymidin (3.3 μg/ml, Sigma) for 5-6 hours. Before harvesting the cells were treated with colcemid (0.21μg/ml, Sigma) for the last 10 minutes. After a hypotonic treatment at 37ºC with KCL (0.075M, Merck) the cells were fixated three times with methanol/acetic acid (first step 1:9 and two times 1:3, Merck). Slide preparation was performed according to the conventional method. Chromosome analysis was performed on GTG-banded metaphases and 14 cells were analysed.

Array comparative genomic hybridization (CGH)

DNA was isolated from fresh frozen ascitic EATL cells, using the QIAamp DNA mini kit (Qiagen, Leusden, The Netherlands) and QIAamp microkit (Qiagen). The sample contained at least

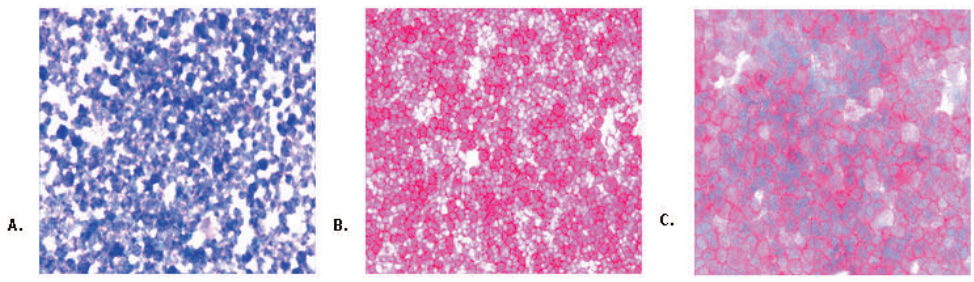
95% tumour cells. Labeling and hybridization procedures were performed according to Buffart et al.[21] Briefly, 500 ng genomic DNA was labeled using a Genomic DNA Labeling kit and according to the manufacturer's protocols (Enzo Life Sciences BVBA Zandhoven, Belgium). Cy3 label was used for the patient sample and Cy5 label was used for the reference which consisted of DNA isolated from blood obtained from 18 healthy females. Hybridizations were performed on custom designed Agilent Sure print arrays (Agilent Technologies, Palo Alto, California, USA) containing about 180.000 probes, designated 180K-CC. This design encompasses 169.793 probes distributed over the genome at 17kb intervals and is enriched with 4548 additional probes on 238 of the genes in the Cancer Census list[22] in addition to standard control probes. The exact design is available in the Gene Expression Omnibus (GEO) platform GSE17715 http://www.ncbi.nlm.nih.gov/geo/. Images of the arrays were acquired using a micro array scanner G2505C (Agilent Technologies) at 2μm resolution and image analysis was performed using feature extraction software (version 10.1.1.1; Agilent Technologies). Aberrations were subsequently called used CGHcall.[23] Raw and processed data are available under GEO accession number GSE17715 http://www.ncbi.nlm.nih.gov/geo/.

RESULTS

Cytological analysis

Cytological analysis of the leukemic ascites showed lymforeticular cells with hyperchromatic nuclei, some of which were multinuclear and Reed-Sternberg-like (Figure 1A). Immunocytochemical staining of these cells showed expression of CD3, CD30 and CD45, in agreement with a mature malignancy of T-cell origin, including EATL (Figure 1B and 1C). No expression of the T-cell associated markers CD4, CD5 and CD8 was detected. Additionally, neither expression of the myeloid marker CD15, nor of the B-cell markers CD20 and CD79a was observed (data not shown).

Figure 1 | Morphology and immunohistochemical analysis of the ascitic fluid EATL cells.
(See page 214 for colour figure)



MGC staining CD3 CD39

Flow cytometric analysis

An extensive panel of surface and intracellularly expressed markers was evaluated by multiparameter flow cytometry. Table 1 shows an overview of all markers analysed and their respective expression by the ascitic cells, both EATL and normal lymphocytes. All cells in the ascitic fluid showed strong positive expression of CD45 and low to intermediate sideward scatter, indicating a relatively homogeneous population of lymphocytic cells (Figure 2A). Analysis of CD3 expression showed that approximately 95% of these cells had lost surface expression of CD3, but remained positive for cytoplasmic CD3, in agreement with the previously described immunophenotypically aberrant intestinal T-cells (Figure 2C). The remaining 6% of cells displays surface CD3 expression and represent phenotypically normal T-cells. In keeping with the generally accepted aberrant immunophenotype found in the intestinal T-cells in RCD patients, the aberrant ascitic fluid cells lacked surface CD4 and CD8 expression in addition to CD3. Furthermore, CD5 expression was not detected, however is not expressed by aberrant intestinal T-cells either (unpublished results by MWJS and BMEvB). In contrast to aberrant intestinal T-cells, the 95% EATL cells had lost expression of CD2, CD7 (Figure 2B), CD52 and CD103 (Figure 2D), illustrating progression towards an "elevated level" of immunophenotypic aberrancy. Expression of the beta-2-integrin adhesion molecule CD11a/CD18 (LFA-1) was also undetectable, whereas its ligand CD54 (ICAM-1) was still expressed.

Consistent with the usual immunophenotype of EATL, expression of CD30 was readily observed on all ascitic EATL cells (Figure 2E). Interestingly, we observed a positive correlation between the CD30 expression level and sideward scatter, showing the highest CD30 expression on the cells with the highest cytoplasmic granularity and/or nuclear anomaly (not shown). In addition to CD30, granzyme-B is usually expressed by EATL, illustrating its cytotoxic phenotype. In contrast to the normal ascitic T-cells however, granzyme-B expression was not detected in the current EATL presentation. We did find expression of CD45RA on all cells, but not CD45RO, which is known to be associated with cytotoxic activity in (antigen experienced) T-cells. In agreement with the recognised role of IL-15 in the pathogenesis of RCD, and presumably in the development of EATL in RCD II patients, we found IL-15R-alpha expression on one-third of the EATL cells (Figure 2F).

Analysis of cell cycle activity of the leukemic cells using nuclear staining of Ki-67 and proliferating cell nuclear antigen (PCNA) revealed the majority of these cells being positive (Figure 2G and H). The latter indicates highly active cell cycle progression and proliferative activity, consistent with the large amount of tumour cells present in the ascitic fluid.

Intracellular IFN-γ expression in PMA/ionomycin stimulated EATL cells was detected by flow cytometric analysis, be it in a small percentage (6,5%) of the cells. A quarter of the EATL cells showed intracellular IL-4 expression. Intracellular IL-2, IL-10 and IL-13 was not observed. In the supernatant of the leukemic ascitic fluid high levels of sCD25, IL-6, IL-10 and IFN-γ, and intermediate levels of IL-13 were detected by ELISA, but not IL-1β, IL-4 and TNF-α (data not shown).

Table 1 | Immunophenotype of the ascitic fluid EATL and normal T-cells determined by flow cytometric analysis.

	EATL-cells (~94%)	Normal T-cells (~6%)		EATL- cells (~94%)	Normal T- cells (~6%)
CD2	-	+	**CD45RO**	-	-
CD3	-	+	**CD52**	-	+
cytCD3	+	+	**CD54**	+ (99%)	+
CD4	-	+	**CD95**	+ (100%)	-
CD5	-	+	**CD103**	-	+
CD7	-	+	**IL-15R-alpha**	+ (30%)	-
CD8	-	+	**Ki-67**	+ (88%)	-
CD11a	-	+	**PCNA**	+ (70%)	-
CD14	-	-	**NKG2D**	-	-
CD16	-	-	**HLA-DR**	-	+
CD18	-	+	**granzyme-B**	-	+
CD19	-	-	**IL-2**	-	NA
CD25	+ (40%)[1]	-	**IL-4**	+ (25%)	NA
CD30	+ (90%)	-	**IL-10**	-	NA
CD45	+	+	**IL-13**	-	NA
CD45RA	+ (90%)	+	**IFN-γ**	+/- (6.5%)	NA

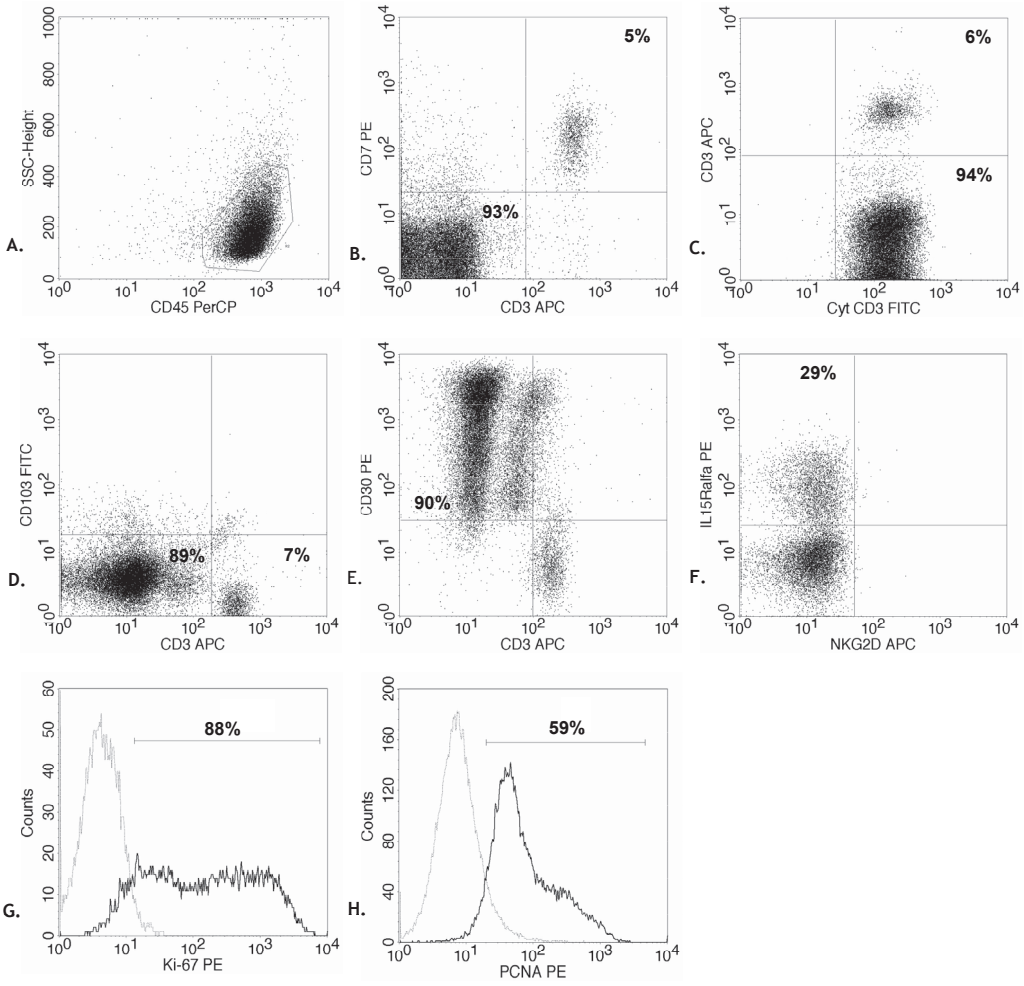
Abbreviation: NA, not analysed.
[1] Percentages indicate the relative fraction of the ascitic EATL cells.

Genomic analysis

Chromosome analysis of the ascitic fluid cells revealed the following karyotype: 46-48, -X, add(X)(q2?6), +1, i(1)(q10), der(1;15)(p10;q10), 3, add(3)(q2?7), del(3)(q21), add(4)(q2?1), del(6)(q13q21), qdp(7)(q22q32), add(9)(p24), del(10)(p11.2), -11, ?ins(11)(q13q13q14), -13, del(13)(q14q21), -15, -17, add(18)(q23), 3-4 mar[cp14].

Because of its complexity, this karyotype is reported as a composite karyotype [cp], which means that all clonal abnormalities are mentioned in the karyotype, but not all abnormalities are present in all cells. Subsequently, these multiple genetic aberrations were analysed in more detail by a high resolution (180 K) array CGH. In agreement with the karyotype described above, multiple chromosomal gains and losses with sizes up to 37 Mb were identified by array CGH (Figure 3; Table 2). The major corresponding results included a loss of chromosome X, a gain of chromosome 1q, a gain and a loss within chromosome 3, an additional part to chromosome 9 and a deletion of part of chromosome 13. In contrast, the karyotype showed trisomy of chromosome 6, a deletion of chromosome 10, insertion in chromosome 11 and an additional part to chromosomes 4 and 7 whereas the array CGH did not show these aberrations.

Figure 2 | Flow cytometric analysis of the ascitic fluid cells.
Almost all cells express CD45 (A). About 6% has a normal immunophenotype expressing CD3, CD7 (B) and CD103 (D) and 94% an aberrant immunophenotype lacking CD3, CD7 (B) and CD103 (D) but expressing cytCD3 (C), CD30 (E), IL-15R-alpha (F), Ki-67 (G) and PCNA (H).



In this case, an array CGH of non-tumour cells of our patient for DNA matching could not be performed due to lack of blood samples. Therefore all identified regions of chromosomal alteration were compared to well-known naturally occurring copy number variants (CNV) in the human genome described in the reference database of genomic variants *[available at: http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg18/].* Consequently, regions of alteration were excluded when identical to a known CNV currently present in this genome

database. In total, 43 CNV were ruled out, 37 losses with a mean size of 0,18Mb (range 0,01-1,35) and 16 gains with a mean size of 0,125 (range 0,01-0,76).

As described for EATL in the current WHO classification of tumours of haematopoietic and lymphoid tissues[5], our results did show a gain of the 9q (9q31.3-q34.3) chromosome region, but not a loss of 16q12.1, which both are described to be important in the oncogenesis of EATL. The MYC oncogene locus at 8q24 showed a gain, as reported for almost 30% of CD associated (type 1) EATL. Although reported for 80% of type 1 EATL, a gain of the 5q34-q35.2 region was not found. In keeping with the majority of type 1 EATL, a 1q gain was detected at region 1q32.1-q41 in the leukemic cells. As compared to other previously reported chromosomal aberrations of EATL cells, our case shows both similarities as well as discrepancies. Whereas some chromosomal regions were amplified in our case as well (gains of 1q23.1-q23.3, 1q23.3-q31.2, 7q21.3-31.1) other aberrations described by De Leeuw et al.[24] were not detected. Interestingly, a substantial number of novel chromosomal aberrations were identified in the current case, as listed in Table 2. In Table 2, only the chromosomal gains and losses larger than 1Mb are shown. All data are available under GEO accession number GSE17715 at http://www.ncbi.nlm.nih.gov/geo/.

Figure 3 | Array CGH of the ascitic fluid EATL cells showing multiple chromosomal gains and losses.

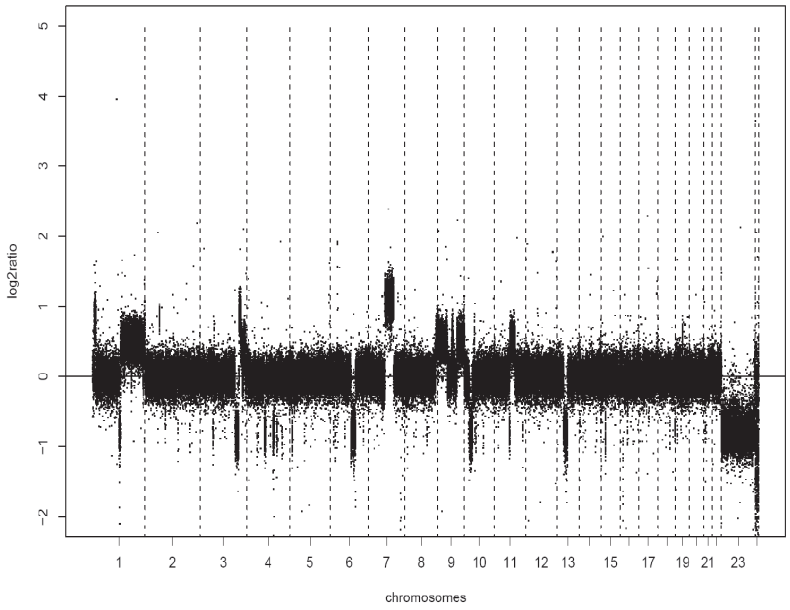


Table 2 | Chromosomal gains and losses (>1 Mb) of the ascitic fluid EATL cells detected by array CGH.

Chromosome	Start	End	Clones	Gain Loss	Cytoband	Size (Mb)
1	114177461	119445721	327	Loss	1p13.2-p12	5.27
1	187482775	188627699	66	Gain	1q31.1-q31.2	1.14
1	239527839	241285152	113	Gain	1q44	1.76
1	153299745	155287847	167	Gain	1q22-q23.1	1.99
1	242308966	246659020	278	Gain	1q44	4.35
1	155591301	160148729	315	Gain	1q23.1-q23.3	4.56
1	7133719	15251130	358	Gain	1p36.23-p36.21	8.12
1	142684203	153211435	556	Gain	1q21.1-1q22	10.53
1	188817376	200074269	673	Gain	1q31.2-q32.1	11.26
1	160659687	187034587	1702	Gain	1q23.3-q31.1	26.37
1	200265176	237873835	2269	Gain	1q32.1-q43	37.61
3	153615576	169368050	952	Loss	3q25.2-q26.2	15.75
3	189514724	192067160	209	Gain	3q28	2.55
3	186142831	189471639	228	Gain	3q27.2-q27.3	3.33
3	169954218	180811404	796	Gain	3q26.2-q26.33	10.86
4	118768823	123758682	305	Loss	4q26-q27	4.99
4	80274266	85861549	333	Loss	4q21.21-q21.23	5.59
5	10839633	11998259	69	Loss	5p15.2	1.16
6	94237970	103827105	557	Loss	6q16.1-q16.3	9.59
6	103873584	115825594	713	Loss	6q16.3-q22.1	11.95
7	97406212	112063267	922	Gain	7q21.3-q31.1	14.66
7	76442283	97338051	1354	Gain	7q11.23-q21.3	20.90
8	137147912	146272353	578	Gain	8q24.23-q24.3	9.12
9	36679951	38526827	150	Gain	9p13.2	1.85
9	33809009	36522241	183	Gain	9p13.3-p13.2	2.71
9	194193	2952674	169	Gain	9p24.3-p24.2	2.76
9	89578197	94690454	326	Gain	9q22.2-q22.32	5.11
9	3173242	33767785	1903	Gain	9p24.2-p13.3	30.59
9	109307305	140241876	2057	Gain	9q31.3-q34.3	30.93
10	32785560	34728920	115	Loss	10p11.21-p22	1.94
10	30748387	32748105	120	Loss	10p11.22-p23	2.00
10	22035743	30660372	538	Loss	10p12.31-p11.23	8.62
11	68093207	69711909	97	Loss	11q13.2-q13.3	1.62
11	69730962	70937887	77	Gain	11q13.3-q13.4	1.21
11	71304306	90873318	1234	Gain	11q13.4-q14.3	19.57
13	47707348	63242326	971	Loss	13q14.2-q21.31	15.53
15	41985456	43037392	69	Loss	15q15.3-q21.1	1.05
19	35592894	36890614	75	Gain	19q12	1.30

DISCUSSION

Classical type 1 EATL is a non-Hodgkin T-cell lymphoma of the gastro-intestinal tract and, in contrast to the monomorphic type 2 EATL, strongly associated with CD.[5] In spite of its low prevalence, EATL is one of the main causes of death in patients with long-lasting untreated CD or RCD, due to its aggressive nature and unresponsiveness to currently available therapies.[15, 18] Therefore it is of utmost importance to gain more insight into the pathogenesis of RCD and EATL in order to develop new therapeutic options. We report on an evolved type 1 EATL with an exceptional presentation as leukemic ascites, and on the unique opportunity to perform an in depth flow cytometric analysis of the immunophenotype and analysis of genomic alterations of these cells.

In agreement with the highly aggressive nature of EATL, our patient showed a very prompt deterioration of her clinical course and subsequently died within a few weeks. Although coeliac serology was still positive, yet decreasing on a GFD for seven months, she was already diagnosed with and treated for RCD. This phenomenon is seen in a minority of RCD patients.[25] In addition, clonal expansion of intestinal aberrant IELs, which is causative for EATL, was readily detected. Importantly, an identical clonal TCR-γ gene rearrangement was detected in the ascitic fluid cells as compared to the duodenal biopsies, proving the intestinal origin of this apparently evolved type 1 EATL.

Flow cytometric analysis showed that the immunophenotype of this EATL is substantially different from the corresponding aberrant T-lymphocytes in the small intestine, most probably as a consequence of further transformation. Whereas aberrant IELs found in RCD patients have lost some but not all (intestinal) T-cell lineage associated markers and generally still do express CD2, CD7, CD52 and CD103,[2, 5, 19] these markers could not be detected on the leukemic ascitic fluid derived EATL cells. Importantly, lack of CD7 and CD103 expression on EATL has not been reported so far and is not included in the current WHO classification of haematological malignancies.[5] Absence of CD103, an αEβ7 integrin contributing to tissue-specific retention of lymphocytes within the intestinal epithelial layer by binding its ligand E-cadherin expressed on all epithelial cells[26], may have contributed to migration of EATL cells away from the gastro-intestinal tract. The lack of LFA-1 expression observed on the same EATL may play a role in this as well. Although gastro-intestinal localisation of EATL has not been confirmed by histological examination in this case, MRI-enteroclysis did show diffuse wall thickening of the jejunal-ileal border, suggesting infiltration of tumour cells into the intestinal wall towards the peritoneum.

In contrast to most EATL cases exhibiting a cytotoxic immunophenotype[5, 19] the current EATL presentation showed absence of granzyme-B expression. Yet, the observed expression of CD45RA is associated with cytolytic effector cell activity.[27] The lack of CD45RO positive EATL cells is in contrast to other studies which showed that EATL often expresses this T-cell memory associated surface marker.[28, 29] The EATL cells showed extensive proliferative activity indicated by high expression levels of Ki-67 and PCNA. Similar to CD30 expression[30, 31],

high proliferative activity of EATL cells is supposed to be an adverse prognostic factor[32] which is in agreement with the clinical outcome of our patient.

The high levels of IL-2R-alpha (sCD25) and cytokines IL-4, IL-10, IL-13 and IFN-γ detected in the supernatant of the leukemic ascites indicate local T-lymphocyte activation. Since only IL-4 and IFN-γ expression was detected in the EATL cells themselves, other cytokines detected may have been produced by the reactive normal T-cells shown to be present in the ascites as well. Indeed, the latter population showed clear expression of the T-cell activation marker HLA-DR, not expressed by the EATL cells. Expression of CD25 was detected on EATL cells, in agreement with previous work indicating that sCD25 is a sensitive marker for tumour burden in some lymphomas.[33] Illustrative for the local pro-inflammatory environment, IL-6 was clearly detected in the ascites, explaining the B-symptoms fever and night sweats in our patient.

Over expression of IL-15 by enterocytes and resulting up regulation of natural killer (NK) associated receptor NKG2D by IELs are both assumed to be involved in the pathogenesis of (R)CD.[34, 35] Since IL-15 promotes the clonal expansion and activation of IELs[36], it may even have the potential to induce further transformation of aberrant IELs towards EATL upon continued over expression. Our results show that approximately one-third of the ascitic EATL cells expressed the IL-15R-alpha, which indeed suggests a role for IL-15 in the pathogenesis of the current EATL. Interestingly, up regulation of NKG2D expression was not detected.

The cytogenetic analysis identified multiple chromosomal alterations not included in the current WHO classification, which might be a contributing factor to the observed extensively aberrant immunophenotype of this EATL and its exceptional clinical presentation as leukemic ascites. It should be pointed out that the use in this study of the currently most sensitive, 180 K, array CGH may contribute to the many chromosomal aberrations found. Limited studies about the karyotype of aberrant IELs in RCD patients suggested a partial trisomy of the long arm of chromosome 1 as an early marker of EATL transformation and a gain in chromosome 9q as a late event in such a malignant transformation.[37] In agreement with this, we also observed a gain in the 9q region as well as a trisomy of chromosome 1. De Leeuw et al.[24] postulated a stepwise genetic model for the whole spectrum of CD including a 9q gain or a 16q loss responsible for inducing transition from RCD into EATL, and gains of 1q/5q and 8q distinguishing between most cases of type 1 and 2 EATL, respectively. The aforementioned genomic alterations are included in the current WHO classification.[5] We found both 1q and 8q gains, suggesting an increased level of chromosomal instability. Interestingly, the observed lower expression of PCNA as compared to Ki-67 appears to be in agreement with the latter, since PCNA is a known factor involved in DNA repair.[38] The described immunophenotypical aberrations of the current EATL cells do not fully correspond to the observed chromosomal gains and losses, except for the lack of CD2 expression corresponding to loss of chromosome 1p12-1p13.2 and the high expression of CD30 in line with a gain of 1p36.21-23. Although CD7 was not expressed on the EATL cells, a gain of chromosome 7q25.5, harbouring the CD7 gene, was found (http://www.ncbi.nlm.nih.gov).

We report on a unique type 1 EATL case, primarily presenting as ascites and displaying a significantly elevated level of immunophenotypic and genomic aberrancy as compared to the current WHO classification. The combined abundant absence of T-cell lineage-, activation- and cytotoxicity-associated markers, high proliferative capacity, as well as substantial level of chromosomal instability of the current EATL may explain the clinical behaviour of this apparently evolved malignancy. Further insight into the mechanisms responsible for the development of these characteristics may be further elucidated in future research and will contribute to a better understanding of the pathogenesis of EATL and may aid in predicting its prognosis. In addition, functional and expression profiling could be supportive in the search for new, more sensitive markers to predict the EATL occurrence in an early stage, in order to improve prognosis and development of new treatment approaches.

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PART III

THERAPEUTIC OPTIONS

CHAPTER 6

Consumption of gluten with gluten-degrading enzyme by coeliac patients: a pilot-study

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ABSTRACT

Objectives: To assess the safety and efficacy of AN-PEP to mitigate the immunogenic effects of gluten in coeliac patients. *Aspergillus niger* prolyl endoprotease (AN-PEP) effectively degrades gluten *in vitro*.

Methods: In a randomised double-blind placebo-controlled pilot-study, 16 patients consumed toast (~7 g/d gluten) with AN-PEP for two weeks (safety phase). After a two-week washout period, 14 patients were randomised to gluten intake with either AN-PEP or placebo for two weeks. Measurements included complaints, quality-of-life, serum antibodies, immunophenotyping of T-cells and duodenal mucosa immunohistology (primary endpoint).

Results: During the safety phase, 14 patients remained stable on gluten and subsequently completed the efficacy phase. Only minor deteriorations from baseline were observed for all parameters in both AN-PEP and placebo group. However, small intestinal IgA-tTG deposits were observed slightly more frequent in the placebo group compared to AN-PEP.

Conclusion: Administration of AN-PEP appears to be safe and well tolerated in coeliac patients. However, the primary endpoint was not achieved due to lack of clinical deterioration on gluten in the placebo group, impeding the identification of a possible effect of AN-PEP. Further studies with longer gluten challenge are warranted to support the potential of AN-PEP in degrading gluten and reducing the patient's burden of disease.

The trial protocol has been registered in the Dutch Trial register (NTR1281) and the FDA Clinical Trial register (NCT00810654).

INTRODUCTION

Coeliac disease (CD) is a major health care issue affecting people of all ages, with a worldwide prevalence of approximately 1%.[1] This immune-mediated small intestinal enteropathy is triggered by gluten proteins derived from wheat, barley and rye. CD is characterised by an inflammatory immune response, resulting in small intestinal mucosal injury and malabsorption in genetically susceptible individuals.[2] Currently, the only safe and effective treatment is a strict gluten-free diet (GFD) combined with nutritional support, which improves the health and quality of life in the vast majority of patients.[3] However, a GFD is perceived as a substantial burden, particularly due to high costs, dietary restriction, reduced social activity, and increasing health worries.[4]

Gluten proteins are highly abundant in proline (15%) and glutamine (35%) residues, particularly in those regions identified as immunogenic in CD.[5] The proline- and glutamine-rich peptides in gluten are relatively resistant to proteolysis by gastric, pancreatic and intestinal enzymes.[6, 7] Consequently, digestion-resistant proline- and glutamine-rich peptides can reach the intestinal epithelium intact and can trigger an immune response that eventually results in mucosal damage. To eliminate such proline-rich gluten peptides, prolyl oligopeptidases, enzymes that can cleave after a proline residue in peptides, have been investigated by Shan and colleagues.[6] Such enzymes, derived from bacteria like *Flavobacterium meningoseptum, Shingomonas capsulate* and *Myxococcus xanthus*, were capable of breaking down toxic gluten *in vitro*.[6, 8, 9] However these prolyl oligopeptidases are not stable and functional under acidic conditions of the stomach[9, 10] and are unlikely to degrade gluten epitopes before they reach the small intestine. Alternative enzymes that can break down gluten are derived from germinating barley and the fungus *Aspergillus niger*. From the latter a prolyl endoprotease termed AN-PEP (*Aspergillus niger-derived* prolyl endoprotease) is derived which has distinct advantages over the bacterial prolyl oligopeptidase as it degrades both whole gluten and gluten peptides into non-immunogenic residues within minutes.[11, 12] Moreover, the enzyme is active between pH 2 and pH 8, with an optimum activity at pH 4-5, and is therefore effective at the pH levels present in the stomach and beyond.[11, 13] Importantly, the enzyme is not degraded by pepsin in the stomach and thus remains fully functional. Mitea et al.[12] extended these findings by showing that AN-PEP degraded toxic gluten proteins in a food matrix into non-immunogenic gluten fragments in an *in vitro* digestion model that mimics the human gastrointestinal tract.[12] After these promising *in vitro* results, it remains to be established whether AN-PEP can reduce the clinical response to gluten in CD patients. The aim of this two-phase proof of concept-study was to demonstrate the safety of AN-PEP in the first phase and the ability of AN-PEP to reduce the clinical response to gluten consumption by CD patients in the second phase of the study. This information will be important to further develop AN-PEP as a future digestive aid for unintentional ingestion of gluten by CD patients.

MATERIALS AND METHODS

Patients

Sixteen adults with CD were recruited at the outpatient clinic of the department of Gastroenterology and Hepatology of the VU Medical Centre Amsterdam, The Netherlands. Inclusion criteria were an initial diagnosis of CD as confirmed by histological abnormalities on duodenal biopsies classified as a Marsh IIIB or IIIC lesion and supported by positive serology; endomysium IgA antibodies (IgA-EMA) and/or tissue transglutaminase IgA antibodies (IgA-tTG). Patients were required to have well-controlled CD as evidenced by Marsh 0 or I, and normalised IgA-EMA and IgA-tTG on a strict GFD for at least one year. Women at fertile age were required to take adequate contraception measures. Reasons for exclusion were: use of any anticoagulant or immunoregulatory drug within the last six months; clinically suspected bleeding tendency; pregnancy or breast feeding; presence of any concurrent active infection; and IgA-deficiency.

The study was approved by the Medical Ethics Committee of the VU Medical Centre and conducted in accordance with the guidelines of the Declaration of Helsinki. The trial has been registered in the Dutch Trial register (NTR1281) and the FDA Clinical Trial register (NCT00810654). A written informed consent was obtained from each patient before enrolment.

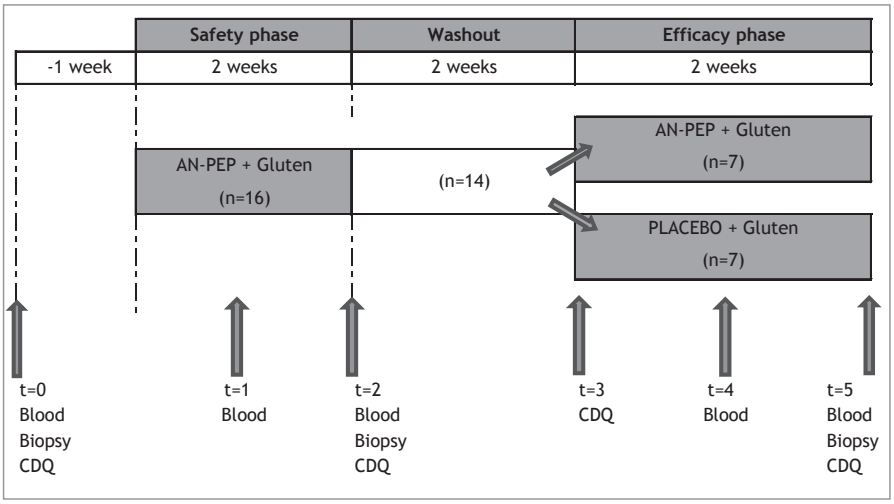
Design and intervention

The intervention was performed between May 2008 and April 2009. The intervention consisted of two periods, each lasting two weeks (Figure 1). The first study phase was an open-label period designed to assess the safety of high gluten intake with AN-PEP (safety phase). The second phase was a randomised, double-blind, placebo-controlled parallel-group study to assess the effect of AN-PEP on gluten-induced clinical response (efficacy phase). Sixteen patients with diagnosed CD were enrolled in the safety phase. Patients were asked to consume five pieces of toast (in total ~7 g gluten, Bolletje®, The Netherlands) with AN-PEP-containing topping daily in the morning for two weeks. Patients were allowed to consume a glass of water (250 mL) with their toast. They were asked to continue their usual GFD. For ethical reasons, patients deteriorating ≥2 scales on the histological Marsh classification during this safety phase were not included in the efficacy phase. Between the study phases, a two-week washout period was introduced in which patients continued their usual GFD. Subsequently, 14 patients were randomised in a 1:1 ratio in blocks of four in a double-blind fashion to the same amount of toast with AN-PEP-containing topping (n=7) or placebo topping (n=7) for two weeks while remaining on their usual GFD. Patients' compliance with the product intake was checked by regular telephone contact.

Before and during the study phases, the patients visited the outpatient clinic five times (Figure 1). During the safety phase, blood was collected one week before (baseline), and one and two weeks after start of gluten with AN-PEP consumption. During the efficacy phase, blood was collected at one and two weeks after start of gluten with AN-PEP or placebo

consumption. Duodenal biopsies were taken at baseline and at the end of the safety phase and the end of the efficacy phase. Both in the safety and efficacy phase, participants were asked to complete a coeliac disease-specific health-related quality of life questionnaire for adults[14] at baseline and after two weeks of intervention. Biopsies and blood sampled at the end of the safety phase were used as baseline values to limit the burden for the patients.

Figure 1 | Study design and flow chart.



In the safety phase, 16 coeliac patients daily consumed five pieces of toast with AN-PEP for two weeks while continuing their GFD. Two patients deteriorated on Marsh grades and were excluded. After a 2-week wash-out period with their usual GFD, the remainder of 14 patients were randomised to the efficacy phase to receive two weeks of toast with either AN-PEP or placebo while continuing their GFD.

AN-PEP enzyme

The AN-PEP and placebo topping were prepared by DSM Food Specialties, Delft, The Netherlands. Both toppings (18.5 g) contained 8.2 wt% sucrose, 8.2 wt% saccharine solution (400 mg/L saccharine plus 4000 mg/L cyclamate), 0.4 wt% citric acid (Jungbunzlauer, Basel, Switzerland), 0.08 wt% potassium sorbate (Interland Chemie, Oosterhout, The Netherlands), 0.31 wt% sodium benzoate (Prolabo, Leuven, Belgium), and 1.23 wt% xanthane gum Keltrol RD (CP Kelcko, Nijmegen, The Netherlands). The AN-PEP topping contained 81.5 wt% AN-PEP enzyme concentrate corresponding with 168 Proline Protease Units of enzyme activity. The placebo topping contained 81.5 wt% distilled water with 0.06 wt% Plantex® MDA31 (colouring agent, DSM Food Specialties, Delft, The Netherlands) to match for colour differences. The aroma, flavour and consistency of the topping with AN-PEP were identical to those with placebo and both toppings could not be distinguished. Microbial counts and enzyme activity of the AN-PEP and placebo toppings were analysed monthly. All microbial counts remained below 10 CFU/g and the activity of the enzyme was maintained at 9.1±0.3 PPU/g topping

during 12 months shelf life at 4°C. The AN-PEP and placebo toppings were identical in taste and appearance. They were pre-packed in containers (14 per box) by DSM and consecutively numbered for each patient according to the randomisation schedule (prepared by the DSM statistician).

Blinding

Each patient was assigned a random order number and received from the physician the containers in the corresponding non-transparent pre-packed box. The allocation sequence was concealed from the researcher enrolling and assessing participants in sequentially numbered sealed non-transparent envelopes. Envelopes were opened only after completion of the trial and assessments. All patients, investigators, care providers, and staff assessing outcomes were kept blind to treatment assignment.

Measurements

Mucosal biopsy immunohistology and immunophenotyping of lymphocytes, and serum antibodies were measured in the service laboratories of the VU University Medical Centre Amsterdam, The Netherlands. Mucosal biopsy gluten-specific T-cell lines were measured in the research laboratory of the Leiden University Medical Centre Leiden, The Netherlands. Mucosal biopsy IgA-tTG deposits were analysed at the University of Debrecen, Hungary.

Adverse event reporting

Tolerability of the gluten intake with AN-PEP or placebo was assessed by adverse event reporting to the physician during visits. All complaints were documented throughout the study. The study design did not allow for differentiation between complaints resulting from gluten or treatment. A difference in complaints between the AN-PEP and placebo group during the efficacy phase may give an indication of treatment-related effects.

Coeliac disease quality of life

All participants were asked to complete at home the CD quality of life questionnaire, which was translated into Dutch. The CD quality of life questionnaire included four disease-specific and health-related categories (emotional problems, social problems, disease-related worries, and gastro-intestinal symptoms) with seven items each. Each question was weighed on a scale of 1-7 points, a high score corresponding to a high level of well-being. In total 196 points could be obtained, with a maximum of 49 points for each separate category. A change of 12 or more points on the total score or of 3 or more on the different categories was considered a clinically relevant change.[14]

Mucosal biopsy immunohistology

Twelve duodenal mucosal spike biopsies were taken through upper gastro-intestinal endoscopy. Four paraffin-embedded biopsies were sectioned and hematoxylin-eosin-stained

for histological evaluation according to the modified Marsh classification.[15] At least two grades increase in the Marsh scale was considered a clinically significant deterioration. Six fresh biopsies were used for flow cytometric analysis and two were snap frozen in liquid nitrogen and stored.

Mucosal biopsy immunophenotyping of lymphocytes

Multiparameter flow cytometric immunophenotyping of mucosal intraepithelial and lamina propria lymphocytes was performed. These lymphocytes were isolated from six duodenal biopsy specimens per time point through chemical and enzymatic dissociation.[16] The cells were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC)-labelled monoclonal antibodies directed against CD3, CD4, CD8, CD16/56, CD19, CD45, CD45RA, HLA-DR, NKG2D, CD25 and TCR- γδ (all from BD Biosciences, San Jose, California, USA), and appropriate isotype controls were included. Stained cells were analysed on a 4-colour flow cytometer (FACSCalibur™, BD Biosciences) and the data were analysed using Cellquest™ software (Becton Dickinson, San Jose, California, USA). Care was taken to analyse only viable cellular events based on light scatter properties. The mean fluorescence intensity index as compared to isotype controls was calculated for the markers included.

Mucosal biopsy gluten-specific T-cell lines

Gut-resident, gluten-reactive T-cells are a hallmark of CD. To demonstrate that all patients possessed such cells polyclonal T-cell lines were generated from small intestinal biopsies as described.[17] The resulting T-cell lines were tested for reactivity against a pepsin/trypsin digest of gluten and a pepsin/trypsin digest of gluten that had been treated with tissue tranglutaminase in a T-cell proliferation assay as described.[17] In all patients gluten reactivity could be demonstrated (not shown).

Mucosal biopsy IgA-tTG deposits

Biopsies at the end of the randomisation study phase were stained for tTG-related extracellular IgA deposits and, in case of positivity, baseline biopsies were stained as well. Twelve unfixed, 5 µm-thick frozen sections were examined per patient by double immunofluorescent labelling of IgA (green) and tTG (red) as previously described.[18] IgA is normally detected only inside plasma cells and at the luminal surface, whereas in active CD, subepithelial deposits composed of IgA-tTG are found along the surface and crypt basement membranes and around mucosal vessels, corresponding to the intestinal localisation of tTG. The CD-type IgA deposits were graded from 0 to 3 according to their intensity along the basement membranes in the villous-crypt area. As this study of the small intestinal IgA-tTG deposits is highly subjective, it was performed by an independent specialist in this field in a blind manner to greatly increase its accuracy.

Serum antibodies

Blood samples were collected by venapuncture to analyse CD associated antibodies. Levels of IgA-tTG, gliadin IgA antibodies (IgA-AG) and gliadin IgG antibodies (IgG-AG) were determined with a standard in-house enzyme-linked immunosorbent assay (ELISA), using recombinant human tissue transglutaminase (Diarect AG, Freiburg, Germany) and gliadin extract (Sigma-Aldrich, Zwijndrecht, The Netherlands) as substrates, respectively. IgA-EMA antibodies were determined by an in-house indirect immunofluorescence test according to Lerner using monkey oesophagus as substrate.[19] IgA-deficiency was excluded to avoid false-negative serology. In addition, in retrospect a combined test for IgA and IgG antibodies directed against human tissue transglutaminase and deamidated gliadin-derived peptides (IgA/G-DGP-tTG; tTG/DGP Screen ELISA, INOVA Diagnostics, San Diego, USA) was performed.[20] References values for antibodies were categorised into negative, dubious, weak positive, positive, and strong positive. References values for antibodies were categorised into negative, dubious, weak positive, positive, and strong positive. Reference ranges for IgA-AG were <2.4, 2.5-3.9, 4.0-20, 20-80, and >81 U/mL, for IgG-AG, <11, 12-20, 21-40, 41-100 U/mL, for IgA-tTG, <2.9, 3.0-5.9, 6.0-20, 21-50, >51 U/mL, and for IgA/G-DGP <6.9, 7.0-10.9, 11-30, 31-100 and >100 U/ml, respectively.

Statistical analysis

Data were analysed by OCS Biometric Support (Leiden, The Netherlands). Difference from baseline in mucosal immunohistology between the two groups after two weeks as measured by Marsh classification was considered the primary outcome measure. All other parameters were considered secondary endpoints. Power analysis revealed that for the detection of a two-grade difference in the Marsh score with a power of 0.80 and a one-sided α level of 0.05, 14 patients were needed to finalise the study. Data were analysed in the SAS version 9.1, using both parametric and non-parametric tests depending on the nature of the data. The quality of life data were analysed with paired t-tests to test for differences between data before and after the 1st (safety) and 3rd (efficacy) period of the study. Serological and histopathological outcome parameters were analysed with Wilcoxon signed-rank tests to determine differences between data before and after the 1st period and the Wilcoxon rank sum tests to test the treatment differences in change from baseline in the 3rd period of the study. In order to explore whether patients' baseline characteristics would predict their response to gluten (and hence to increase the chances of success in a future trial), rank correlations between baseline characteristics and outcome variables were explored in the placebo group using the Spearman Rank Correlation Coefficient (r) of the ranked data. A p-value of <0.05 was considered significant.

RESULTS

The demographic and baseline characteristics of the patients are presented in Table 1. In total, 16 adults on a GFD diagnosed as having CD (median age 55 year (range, 20-68)) were enrolled in the study. The demographic characteristics of both treatment groups were comparable with exception of the median age at diagnosis of CD, which was 20 year higher in the AN-PEP compared to the placebo group. The median time on GFD treatment was similar in both groups. Two patients were excluded after the safety phase because of a histological deterioration of two and three Marsh grades, respectively, which for one patient returned to normal (Marsh 0) after four weeks of exclusion. The patient that did not return to normal started the study with high IgA/G-DGP-tTG values. However, other CD related antibodies remained undetectable in these two patients. The remaining 14 patients entered and completed the efficacy phase.

When correlating the patients' baseline characteristics with their response to gluten, highly significant inverse relationships were found between the patients' time since diagnosis or time spent on a GFD and their response to gluten as measured by IgG-AG, IgA-tTG and IgA/G-DGP-tTG, and Marsh grades (data not shown).

Adverse events

No serious adverse events occurred during the trial; patients reported no severe adverse events and no patients withdrew during the trial. Complaints that were reported during the safety and efficacy phase were of gastro-intestinal nature and mostly mild and transient. The number of reported gastro-intestinal complaints did not differ between the AN-PEP and placebo group (Table 2).

Table 1 | Demographic and baseline characteristics of the safety and efficacy phase.

	Safety phase	Efficacy phase	
	Gluten + AN-PEP	Gluten + Placebo	Gluten + AN-PEP
Patients, n	16	7	7
Gender			
Male	4	2	1
Female	12	5	6
Median age at inclusion (yr), range	55 (20-68)	44 (20-68)	57 (30-64)
Median age at diagnosis (yr), range	44.5 (0-62)	29 (0-62)	49 (26-53)
Median time on a GFD (yr), range	7.5 (2-40)	9 (2-40)	8 (4-12)
HLA class, n			
DQ2/X	12	5	5
DQ2/DQ2	2	1	1
DQ2/DQ8	1	0	1
Unknown	1	1	0
Marsh at inclusion, n			
Marsh 0	10	4	3
Marsh I	6	3	4

Table 2 | The number of adverse events experienced during the 2-week safety phase and 2-week efficacy phase.

Gastro-intestinal symptoms	Safety phase	Efficacy phase	
	Gluten + AN-PEP n=16	Gluten + Placebo n=7	Gluten + AN-PEP n=7
Abnormal bowel sounds	4	-	2
Abdominal pain	5	3	2
Bowel distension	5	3	1
Change of defecation	4	2	6
Constipation	3	2	-
Diarrhoea	3	1	1
Dysgeusia	1	1	-
Flatulence	6	1	2
Nausea	4	2	-
Reflux	2	-	1
Vomiting	1	1	-
Weight loss	-	1	-
Total number of symptoms	**38**	**17**	**15**

Coeliac disease quality of life

The mean total scores of the four categories on the CDQ were relatively high (145-156 out of a total score of 196) in the total group and throughout both study phases. In the safety phase, the total CDQ score significantly (p=0.04) increased by 6 points during gluten with AN-PEP treatment. This increase was, however, lower than the 12 points increase that is considered a clinically relevant quality of life improvement.[14] In the efficacy phase, the individual or total CDQ scores of patients consuming gluten with placebo or gluten with AN-PEP did not significantly deteriorate. No differences between the groups were observed. The mean score for the gastro-intestinal CDQ was relatively high throughout the study, indicating that gluten with AN-PEP was well tolerated.

Mucosal biopsy immunohistology

In the patients receiving gluten plus AN-PEP treatment in the safety phase, several patients showed variability in Marsh scores but overall no significant change in degree of mucosal damage, as indicated by changes in the Marsh score, was observed (Table 3). Two of 16 patients were excluded from entering the efficacy phase as their mucosa showed an increase of two Marsh steps while 14 patients were considered histologically stable on gluten with AN-PEP. Also after the efficacy phase, no significant deterioration was observed in the group consuming gluten with placebo compared to the group receiving AN-PEP.

Mucosal biopsy immunophenotyping of lymphocytes

Flow cytometric analysis of intestinal lymphocyte subsets showed no significant changes in the expression of the T-cell lineage associated markers CD3, CD4, CD8 and TCR-γδ, in either the intraepithelial lymphocyte or the lamina propria lymphocyte populations of both treatment groups during the efficacy phase. The mean fluorescence index of the activation markers CD25, HLA-DR, the NK receptor NKG2D as well as CD45RA, a marker for naïf T-cells, showed no significant change in either group.

Mucosal biopsy IgA-tTG antibody deposits

Mucosal tTG-related extracellular IgA deposits are hypothesised to be an early marker for CD activity.[21] Despite a GFD, two of seven patients started with positive staining for IgA-tTG at baseline (Table 3). Compared to baseline, IgA-tTG deposit staining increased after two weeks of gluten intake in four out of seven patients on placebo. In the seven patients receiving AN-PEP, one patient showed increased and one showed decreased IgA-tTG deposits (Table 3, Figure 2).

Serum antibodies

Serum CD associated antibodies (IgA-tTG, IgA-EMA, IgA-AG, IgG-AG and IgA/G-DGP-tTG) were not detectable in the serum of enrolled patients at baseline (Table 3) except for one patient in which borderline levels of IgA/G-DGP-tTG were detected, which became negative after two weeks of gluten with AN-PEP consumption. The IgA-tTG, IgG-AG, IgA/G-DGP-tTG, and IgA-EMA antibody titers remained negative on gluten with AN-PEP. Three out of 16 patients developed detectable or borderline IgA-AG levels, while 13 patients remained negative during two weeks of gluten with AN-PEP (Table 3).

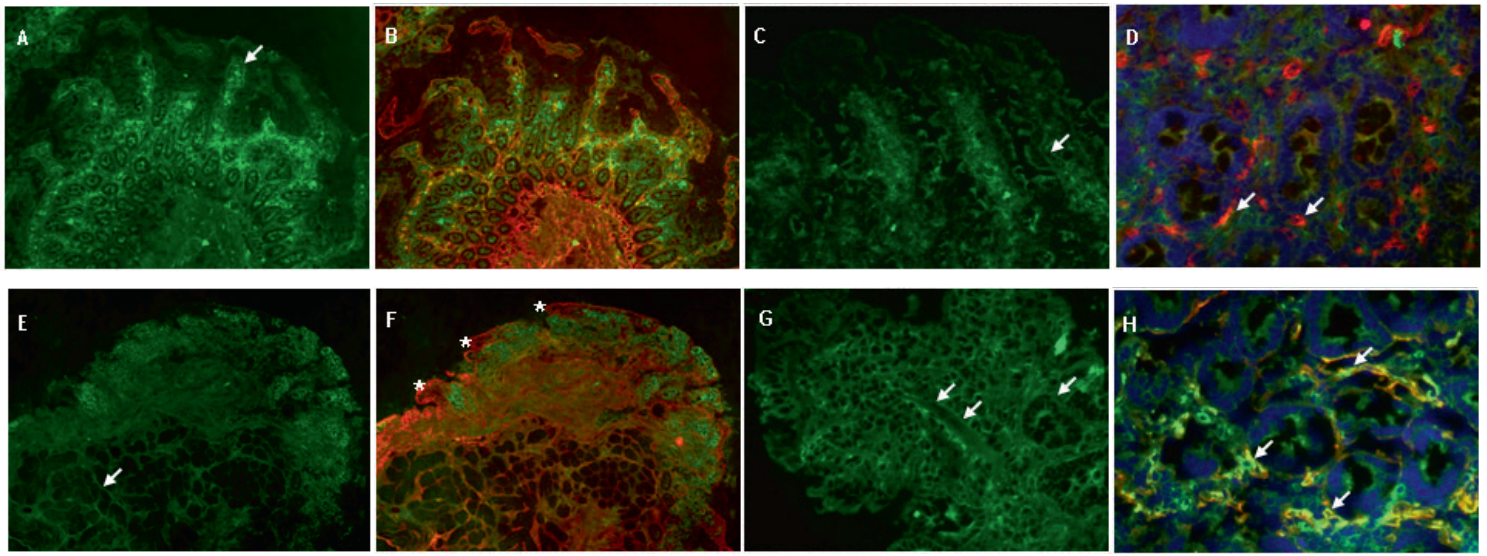
During the efficacy phase, neither the placebo nor the AN-PEP group developed significant antibody titers (Table 3). The median antibody titers after two weeks gluten intake did not significantly differ between AN-PEP and placebo treatment. The IgA-EMA concentrations remained negative in both groups.

Table 3 | Serum antibodies, duodenal immunohistology by Marsh scores and IgA-tTG antibody deposits for each patient before and after two weeks of gluten with AN-PEP or placebo.

	Baseline						Safety phase 2 wk gluten + AN-PEP						Efficacy phase 2 wk gluten + AN-PEP or placebo						
	Serum				Biopsy			Serum				Biopsy		Serum				Biopsy	
	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	IgA-tTG deposits	Tx	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	Tx	IgA-tTG	IgA-AG	IgG-AG	IgA/G-DGP-tTG	Marsh	IgA-tTG deposits
1	-	-	-	-	I	ND	A	-	-	-	-	0	P	-	-	-	-	I	0
2	-	+/-	-	-	0	0	A	-	+	-	-	I	P	-	++	+	-	IIIA	1-2
3	-	-	-	-	I	0	A	-	-	-	-	I	P	-	-	-	-	IIIA	1-2
4	+/-	-	-	+	I	1	A	-	-	-	+	0	P	+	-	-	++	I	2-3
5	-	-	-	-	0	ND	A	-	-	-	-	0	P	-	-	-	-	0	0
6	-	-	-	-	0	0	A	-	-	-	-	0	P	-	-	-	-	0	1
7	-	-	-	-	0	0	A	-	-	-	-	0	P	-	-	-	-	I	1-2
8	-	-	-	-	I	ND	A	-	-	-	-	II	A	-	-	-	-	I	0
9	-	-	-	-	0	3	A	-	-	-	-	I	A	-	+/-	-	+	I	1
10	-	-	-	-	0	ND	A	-	+/-	-	-	I	A	-	+/-	+	+	I	0
11	-	-	-	-	I	0	A	-	+	-	-	II	A	-	+/-	-	-	IIIA	1
12	-	-	-	-	I	ND	A	-	-	-	-	I	A	-	-	-	-	I	0
13	-	-	-	-	0	ND	A	-	-	-	-	0	A	-	-	-	-	0	0
14	-	-	-	-	0	ND	A	-	-	-	-	0	A	-	-	-	-	0	0
15	-	-	-	++	0	ND	A	-	-	-	++	II	E	-	-	-	++	IIIA	ND
16	-	-	-	-	0	ND	A	-	-	-	-	IIIA	E	-	-	-	-	0	ND

Abbreviations: Tx, treatment; A, AN-PEP; P, placebo; E, excluded; IgA-tTG, anti-tissue transglutaminase IgA antibodies; IgA-GA, anti-gliadin IgA antibodies; IgG-GA, anti-gliadin IgG antibodies; IgA/G-DGP-tTG. anti-tissue transglutaminase and deamidated gliadin-derived peptide IgA and IgG antibodies; I-FABP, intestinal fatty acid binding protein; ND, not determined; -, negative; +/-, dubious; +, weak positive; ++, positive; +++, strong positive. Mucosal tTGA-A deposits were graded from 0 to 3. Serum IgA-EMA remained negative in all patients during the entire study. Mucosal tTGA-A deposits were graded from 0 to 3.

Figure 2 | Small intestinal tissue transglutaminase IgA antibody deposits in two patients; in one patient at baseline (A, B) and after randomisation to AN-PEP (C, D) and in one patient at baseline (E, F) and after randomisation to placebo (G, H).
(See page 215 for colour figure)



Baseline evaluation (A, B and E, F) showed preserved villous architecture (asterisks) in both cases. Intense, grade 3 IgA deposition (green) subepithelially and around crypts (A) merge to yellow indicating co-localisation with tTG shown in red (B). IgA deposits diminished after two weeks AN-PEP treatment to grade 1, when only faint and patchy antibody deposition was seen (C, arrow). In the patient on placebo at baseline, faint, grade 1 IgA depositions were seen in the deep mucosal layer around Brunner glands (E, arrow) which were not sufficient to obtain a yellow colour at merging with tTG shown in red (F). IgA deposition increased to grade 2 subepithelially (G, arrows) and to grade 3 in the crypt region (H, arrows) after two weeks placebo. The IgA deposition after a 2-week placebo-treatment (H) co-localised with tTG to intense yellow (arrows) while tTG appeared in red in the AN-PEP-treated patient (D, arrows) in the absence of IgA deposition. The cell nuclei were stained with DAPI in D and H (blue).

DISCUSSION

The enzyme AN-PEP might possibly assist in digesting unintentionally ingested amounts of gluten in those who cannot tolerate gluten. However, demonstrating a treatment effect on (small) clinical deterioration induced by small amounts of gluten in the placebo group may be difficult. Therefore, in this proof of principle study, the enzyme was given to patients consuming large amounts of gluten in a relative small period of time. A 2-week safety phase (AN-PEP + gluten) preceded the randomisation for AN-PEP or placebo as requested by the medical ethical commission due to concerns about such a high dose of gluten consumption. Unfortunately, the primary aim of the study was not met as the placebo group did not show any deterioration after two weeks of gluten consumption. With hindsight, the study should possibly have been designed for a much longer period of time with many more patients.

The baseline characteristics were balanced between groups except for median age at diagnosis, which was 20 years higher in the AN-PEP compared to the placebo group. However, this is unlikely to have influenced the study outcome as no relationship between the age of diagnosis and the response to gluten was observed (data not shown).

The safety phase showed that AN-PEP treatment, when consumed with a high dose of about 7 g of gluten for two weeks, was safe in patients and no severe adverse events were reported. The CDQ scores remained relatively high during two weeks consumption of gluten and AN-PEP indicating that patients' general well-being remained high. Serum antibodies of the 16 patients did not increase when consuming AN-PEP with 7 g of gluten for two weeks. Also, histology of the biopsies of the majority of patients (14) showed no deterioration while two patients developed increased Marsh grades, however not accompanied by increased antibodies. The safety phase was subject to a so-called "ceiling effect" because patients entered the study on a GFD reflecting relatively healthy baseline values, limiting the ability to demonstrate any further improvement by AN-PEP.

Patients in the placebo group did not show significant deterioration on any of the measured clinical variables after a 2-week gluten challenge, indicating that two weeks of gluten challenge is insufficient to induce a clear clinical response in this population of coeliac patients. Due to lack of response to gluten in the placebo arm, no treatment effect of AN-PEP could be detected. The measured serum levels of IgA-tTG, IgA-EMA, and IgA/G-DGP-tTG antibodies are considered sensitive markers of CD and should be able to detect subtle immunogenic effects of gluten. Similarly, the CD specific quality of life questionnaire is considered a CD specific measure of quality of life and should also be able to pick up relevant changes in health.[14] However, histological examination of small intestinal biopsies may be less reliable than CD associated antibodies due to heterogeneous distribution of lesions, low grade histopathology, and intra- and interobserver variability.[22] Interestingly, measures of clinical response to gluten (Marsh grades, antibody titres, quality of life scores) did not correlate in this study, which may in part be explained by the lack of response to gluten. The IgA antibody reactivity to small intestinal mucosa tTG has been considered

to be an early marker for gluten-induced pathology in CD patients.[23] It was observed that intestinal IgA-tTG deposits can be detected in latent CD patients in which the mucosal villous architecture is still intact, and that the intensity of these mucosal deposits decreased after adherence to a GFD and increased after gluten consumption. Although numbers were low, mucosal IgA-tTG deposits increased in four patients on placebo and one on AN-PEP and decreased in one patient on AN-PEP, compared to baseline values, suggesting that AN-PEP may mitigate gluten exposure.

Some gastro-intestinal related symptoms, mostly mild and transient, were reported during gluten challenge, and symptoms between the two groups were comparable suggesting no treatment-related effects. Besides the substantial gluten intake, emotional stress as a consequence of having to ingest gluten might have triggered some of the reported gastro-intestinal complaints.

The coeliac patients consumed approximately 7 g gluten daily, which is about half of the average adult daily gluten intake in The Netherlands.[24] Despite this high gluten dose, no substantial histological, serological, or symptom changes were observed on placebo after two weeks. In another study[25] in which adult CD patients consumed approximately 3.5 g/day of gluten from cracker biscuits for two weeks, only few patients consuming gluten on placebo showed deterioration on histology, serology and symptoms. Two other studies investigating a gluten challenge in adult CD patients, based on either lower gluten intake (2.5 – 5.0 g/day) for at least three months[26] or comparable gluten intake (four slices of white bread daily; ~8 g/d)[27] showed that a moderate gluten intake can be tolerated by some patients for several weeks-to-months without significant changes in symptoms[27], serology[26] and histology[26, 27]. The time to serological and mucosal relapse and recovery after gluten reintroduction and elimination, respectively, can be highly variable among adult CD patients from several weeks up to many years.[27-29] Excluding two out of 16 patients that may have been more sensitive to gluten from the efficacy phase may, to a small extent, have caused sample bias by selecting patients being less sensitive to gluten. Nevertheless, the same population of patients that entered the efficacy phase were randomly allocated to the AN-PEP or placebo arm. Also attrition bias can be excluded since all patients remained in the study. The lack of substantial clinical response to gluten observed in this study indicates that a longer gluten challenge is likely necessary to induce a significant clinical response to gluten in the majority of patients. Moreover, unresponsiveness to gluten of patients being diagnosed for more than 10 years ago, suggests that future studies may benefit from selecting more recently diagnosed patients.

In conclusion, AN-PEP appeared to be safe in coeliac patients. More patients and gluten challenge for a longer period of time seem to be required to induce significant clinical changes and to confirm whether the tendency of AN-PEP to reduce small bowel IgA-tTG deposits is of clinical significance. These results together with previous *in vitro* evidence that AN-PEP efficiently degrades gluten under simulated gastro-intestinal conditions warrant confirmation in a larger trial.

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CHAPTER 7

Tioguanine in the treatment of refractory coeliac disease: a single centre experience

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ABSTRACT

Objectives: Refractory coeliac disease type I (RCD I) is a complicated form of coeliac disease characterised by primary or secondary resistance to a gluten-free diet (GFD) with persisting or recurring intestinal villous atrophy and symptoms of malabsorption. Besides corticosteroids, azathioprine has been advocated for the treatment of RCD I. However, tioguanine (TG) might be better tolerated and more efficacious owing to a simpler metabolism towards bioactivation. We evaluate tolerability and effectiveness of the non-conventional thiopurine derivative TG in RCD I.

Methods: RCD I patients treated with TG between June 2001 and November 2010 with a follow-up period of at least one year were included. Adverse events, laboratory values, 6-thioguanine nucleotide concentrations and rates of both clinical and histological response were evaluated at baseline and during follow-up.

Results: Twelve adult RCD I patients were included. The median TG treatment duration was 14 months. Ten patients tolerated TG treatment on the long-term, whereas two patients withdrew treatment due to adverse events. No nodular regenerative hyperplasia of the liver was observed. During follow-up clinical and histological response was observed in 83% and 78%, respectively. Corticosteroid dependency decreased by 50%.

Conclusion: Tioguanine appears to be a convenient drug for the treatment of RCD I based on higher histological and similar clinical response rates as compared to historical conventional therapies.

INTRODUCTION

A small percentage of the adult-onset coeliac disease (CD) patients displays a primary or secondary lack of clinical and histological response upon strict adherence to a gluten-free diet (GFD) for more than 12 months despite normalised CD associated antibodies. These patients are diagnosed as having refractory coeliac disease (RCD) when dietary consumption of gluten and other causes of malabsorption with villous atrophy are ruled out.[1, 2] This syndrome can be subdivided into RCD type I and RCD type II, with immunophenotypically normal and aberrant intraepithelial T-lymphocytes (IELs) in the small intestinal mucosa, respectively. Aberrant IELs express cytoplasmic CD3, but lack the surface markers CD3, CD4 and CD8. A clinically validated cut-off value of 20% aberrant IELs, detected by using flow cytometric analysis of small intestinal biopsies, is currently used to discriminate between RCD I and RCD II.[3] Although the prognosis of RCD I is much more favourable than that of RCD II, as reflected in 5-year survival rates of around 90% and 44-58%, respectively[4-6], treatment is believed to be important in preventing complications of longstanding malabsorption. The poor prognosis of RCD II is due to a high risk of clonal expansion and further transformation of aberrant mucosal T-lymphocytes into an aggressive enteropathy associated T-cell lymphoma (EATL).[1, 2]

Apart from a GFD and nutritional support, corticosteroids are the mainstay of treatment in RCD I. However, an 80% corticosteroid dependency associated with systemic side effects has been reported.[5] To reduce corticosteroid dependency and in case of corticosteroid refractoriness, the immunomodulatory purine analogue azathioprine (AZA) has been used.[7, 8] Although clinical response rates following AZA therapy were promising, histological remission was lagging behind.[5] In addition, AZA therapy is regularly complicated and discontinued due to adverse events, as has been shown in the treatment of inflammatory bowel disease (IBD) but also in small RCD I series.[7, 9] Both therapeutic failure as well as adverse events may be due to an unfavourable metabolism of AZA. Azathioprine is metabolized via mercaptopurine (MP) into the pharmacologically active 6-tioguanine nucleotides (TGN), and into multiple other metabolites including 6-methyl mercaptopurine (6-MMP) (Figure 1). Despite a weight-adjusted dosing regimen, there is a wide interindividual variety in TGN and 6-MMP concentrations, which reflects individual differences in the activities of involved enzymes.[10] High concentrations of 6-MMP are associated with adverse events often necessitating drug withdrawal.[11] In view of the aforementioned limitations of corticosteroid and immunosuppressive therapies, it is important to evaluate therapeutic alternatives for RCD I.

The non-conventional thiopurine derivative tioguanine (TG) might be a suitable alternative treatment option for RCD I, either as first-line treatment, second-line treatment in patients unresponsive to corticosteroids or AZA, or as a steroid-sparing drug. Tioguanine only needs one enzymatic conversion towards the formation of the active metabolites TGN and when metabolized, it is not converted into 6-MMP. In addition, TG was shown to be

well-tolerated and efficacious in the treatment of IBD.[12, 13] We report a single centre series concerning tolerability and effectiveness of TG in RCD I.

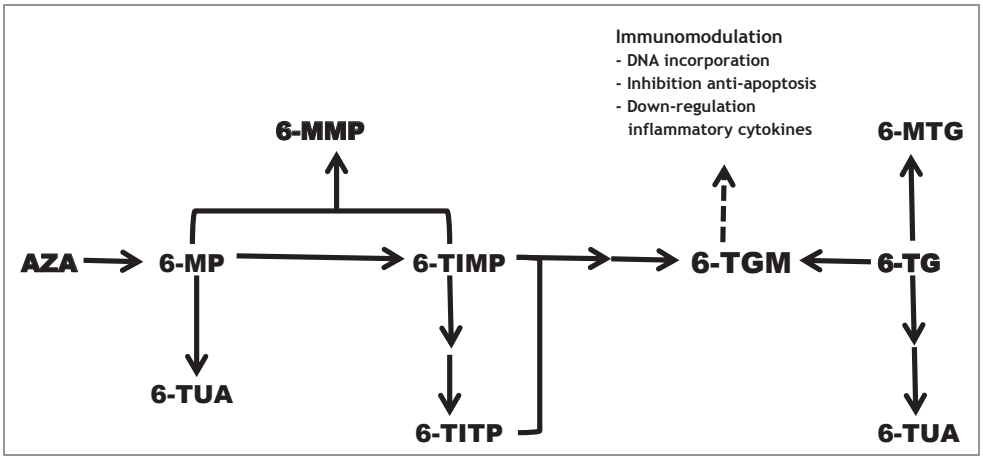
MATERIALS AND METHODS

All patients who were diagnosed with RCD I and received first or second line TG therapy between June 2001 and November 2010 at the gastroenterology department of a tertiary referral centre were identified.

Diagnosis of RCD I was based on persisting or recurring symptoms and small intestinal villous atrophy despite strict adherence to a GFD for at least one year ascertained by negative serology and a specialised dietitian.[1] Furthermore, the proportion of aberrant IELs detected by using flow cytometric analysis of small intestinal biopsies had to be less than 20 percent.[3] In all patients EATL was excluded.[14]

After informed consent, TG was prescribed in a daily dose of approximately 0.3 mg/kg and administered as 18, 21 or 24 mg capsules (generic) or 20 mg tablets (Lanvis® GlaxoSmithKline, Middlesex, United Kingdom). Patients visited the outpatient clinic on a regular basis, usually every three to six months. Patient demographics, date of onset of coeliac disease, date of onset of RCD I, clinical details, HLA-DQ haplotype, history of prior immunosuppressive therapy, reason for and date of initiating TG therapy, and laboratory results were collected from medical records. In addition, where available clinical and histological details were also obtained after cessation of TG.

Figure 1 | Simplified thiopurine metabolism.



Azathioprine (AZA) is converted into mercaptopurine (MP), which in turn is oxidized into 6-thiouric acid (6-TUA), methylated into 6-methyl mercaptopurine (6-MMP) or metabolized into 6-thioinosine monophosphate (6-TIMP). Then, 6-TIMP is metabolized into the pharmacologically active 6-tioguanine nucleotides (TGN), methylated into 6-MMP or is phosphorylated into 6-thioinosine triphosphate (6-TITP). On the other hand tioguanine (TG) is directly metabolized into TGN, methylated into 6-methyl thioguanine (6-MTG), or oxidized into 6-TUA.

Tolerability

Adverse events that had occurred during the use of TG were reported by the treating physicians and described by the researchers as being certainly related, possibly related or not related to TG therapy. In addition, laboratory values regarding the safety of TG therapy that had been determined at baseline and at different time points during follow-up were collected. These values included hemoglobin concentrations, platelet counts, leukocyte counts and liver biochemistry, usually at three month interval.

Therapeutic response

Both clinical and histological characteristics were evaluated at baseline and at different time points during follow-up of TG treatment. Gastro-intestinal symptoms, weight and laboratory values, including haemoglobin and albumin were reported. Multiple duodenal biopsies were taken by upper gastro-intestinal endoscopy to detect histopathological abnormalities. Clinical response was defined as amelioration of gastro-intestinal symptoms, combined with at least two out of the following parameters within their reference range or with an improvement of ≥1 point: body mass index (BMI), albumin and haemoglobin.[15] Complete histological response was characterised by normalisation of the architecture of the small intestinal mucosa, defined as a Marsh 0 or I score according to the modified Marsh criteria, as assessed by an experienced coeliac disease pathologist.[16] A partial histological response was defined as an improvement of the Marsh classification of two or more steps.

Corticosteroid dependency was arbitrarily defined as the use of prednisone or budesonide during follow-up (≥6 months) in a daily dose of at least 10 mg or 9 mg, respectively. The primary end of follow-up was set at the time of TG withdrawal. If remission was the reason for TG cessation, a secondary end of follow-up was set at the time of the last duodenal biopsy performed after TG discontinuation to evaluate ongoing remission.

6-Tioguanine nucleotide concentration

Red blood cell (RBC) TGN concentrations obtained during follow-up were measured by reversed-phase high-performance liquid chromatography according to a slightly modified method described by Dervieux and Boulieu and expressed in pmol/8x10^8 RBC.[17] The mean values of these measurements were used for comparison between patients. For comparability with the frequently used method of Lennard the observed values must be divided by 2.6.

Statistical analysis

Data are tabulated or presented descriptively. Continuous variables were expressed as median values with their range. Based on the skewed distribution of the included variables the non-parametric Mann-Whitney U-test was used for comparison between groups. P-values less than 0.05 were considered statistically significant. SPSS 15.0 for Windows (SPSS Inc, Chicago, Illinois, USA) was used for statistical analysis.

Table 1 | Patient characteristics.

Patient	Gender	Age CD (yr)	GFD (yr)	Initial response GFD	Age RCD (yr)	HLA-DQ status	EMA-Dx	tTGA_ Dx	Marsh before start TG	Marsh 12 mo	Marsh 24 mo	CR	HR	Time HR (mo)	Stop TG	Time TG (mo)	Reason stop TG
1	M	37.1	23.1	Y	60.5	DQ2 homozygous	neg	neg	IIIA	0		Y	Y	12	Y	17.0	Remission
*2	M	55.1	3.6	Y	58.7	DQ2 heterozygous	neg	neg	IIIB	II	0	Y	Y	24	Y	26.6	
3	M	48.7	1.2	Y	49.9	DQ2 heterozygous	neg	neg	IIIB	0		Y	Y	6	Y	7.0	Remission
4	F	41.8	8.8	Y	50.6	ND	dubious	neg	IIIA	IIIA	IIIA	Y	Y **	48	Y	92.2	Remission
*5	F	62.7	1.0	Y	63.7	DQ2 heterozygous	neg	neg	IIIA	ND	ND	N	ND		Y	3.9	Death
6	F	46.8	5.9	N	52.7	DQ2 heterozygous	neg	neg	IIIA	0		Y	Y	12	Y	14.6	Remission
7	F	17.5	32.9	N	51.4	DQ2 homozygous	dubious	dubious	IIIC	IIIB	ND	Y	N		N	11.7	
*8	F	63.9	4.5	N	68.4	DQ2 homozygous	neg	neg	IIIA	ND	ND	N	ND		Y	0.8	
*9	F	18.0	16.4	N	34.4	DQ2/8	neg	neg	IIIA	IIIA		Y	N		N	14.1	
10	F	59.0	4.0	Y	63.0	DQ2 heterozygous	neg	neg	IIIA	ND	ND	Y	ND		N	14.9	
11	M	52.8	4.7	N	57.5	DQ2 homozygous	neg	neg	IIIA	0		Y	Y	12	Y	10.9	Remission
12	F	55.5	1.0	N	56.4	DQ2 homozygous	neg	neg	IIIA	0		Y	Y	12	N	18.5	

Abbreviations: F, female; M, male; CD, coeliac disease; RCD, refractory coeliac disease; GFD, gluten-free diet; EMA, endomysial antibodies, tTGA, tissue transglutaminase antibodies; CR, clinical response; HR, histological response; Dx, at time of diagnosis of refractory coeliac disease; ND, not determined.

* Pre-treatment with azathioprine and/or prednisone.

** Histological response (Marsh I) was achieved after 48 months of follow-up.

RESULTS

Overall, 13 RCD I patients received TG therapy between June 2001 and November 2010. One patient was excluded due to loss to follow-up. The remaining 12 RCD I patients, four males and eight females, were included in the analysis. Their median age at RCD I diagnosis was 58 years (range 35–68), the median time adhering to a GFD was 4.6 years (range 1-33) and median daily dose of TG was 19 mg (range 18-40), corresponding with 0.36 mg/kg (0.26–0.69). Eight patients were treated with TG as first-line treatment, whereas four patients received TG as second-line treatment due to intolerance or resistance to AZA or corticosteroid dependency. The median TG treatment duration was 14 months, ranging from three weeks up to almost eight years. Table 1 shows additional patient characteristics.

Tolerability

Three patients experienced an adverse event during TG treatment, two of which supposedly related to the use of TG. One patient died within four months of therapy due to progression of RCD I with severe diarrhoea not responding to prednisone induction therapy. Subsequently, severe metabolic dysregulation consisting of metabolic acidosis, hypokalemia, and hypoalbumenia occurred and was complicated by septic shock and multiple organ failure. Permission for autopsy to exclude EATL was not obtained. This serious adverse event was not likely to be related with TG treatment itself, supported by the fact that there was no myelotoxicity preceding septic shock. The second patient withdrew TG therapy only after three weeks due to muscle spasms without laboratory abnormalities. This patient was refractory to previous AZA treatment. The spasms resolved after cessation of TG and initiation of corticosteroids. This adverse event was classified as possibly related. The third patient experienced liver test abnormalities after nine months of therapy. Gamma glutamyltransferase, alanine aminotransferase and aspartate aminotransferase concentrations increased up to twice the upper limit of the normal range, yet with alkaline phosphatase within the normal range. Despite a dose reduction from 21 to 10 mg TG daily laboratory findings remained steadily elevated. In this particular patient TG was withdrawn after 17 months as both clinical response and complete histological remission was achieved. Following the complete cessation of TG the deviant laboratory values returned to within the normal range during follow-up. Apart from these transitory, mild non-specific elevation of biochemical liver tests, no other clinical clues for nodular regenerative hyperplasia were observed during follow-up, including a normal alkaline phosphatase level and absence of esophageal varices at gastro-duodenoscopy, ascites and trombopenia indicative for portal hypertension. As these nodular abnormalities are usually isoechoic on ultrasonography this investigation was not performed. Diagnosis requires a liver biopsy and might have revealed (early) signs of nodular regenerative hyperplasia, but was not performed owing to the low suspicion. This adverse event was classified as possibly related.

The two patients initially intolerant of AZA did tolerate TG. No episodes of trombopaenia, leucopaenia or anaemia were observed in any of the patients. In addition, apart from the one patient described above, no episodes of hepatotoxicity, as indicated by abnormal liver tests, occurred in any other patient. Overall, TG was tolerated in nine out of twelve patients (75%).

Therapeutic response

Prior to treatment with TG all patients had weight loss and gastro-intestinal symptoms including diarrhoea. One year after initiation of TG, clinical response was observed in ten out of all twelve patients (83%), corresponding to ten out of ten patients who tolerated TG (Table 1). Median weight, BMI, haemoglobin concentration and albumin concentration increased from 56,5 kg (46-86), 19.5 kg/m2 (16.7–27.8), 7.7 mmol/L (6,5-9.7) and 38 g/L (27–44) at baseline, to 65 kg (53-84), 22.4 kg/m2 (19.7– 27.1), 8.0 mmol/L (7.3 -9.9) and 40 g/L (32-45), respectively. None of these parameters showed a statistically significant difference at one year of follow-up as compared to baseline.

At baseline, duodenal biopsies of all patients revealed intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy. After one year, nine out the ten patients who tolerated TG had been endoscopically evaluated. Complete histological response, was observed in seven patients (78%). In the other two patients no histological response was observed after 12 to 14 months of follow-up despite showing a clinical response (Table 1; Figure 2). Neither clinical nor histological response was related to baseline Marsh grade.

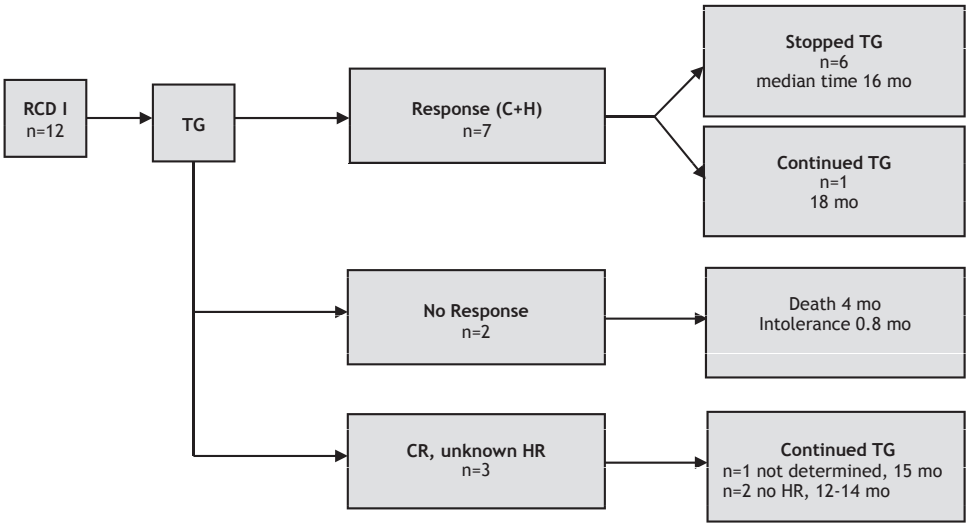
Six patients were withdrawn from TG treatment following both clinical and complete histological respons after a median treatment duration of 17 months (7-92). In four out of these patients, endoscopic evaluation was also performed during follow-up after cessation of TG therapy. One of them again needed TG after five months when symptoms of diarrhoea arose and duodenal histology revealed a Marsh II. Clinical response persisted in the remaining three patients, whereas an ongoing histological response was found in two patients after 6 and 20 months, and evolvement from a Marsh I into a Marsh IIIA in one patient within six months of the extended follow-up.

Four out of the ten patients tolerating TG for at least six months, corticosteroids (prednisone ≥10mg/budesonide ≥9mg) had been using at baseline. Out of these four, two (50%) were corticosteroid dependent during follow-up. All of these patients showed a clinical response, whereas a histological response was only observed in the two corticosteroid dependent patients.

6-Tioguanine nucleotide concentration

During follow-up, the median RBC TGN concentration was 734 pmol/8x10^8 RBC (378-1180). There was no statistically significant difference in TGN concentration between patients who experienced adverse events and those who did not (p=1.0), nor was there a difference between patients with a histological response and those without (p=0.29).

Figure 2 | Flow chart of RCD I patients treated with tioguanine.



Abbreviations: C, clinical; H, histological; CR, clinical response; HR, histological response; RCD, refractory coeliac disease; TG, tioguanine.

DISCUSSION

Refractory coeliac disease type I is a complicated form of CD characterised by primary or secondary resistance to a GFD with persisting or recurring intestinal villous atrophy and symptoms of malabsorption.[1, 2] Although the prognosis is good, adequate treatment is believed to be important in preventing complications of longstanding malabsortion. However randomised trials are lacking and treatment is largely empiric, limited by case-reports, small observational series and a few prospective open-label studies. This study reports on the effectiveness of the non-conventional thiopurine derivative TG in 78% of patients with a well-defined diagnosis of RCD I. In addition, TG seems to have an acceptable safety profile.

Tolerability

The short-term tolerability profile of TG in this study is comparable with that reported in the treatment of IBD, varying between 70% and 80%.[13, 18] However, almost all IBD patients from previous studies had proven to be intolerant of or resistant to AZA or 6-MP prior to TG treatment, whereas most of the RCD I patients in this study were thiopurine naïf. Importantly, the two patients who were intolerant to AZA did tolerate TG. The suggested advantageous tolerability of AZA in the treatment of RCD I in previous studies is remarkable, whereas around 40% of the IBD patients fail therapy due to adverse events.[7, 8, 11] This difference might indicate disease specific adverse event profiles, although contrasting reports have been published in this regard[19, 20], or adverse events in RCD I treatment may not have been

put down in writing. Although not explicitly reported in the treatment of RCD I, potential adverse effects including gastro-intestinal complaints, hepatotoxicity and myelotoxicity may hamper the use of AZA.[11] The complex metabolism of AZA with production of unwanted and potentially toxic metabolites might play an important role in the occurrence of these adverse events.[11] A well-known and dreaded adverse event of TG treatment is the sinusoidal obstruction syndrome, which may induce nodular regenerative hyperplasia of the liver, with in its clinical end stage portal hypertension and its complications. In this study two adverse events were classified as potentially or probably related to TG treatment. One patient with muscle spasm and another with liver test abnormalities. In the latter patient with nonspecific mild elevated liver biochemical tests it is unlikely to be a result of an evolving sinusoidal obstruction syndrome as this usually coincides with signs of portal hypertension, including thrombopaenia and esophageal varices, and elevated levels of alkaline phosphatase. In addition, after cessation of TG the biochemical liver tests normalised during follow-up, consequently a liver biopsy was not performed, however, this investigation is required to fully exclude nodular regenerative hyperplasia. Interestingly, in addition to TG treatment, coeliac disease itself has been associated with the occurrence of nodular regenerative hyperplasia[21] and hypertransaminasaemia[22].

Therapeutic drug monitoring of thiopurine treatment (i.e. azathioprine and mercaptopurine) by measuring erythrocyte TGN concentrations is of growing interest in the treatment of IBD. However, routine measurement to optimise treatment cannot be recommended.[23, 24] With regard to TGN measurement during TG therapy there is even less evidence of its usefulness. In this study we evaluated erythrocyte TGN concentrations obtained during TG treatment. As anticipated, no relation between TGN concentration and toxicity or efficacy was observed.

Therapeutic response

The 83% clinical response rate observed in this study seemed to be at least as effective as that of previous reported therapies.[5, 6, 8, 25, 26] Furthermore, we observed a histological response in over half of the cases, compared to 30% previously reported.[5, 6] Moreover, a 50% reduction in corticosteroid dependency was achieved.

Systemic corticosteroids are the mainstay of treatment in RCD I with clinical response in the majority (~80%) of patients[5, 6], however, potentially severe side effects comprise the long-term use of these agents. Moreover, steroid-dependency is observed in the majority of cases.[5] Oral budesonide is suggested to be an attractive alternative treatment option owing its topical effect and low systemic bioavailability. Daum and colleagues[26] reported good tolerability and stable disease for a median time of 28 months (range 14-56) in four RCD I patients treated with budesonide (9-12 mg/day) after a clinical response to induction therapy with prednisone (median 12 months). However, three patients were eventually treated with AZA, suggesting that long-term use of budesonide monotherapy might not be

effective. In spite of clinical improvement, complete small intestinal mucosal repair was not observed.[25, 26] Furthermore, in the light of oral budesonide lacking long-term efficacy, a recent open-label study investigated small intestinal release mesalamine (SIRM; 4mg/kg) with or without concomitant budesonide in ten RCD I patients. Overall clinical response was 50%, hence with SIRM monotherapy a complete response was observed in three out of the four patients. Moreover, SIRM was generally well-tolerated.[27]

The immunomodulatory agent AZA has been used for its steroid-sparing effect and in case of steroid-refractoriness. Goeres et al.[8] demonstrated that prednisone with concomitant AZA (2 mg/kg) for one year is usually sufficient to gain clinical response in RCD I, however, histologic response rates (partial ~30%; complete ~10%) were lagging behind. Case-reports suggest that other immunosuppressive agents, such as cyclosporine[28] and infliximab[29] might induce prompt clinical and histological improvement as well. Although this study suffers from several limitations, including its retrospective design, its small sample size and the absence of a control group, our experience suggests that TG is effective in obtaining a clinical response in RCD I patients within one year. As most patients in our series were treated with first-line TG, it still remains to be established whether this drug might be a good alternative option in patients intolerant of or refractory to other immunosuppressive drugs as well. In addition, the optimal treatment duration of TG should be established.

In conclusion, TG is relatively safe, potentially steroid-sparing, and efficacious in obtaining clinical and histological improvement in the treatment of RCD I. Although randomised trials are warranted, this particular agent might be an additional or alternative therapeutic option.

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CHAPTER 8

Evaluation of cladribine treatment in refractory coeliac disease type II

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ABSTRACT

Objectives: This study aimed to evaluate cladribine (2-chlorodeoxyadenosine [2CdA]) therapy in refractory coeliac disease (RCD) II.

Methods: An open-label cohort-study of RCD II patients treated with 2-CdA was performed between 2000 and 2010. Survival rate, EATL occurrence, clinical course, and histological and immunological response rates were evaluated.

Results: Overall, 32 patients were included with a median follow-up of 31 months. Eighteen patients responded well to 2-CdA. Patients responsive to 2-CdA have a statistically significant increased survival compared to those who were unresponsive. The overall 3- and 5-year survival was 83% in the responder group and 63% and 22% in the non-responder group, respectively. The overall 2-year clinical, histological and immunological response rates were 87%, 48% and 44%, respectively. Progression into EATL was reported in 17%, all of them died.

Conclusion: Treatment of RCD II with 2-CdA holds promise, showing excellent clinical and histological response rates, and probably less frequent transition into EATL.

INTRODUCTION

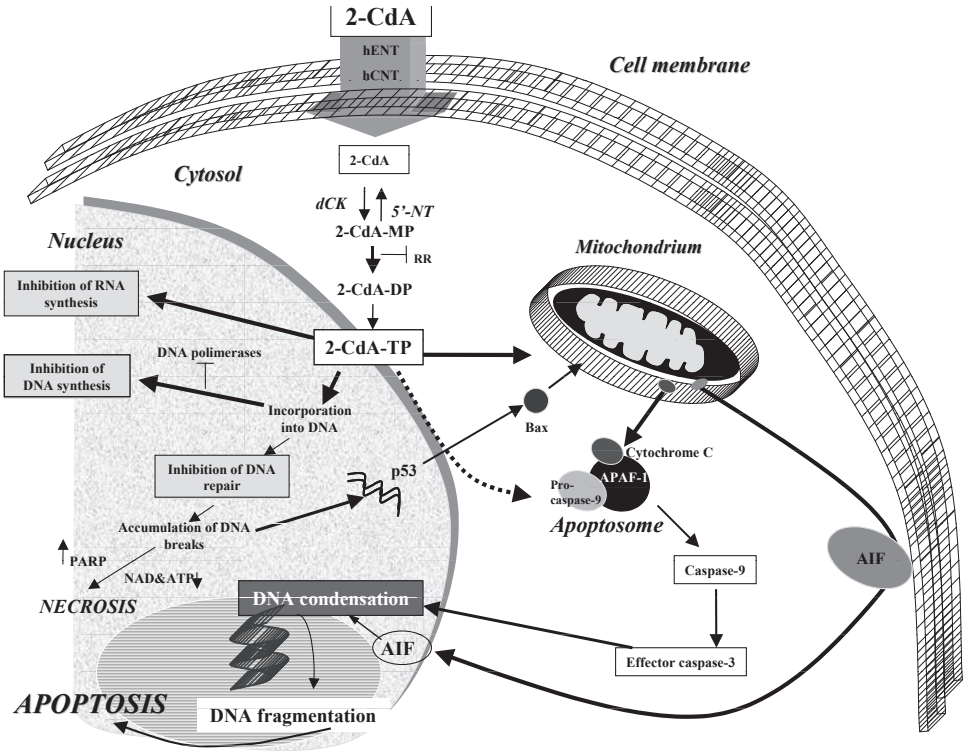
Coeliac disease (CD) is characterised by a permanent state of intolerance to dietary gluten leading to an inflammatory T-cell response in the small intestine with mucosal injury in genetically susceptible individuals.[1, 2] Although reduction of this intestinal inflammatory activity is usually seen upon strict adherence to a gluten-free diet (GFD), a small percentage (2%-5%) of the adult-onset CD patients, especially those diagnosed above the age of 50 years, displays a lack of response to such a diet. They are regarded as suffering from refractory coeliac disease (RCD) when clinical and histological symptoms persist or recur after a former good response to a strict GFD, despite strict adherence to the diet for more than 12 months, unless earlier intervention was necessary.[3, 4] Immunologically, this syndrome can be subdivided into RCD I and RCD II, with immunophenotypically normal and aberrant intraepithelial T-lymphocytes (IELs) in the small intestinal mucosa, respectively. Clonal expansion of these aberrant IELs, lacking the T-cell surface markers T-cell receptor (TCR), CD3, CD4 and CD8[5], but expressing cytoplasmic CD3, is supposed to be responsible for the occurrence of enteropathy associated T-cell llymphoma (EATL).[4-6] This type of lymphoma occurs in 60%-80% of the RCD II patients within 5 years and has a poor prognosis with a 2-year survival of only 15%.[7, 8] The latter is mainly due to incomplete response to currently available therapies, high rates of life-threatening complications such as perforation of the gut and poor nutritional conditions.[7, 8] Therefore, it is of utmost importance to evaluate new treatment strategies for RCD II in order to improve clinical course and prevent or delay progression to overt EATL.

Since the late 1990's, researchers have become increasingly interested in therapeutic alternatives for treating RCD II, however, as far as has been published there is no standardised approach yet.[9, 10] RCD II is, at least in part, resistant to most evaluated therapies so far.[10-12] Until 2005 RCD II, patients were initially treated in our tertiary referral centre with conventional immunosuppressive drugs, mainly azathioprine and prednisone, and if clinically and histologically unresponsive cladribine (2-chlorodeoxyadenosine [2-CdA]) was prescribed. Since then, a modified treatment strategy was initiated with cladribine being drug of first choice.

Cladribine is a synthetic purine nucleoside homologue being equally toxic to proliferating as to non-dividing lymphoid cells. Because of this unique feature it is supposed to be especially active against low-grade malignancies, including hairy cell leukaemia. Cladribine is metabolised into its pharmacologically active cladribine triphosphate, which induces apoptosis, necrosis and inhibition of DNA/RNA synthesis (Figure 1). Clinically, 2-CdA is of proven value in the treatment of a number of haematological malignancies and selected autoimmune disorders, including multiple sclerosis.[13-15] Our pilot-study showed that 2-CdA therapy is feasible and well tolerated in these patients.[16] Survival at that time seemed promising, but follow-up was short.

This analysis evaluates 2-CdA therapy in a large prospectively studied open-label cohort of RCD II patients, during a mean follow-up time of three years.

Figure 1 | Schematic representation of intracellular pathways involved in cladribine cytotoxicity. *(Adapted with permission from Borak, 2006).*



Abbreviations: 2-CdA-MP, cladribine monophosphate; 2-CdA-DP, cladribine diphosphate; 2-CdA-TP, cladribine triphosphate; dCK, deoxycytidine kinase; 5'-NT, 5'-nucleotidase; hENT, human equilibrative nucleoside transporter; hCNT, human concentrative nucleoside transporter; AIF, apoptosis inducing factor; APAF-1, apoptotic protease activating factor; PARP, poly (ADP-ribose) polymerase.

MATERIALS AND METHODS

This cohort-study includes reports of extended follow-up of 14 out of the 17 RCD II patients included into the open-label prospective phase-I study performed by Al-Toma et al.[16] with 18 new patients added. Three patients out of the previous study were not included in this study as they are followed over time outside our hospital. Between January 2000 and April 2010, 2-CdA was prescribed to 32 RCD II patients at the VU University Medical Centre, Amsterdam, The Netherlands.

Inclusion criteria

Patients diagnosed as having RCD II and treated with one or two courses of 2-CdA at the VU University Medical Centre were included. Cladribine was intravenously given in a dose of

0.1 mg/kg per day for five consecutive days. The diagnosis of RCD II was based on persisting or recurring clinical symptoms and small intestinal villous atrophy after a former good response to a strict GFD, despite strict adherence to the diet for more than 12 months. Furthermore, the clinically validated cut-off value of more than 20% aberrant IELs detected by flow cytometric analysis was used to distinguish RCD type I and type II.[5] Although a clonal TCR-gamma rearrangement determined by PCR is still a widely accepted method to define RCD II, Verbeek et al.[5] showed that the percentage of aberrant IELs detected by flow cytometric analysis is a more accurate way to define RCD II. The presence of EATL was excluded by using selected investigations[17-20] and its diagnosis was confirmed according to the WHO classification of tumours of haematopoietic and lymphoid tissues.[21] Furthermore, pre-treatment with immunomodulatory drugs within six months or any experimental drug within 30 days of the study entry was not allowed.

Follow-up and criteria of response

Before and during follow-up after 2-CdA treatment a clinical assessment was carried out noting in particular signs and symptoms of malabsorption, body mass index (BMI), albumin, and haemoglobin (Hb). A nutritional screening was performed by a dietitian specialised in CD in almost all patients prior to treatment and nutritional support was given when indicated. Clinical response was defined as improvement of the diarrhoea, abdominal discomfort and/or signs of malabsorption, combined with at least two out of the following parameters of intestinal integrity within the normal range or an improvement of ≥1 point: 1) Hb, 2) BMI and 3) albumin. Multiple duodenal biopsies were taken by upper gastro-intestinal endoscopy in order to detect histopathological abnormalities and to perform immunophenotyping of IELs by flow cytometric analysis at different time points (planned at 3, 6 and 12 months, and then every year during follow-up). Isolation of small intestinal T-lymphocytes and staining for immunophenotyping were performed as previously described.[5] Complete histological response has been defined as a normalisation of the architecture of the duodenum, classified as a Marsh 0 or I lesion according to the modified Marsh classification.[22] A decline of 20% or more in the percentage of aberrant IELs was considered a significant immunological response. In addition, survival rate and EATL occurrence were evaluated during follow-up.

Furthermore, following on Verbeek et al.[10] who hypothesised that pre-treatment with common immunosuppressive drugs, including azathioprine and prednisone might influence the response to 2-CdA treatment, this study compared RCD II patients pre-treated with immunosuppressive agents before 2-CdA was prescribed (group I) to those treated with upfront 2-CdA (group II).

Ethical approval and informed consent

Approval for this open label study protocol was obtained from the local ethics committee in 2000 and all patients gave their informed consent.

Statistical analysis

Quantitative data were expressed as medians and means. Kaplan-Meier survival curves were constructed using SPSS software (SPSS Inc., Chicago, Illinois, USA). In addition, the log rank test was used to assess the statistical significance. A p-value of less than 0.05 was considered statistically significant.

RESULTS

In total, 32 RCD II patients who were treated with 2-CdA were included with a median follow-up time of 31 months (range 4-120). Patient characteristics (Table 1) show a median age of >50 years, with a male predominance. Prior to the start of 2-CdA therapy, ten patients failed to respond clinically and histologically to conventional immunosuppressive drugs (defined as group I), including high dose prednisone in two and azathioprine or 6-tioguanine added to prednisone in eight patients. The remaining 22 patients (defined as group II) were initially treated with 2-CdA following diagnosis of RCD.

Disease status

In agreement with our previous study, 2-CdA was feasible and well tolerated without serious adverse events.[10] Overall, 18 (56%) of the RCD II patients were responsive to one or two courses of 2-CdA based on the clinical, and complete histological and/or immunological response (Figure 2). Seven showed a clinical and histological response, four a clinical and immunological response, and seven a clinical, histological as well as immunological response. Out of the remaining 14 patients unresponsive to 2-CdA, two were admitted for a second course of 2-CdA in the last six months and in anticipation of evaluation of response. Six non-responsive patients were evaluated for high dose of chemotherapy followed by autologous hematopoietic stem-cell transplantation (au-SCT). In two of them stem cells could not be harvested, therefore four patients were actually transplanted.[23] Another six patients had an expectative follow-up, two of them had an exacerbation after an initial response to 2-CdA for almost 2 and 3.5 years.

Table 2 shows the clinical, histological and immunological 1- and 2-year response rates to 2-CdA treatment. In total, clinical response was observed in 26 (81%), complete histological response in 15 (47%) and immunological response in 13 (41%) of the RCD II patients. The median levels of BMI, Hb and albumin increased from 20.9 kg/m^2, 7.8 mmol/L and 36 g/L at baseline to 23 kg/m^2, 7.9 mmol/L and 39g/L at the end of follow-up, respectively. In addition to the fifteen patients in whom complete histological response, defined as Marsh 0 or I, was observed, two patients had a partial histological response from Marsh IIIB and IIIC lesions at baseline to Marsh II and IIIA at the end of follow-up respectively. The median percentage of intestinal aberrant IELs before 2-CdA treatment was 61% and declined to 56% after 2-CdA treatment. The time to a 50% response rate was three years. Approximately 33% of the patients lacked a clonal TCR-γ gene rearrangement, although all patients had

an aberrant IELs of more than 20%. A statistical significance between the percentage of aberrant IELs and the clonality status was not found.

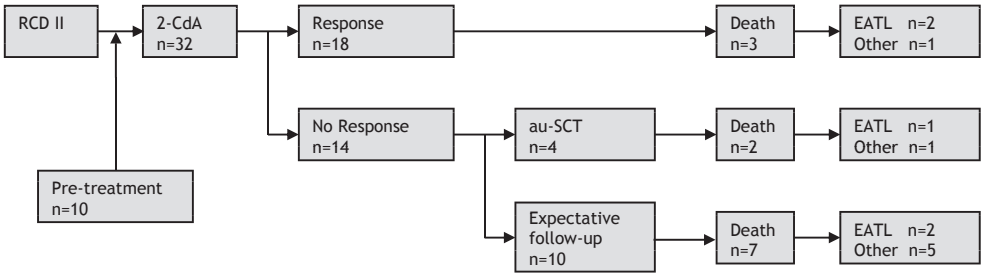
Analysis of the overall response in patients pre-treated with immunosuppressive drugs prior to 2-CdA (group I) and those with up-front 2-CdA treatment showed no statistical significance (log rank, p=0.856). Immunological (log rank, p=0.030) response, however, was significantly higher in group II. In addition, a trend towards a higher clinical response in group II (log rank, p=0.058) was found. Yet, for the histological response rate a statistical significance was not observed (Table 2). Cox-regression analysis showed that the following parameters have no predictive value for response to 2-CdA: age at 2-CdA infusion (p=0.06), gender (p=0.60), TCR-γ clonality (p=0.604), and percentage of aberrant IELs (p=0.646), degree of small intestinal villous atrophy (p=0.610), BMI (p=0.095), albumin (p=0.936) and Hb (p=0.953) before treatment.

Table 1 | Patient characteristics.

Gender		
Male	18	
Female	14	
Median age of CD diagnosis (yr), range	58.5	(38-74)
Median age of RCD II diagnosis (yr), range	64	(42-78)
Median age at the start of 2-CdA treatment (yr), range	64	(45-78)
Treatment prior to 2-CdA		
None	22	
Immunosuppressive drug	10	
Median follow-up time (months), range	31	(4-120)
HLA-DQ status		
DQ2 heterozygous	17	
DQ2 homozygous	12	
DQ2 and DQ8	2	
Unknown	1	
TCR-γ gene rearrangement		
Monoclonal	18	
Polyclonal	9	
Unknown	5	
Marsh grade before 2-CdA		
Marsh IIIA	13	
Marsh IIIB	11	
Marsh IIIC	8	
Median intestinal aberrant IELs before 2-CdA (%), range	61	(21-96)
Median BMI before 2-CdA (kg/m2), range	21	(16-27)
Median albumin level before 2-CdA (g/L), range (Reference value 35–52 g/L)	36	(23-47)
Median Hb level before 2-CdA (mmol/L), range	7.8	(6.0-9.8)

Abbreviations: CD, coeliac disease; RCD, refractory coeliac disease; 2-CdA, cladribine; BMI, body mass index; Hb, haemoglobin.

Figure 2 | Flowchart of the response to cladribine treatment.



Abbreviations: RCD, refractory coeliac disease; 2-CdA, 2-chlorodeoxyadenosine; au-SCT, autologous hematopoietic stem-cell transplantation; EATL, enteropathy associated T-cell lymphoma.

Table 2 | The 1-year and 2-years response rates and the median time to response in the pre-treated (I) and the up-front cladribine (II) group.

	Treatment	(n)	Response (%)		Time to a 50%
			1 yr	2 yr	response rate (mo)
Histological	Group I	10	13	25	36
Response	Group II	22	20	58	24
	Overall	32	10	48	36 (3-96)
Immunological[1]	Group I	10	11	11	>60
Response	Group II	22	52	58	12
	Overall	32	39	44	41 (2-96)
Clinical[2]	Group I	10	67	67	6
response	Group II	22	95	95	3
	Overall	32	81	81	3 (2-72)
Overall	Group I	10	22	38	36
Response	Group II	22	28	43	30
	Overall	32	25	41	36

[1]P=0.030 and [2]P=0.058 between group I and II.

Survival

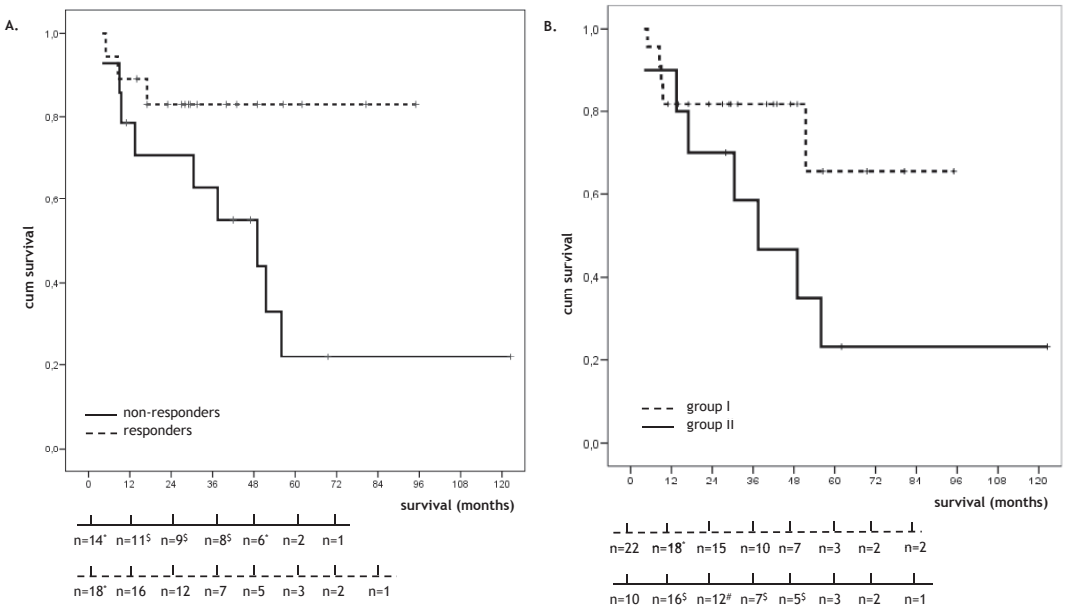
The overall median survival is almost 4.5 years. In total, 37% (12/32) of the included patients died, due to an EATL in 42% (5/12). All patients diagnosed with EATL (15%) died subsequently, having a median survival of only 4.4 months (range 0-12) after diagnosis.

The survival curve (Figure 3A) shows a statistically significant difference (p=0.037) between responders and non-responders to 2-CdA treatment. The 3- and 5-years survival is 83% in the responding group and 63% and 22% in the non-responding group, respectively. One-sixth (3/18) of the responders died, one due to refractory disease status and two were diagnosed with EATL within three and nine months after the last infusion of 2-CdA.

Approximately 65% (9/14) of the non-responders died, three as a consequence of developing an EATL within 8, 12 and 44 months after 2-CdA treatment (Figure 2). The exceptionally delayed progression into EATL in the latter patient might be explained by treatment with high dose of chemotherapy followed by au-SCT eight months after 2-CdA treatment. The remaining six unresponsive patients deceased as a result of refractory disease status; one treated with chemotherapy and au-SCT died after one year of follow-up and five with expectative follow-up despite lack of response to 2-CdA after a median follow-up of 18 months (range 4.5–60). The patient in the latter group who died five years after 2-CdA treatment, had an exacerbation after an initial remission to 2-CdA for 3.5 years and then refused further treatment.

In addition, there was no statistical significance (p=0.23) between the overall survival in the pre-treatment group (I) and the upfront 2-CdA treatment group (II) as depicted in Figure 3B.

Figure 3 | Kaplan-Meier survival curves.



A: Survival rate of responders and non-responders to cladribine treatment (Log Rank: p=0.037).
* Three patients died in the 1st and 5th year; $ one patient died in the 2nd, 3rd and 4th year of follow-up, respectively.
B: Survival rate of the pre-treatment (I) and the upfront cladribine (II) group (Log Rank: p=0.23).
* Four patients died in the 1st year; $ one patient died in the 1st, 3rd and 4th year of follow-up, respectively; # two patients died in the 2nd year of follow-up.

DISCUSSION

Approximately half of the RCD II patients have an unfavourable clinical course as a consequence of non or partial response to current available therapies and subsequently the progression into EATL[7, 8] For that reason clinicians have become increasingly interested in treating this condition alternatively and more effectively. However, there is no standardised approach reported so far. In this study a large cohort of RCD II patients treated with 2-CdA has been analysed.

In agreement with Al-Toma et al[16], this report describing almost twice as many patients with extended follow-up, showed that 2-CdA is indeed feasible and well tolerated without serious adverse events after short and long-term follow-up. Our data show that RCD II patients responsive to 2-CdA treatment have a statistically significant increased survival compared to those who are unresponsive. Although the median follow-up time was only 30 months, after 1.5 year of follow-up none of the responders died compared to four non-responders, indicating a clear difference in survival rate. The overall 5-year survival of RCD II patients in this analysis is 46% so far, which is in line with other single-centre studies[11, 12], and is expected to improve after extended follow-up. Previous reports included RCD II patients treated with diverse steroid-like drugs[11, 12], whereas in this study ten RCD II patients unresponsive to such immunosuppressive drugs were included as well. However, unexpectedly the overall survival did not significantly differ between patients pre-treated with conventional immunosuppressive drugs (group I) compared to those with upfront 2-CdA treatment (group II). Nevertheless, a clearly increased median survival rate in group II was observed. The lack of statistical significance might be due to the small cohort included in group I. In addition, some of the RCD II patients, treated with conventional immunosuppressive drugs, died before 2-CdA treatment could be initiated as previously reported by our group[24] and were therefore not included in the current analysis. Apart from favouring clinical course and increasing survival, preventing EATL represents the ultimate goal of treating RCD II. Although treatment with 2-CdA could not prevent progression into EATL, compared to previous reports showing EATL in 33–52% of the RCD II patients within five years[7, 11], our results showed a much lower rate of lymphomagenesis (17%), yet approximately 50% has a 5-year follow-up.

Furthermore, our open-label and observational data showed that 2-CdA is an effective treatment in obtaining clinical and immunohistological response in more than half of the RCD II patients. Previous studies reported a good clinical response after treatment, but histological response rates up to only 30% were found depending on the type of treatment.[11, 12] Consistent with these findings, a clinical response rate of 80% was found. The potential advantage of 2-CdA, however, seems to be a good histological response. In fact, histologic healing (Marsh 0 or I) was observed in almost half of the cases and a partial response of at least two Marsh scores in another three cases. Although not statistically significant, the 2-year histological response rate in up-front 2-CdA group (II) was twice that of the pre-

treatment group (I), possibly indicating a beneficial effect of upfront 2-CdA treatment. Furthermore, Malamut et al.[11] recently described a 74% steroid-dependency in a large cohort of RCD II patients treated with corticosteroids. Since there is no need for corticosteroids when 2-CdA therapy is prescribed, steroid-dependency and its complications will not occur.

In this cohort, the proportion of RCD II patients lacking a clonal TCR-γ gene rearrangement was relatively high (33%) compared with that reported in other studies[4, 11, 12], although all patients had more than 20% aberrant IELs determined by flow cytometric analysis. This discrepancy might be the result of some technical aspects of the DNA analysis, for instance a reduced DNA quality due to formalin fixation of the biopsies and a limited sensitivity of the PCR in case of a low percentage of aberrant IELs. Therefore, the tested polyclonal status in this cohort is most likely overestimated. However, we have previously shown[5] that flow cytometry of aberrant IELs is superior to clonality analysis for risk stratification in RCD II.

Approximately 40% of the RCD II patients showed an immunological response after 2-CdA treatment, defined as a decrease of more than 20% of the aberrant intestinal IELs determined by flow cytometric analysis. However, the majority still showed more than 30% aberrant IELs during follow-up. Expansion of these aberrant T-lymphocytes, which reside in the intraepithelial as well as lamina propria layer of the small intestine[25], is generally accepted as the culprit factor in the progression into EATL. Conversely, in our series the persisting high percentage of aberrant mucosal T-lymphocytes did not correlate with the relatively low EATL occurrence found during the time of follow-up so far. Unfortunately, reports from other centres on the immunological response determined by flow cytometric analysis after treatment are lacking. Whereas the percentage of aberrant IELs is established to be important in the diagnostic work-up for distinguishing RCD type I and II[5], apparently it is questionable whether this percentage is a predictive marker for progression into EATL during monitoring of the therapeutic response. Further research is mandatory to further elucidate EATL risk stratification. Immunophenotyping of the aberrant T-cells by flow cytometric analysis of small intestinal biopsies and probably also genotyping seem to be appropriate methods to search for predictive markers. In addition, future studies on quantifying the mass of aberrant IELs using immuno-PET techniques, instead of the currently determined percentage of aberrant IELs, given the relatively equal depletion of normal and aberrant IELs upon 2-CdA treatment, have to be conducted.

In the current analysis approximately half of the RCD II patients was unresponsive to 2-CdA, for yet unknown reasons. A significant association with the degree of mucosal villous atrophy, the percentage of aberrant IELs, and levels of BMI, Hb and albumin before 2-CdA treatment, clonal TCR-γ gene rearrangement and HLA-DQ status was not revealed. Although dose-finding studies with 2-CdA infusion in RCD are not conducted, clinical dose-finding studies in lymphoproliferative diseases showed good response rates with an identical treatment schedule.[13, 26] In addition, compared to intravenous infusions and subcutaneous injections which provide identical plasma 2-CdA levels, oral administration has a much lower

bioavailability (approximately 40%) due to degradation through acid in the stomach and intestinal bacteria.[14] A higher dose and/or a prolonged treatment schedule might result in a higher response rate, yet the maximum tolerated dose established in lymphoproliferative diseases without serious adverse events was 0.1mg/kg/per day for 7 days.[13] A recent clinical trial of oral cladribine for relapsing multiple sclerosis showed that short-course and high dose (3.5mg/kg) therapy is effective, yet lymphocytopenia is frequently reported.[15] Furthermore, the high sensitivity of hairy cell leukaemia (HCL) for treatment with 2-CdA, showing low resistance levels, is hypothesised to be the result of p53-dependent pathways required for killing resting cells and its inhibitory effect on the cholesterol metabolism which is highly active in HCL cells.[14] Physiological conditions such as increased repair of DNA, increased anti-apoptotic effects and decreased activation of deoxycytidine kinase, an enzyme required for the cytotoxicity of 2-CdA, might be contributing factors to resistance as well.[27] Whether the same results regarding administration route, treatment schedule and resistance pattern of 2-CdA also correlate with such good response rates in RCD II, remains to be further elucidated. The registration of oral 2-CdA for multiple sclerosis in Europe, might be a further step forward towards the application of this drug in RCD as well as some other gastro-enterological diseases refractory to currently available therapies, including Crohn's disease, ulcerative colitis and autoimmune hepatitis.

In conclusion, 2-CdA appears to be a promising treatment in RCD II. This analysis showed excellent clinical and histological response rates after 2-CdA treatment. Furthermore, 2-CdA therapy does not necessitate the additional use of corticosteroids and subsequently prevents steroid-dependency and its complications. Although EATL could not be fully prevented, its incidence was restricted to 17%. Multicentre, randomised clinical trials with 2-CdA and/or other new treatment options are mandatory to standardise the treatment strategy for RCD II, in order to further decrease morbidity and mortality in this patient group.

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CHAPTER 9

Autologous haematopoietic stem-cell transplantation in refractory coeliac disease type II patients unresponsive to cladribine therapy

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ABSTRACT

Objectives: Autologous haematopoietic stem-cell transplantation (au-SCT) has been an effective therapy for refractory disease, in both malignancies and severe autoimmune diseases. It seems feasible and safe for refractory coeliac disease (RCD) type II, although long-term results have not been evaluated yet. With current therapies, progression into enteropathy associated T-cell lymphoma (EATL) occurs in 60-80%, having a high mortality rate. Therefore, it is important to evaluate new treatment strategies.

Methods: Between March 2004 and February 2010, 18 RCD II patients were evaluated for au-SCT preceded by conditioning with fludarabine and melphalan, as a consequence of unresponsiveness to cladribine therapy. Adverse events, survival rate, EATL development, and change in clinical, histological and immunological course were monitored.

Results: Thirteen patients were transplanted successfully and followed for >2 years; the 4-year survival rate was 66%. Only one patient died because of transplant-related complications. The majority of patients showed an impressive clinical improvement and five a complete histological response. In five patients, au-SCT could not be performed; they all died with a median survival of 5.5 months. EATL was observed in one transplanted patient, only after four years of follow-up.

Conclusion: Au-SCT after conditioning with high dose chemotherapy in RCD II patients unresponsive to cladribine therapy, is feasible, and seems promising.

INTRODUCTION

Coeliac disease (CD) is a major health care issue world wide, with a prevalence of around 1%, affecting people of all ages.[1] A permanent state of intolerance to gluten-containing food leads to chronic inflammation of the small intestine. Hallmarks of CD are lymphocytic infiltration of the lamina propria, expansion of the intraepithelial lymphocyte (IEL) population, hyperplasia of the crypts, and atrophy of the villi. CD occurs in genetically susceptible individuals who almost always carry the HLA-DQ 2/8 alleles.[2, 3]

Although improvement is usually seen upon life-long withdrawal of dietary gluten, a small percentage (2-5) of the adult-onset CD patients, especially those diagnosed above the age of 50 years, lacks clinical and histological response despite strict adherence to such a gluten-free diet (GFD) for more than 12 months. Patients are classified as having refractory coeliac disease (RCD) if dietary consumption of gluten and other causes of malabsorption with villous atrophy are ruled out.[4, 5] On the basis of immunophenotyping of the IELs, RCD can be subdivided into two groups: type I with phenotypically normal and type II with phenotypically aberrant IELs, aberrance being defined as lack of the surface markers CD3, CD4 and CD8, but with expression of cytoplasmic CD3. Clonal expansion of these aberrant IELs is thought to be responsible for progression to enteropathy associated T-cell lymphoma (EATL).[4, 6, 7] The genesis and expansion of these monoclonal T-cells involve both inappropriate immune responses to gluten and acquisition of genetic abnormalities. RCD II is in most patients resistant to most studied therapies, including corticosteroids, budesonide, infliximab, cyclosporine, IL-10 and azathioprine/prednisone[8] and has a high risk of progressing to EATL (60-80% within 5 years).[9, 10] This peripheral T-cell lymphoma has a very poor prognosis with a 2-year survival of only 15%, mainly because of failure to respond to chemotherapy.[9, 10] Therefore, it is of utmost importance to evaluate new treatment strategies for RCD II patients, and in particular for those unresponsive to conventional immunosuppressive drugs to improve the clinical course and prevent or delay development of EATL.

Until 2005 in our tertiary referral centre for RCD, type II patients were initially treated with conventional immunosuppressive drugs, mainly azathioprine or prednisone or both, cladribine was prescribed if they were clinically and histologically unresponsive. Cladribine is a synthetic purine nucleoside homologue with cytotoxic activity. Since 2005 a modified treatment strategy has been initiated with cladribine being drug of first choice, based on a study showing that azathioprine combined with prednisone is not effective in most RCD II patients.[11] For patients not responding, an alternative was sought. High dose chemo/radiotherapy followed by autologous haematopoietic stem-cell transplantation (au-SCT) has been effective therapy for refractory disease not only in haematological malignancies, but also in severe autoimmune disease.[12-14] In autoimmune disease, au-SCT is supposed to induce immunoablation with subsequent regeneration of naïf T-lymphocytes derived from reinfused haematopoietic progenitor cells.[13-15] Analysis of our first seven RCD II patients treated with

au-SCT showed this treatment approach is feasible and well tolerated in coeliac patients. Survival at that time seemed promising, but follow-up was short.[16]

This analysis evaluates the follow-up of 18 RCD II patients, including seven patients of our pilot study, who were selected for au-SCT as a consequence of unresponsiveness to conventional immunosuppressive and/or cladribine therapy. Fifteen patients were treated in Amsterdam, one in each Italy, Portugal and Germany.

MATERIALS AND METHODS

This study reports extended follow-up of the open-label prospective phase I study performed by Al-Toma et al.[16] with six new transplanted patients added, who were referred after inclusion of the latter study was finished. Between March 2004 and February 2010, 18 cladribine unresponsive RCD II patients were evaluated for au-SCT.

Inclusion and exclusion criteria

Patients aged <70 years and diagnosed with RCD II were included when they showed no response to one or two courses of cladribine, administered intravenously in a dose of 0.1mg/kg per day for 5 consecutive days. Response was defined as clinical (improvement of signs and symptoms of malabsorption and weight gain) and histological (Marsh criteria 0/I) and/or immunological (>20% decrease of aberrant IELs) response. The diagnosis of RCD II was based on persisting or recurring symptoms and small intestinal villous atrophy after a former good response despite strict adherence to a GFD for at least 1 year.[5, 17] Furthermore, the clinically validated cut-off value of >20% aberrant IELs detected by flow cytometric analysis was used to distinguish RCD type I and type II.[7] A lower percentage of aberrant T-cells was allowed in the presence of ulcerative jejunitis. T-cell receptor (TCR)-γ gene rearrangement was performed as previously described.[7] Although this clonal TCR- γ rearrangement is still a widely accepted additional method to define RCD II, Verbeek et al.[7] showed that the percentage of aberrant IELs detected by flow cytometric analysis is more accurate to define RCD II. EATL was excluded by several investigations after CT-scan[18], whole body PET-scan[19], MRI-enteroclysis[20], upper gastro-intestinal endoscopy, video capsule endoscopy[21] and/or double-balloon enteroscopy[22]. This diagnosis was confirmed according to the WHO classification of tumours of haematopoietic and lymphoid tissues.[23] WHO performance status[24] had to be ≤2, and no severe concomitant cardiac, pulmonary, renal or hepatic disease was to be present. Active uncontrolled infection and HIV positivity were also exclusion criteria.

Peripheral blood stem cells mobilisation, conditioning and au-SCT

Mobilisation of haematopoietic progenitor cells from the bone marrow into the peripheral blood was achieved using granulocyte colony-stimulating factor (G-CSF) 2x10 μg/kg by

s.c. injection daily for at least four days without preceding chemotherapy. The minimum amount of CD34+ cells collected was $2x10^6$ per kg. The conditioning regimen consisted of fludarabine administered orally for five days (40 mg/m^2 per day) and an intermediate dose melphalan (administered i.v., 2 days, 70 mg/m^2 per day), as shown in Figure 1. At day 0 stem cells were reinfused. The purpose of this conditioning regimen was both intensive T-cell depletion and myeloablation by a purine analog added to melphalan (total dose 140 mg/m^2). All patients received standard antibacterial and antifungal prophylaxis during neutropenia and trimethoprim-sulfamethoxazole gluten-free syrup 480-960 mg daily until six months after transplantation. Total parenteral nutrition and blood and platelet transfusions were given if indicated.

Follow-up and criteria of response

Before, during and after au-SCT, a clinical assessment was carried out noting in particular signs and symptoms of malabsorption. Body mass index (BMI), WHO performance status, and the need for transfusions, for additional nutritional support and for additional antimicrobial treatment were documented. Clinical response is defined as improvement of diarrhoea and constant or improved WHO performance status, combined with at least two out of the following clinical parameters within the normal range or an improvement of ≥1 point: 1) BMI, 2) albumin and 3) Hb. Multiple duodenal biopsies were taken by upper gastro-intestinal endoscopy to detect histopathological abnormalities and to perform IELs immunophenotyping by flowcytometry analysis[7, 25, 26] at different time points (3, 12 and 24 months) after au-SCT. Isolation of small intestinal IELs and staining for immuno-phenotyping were performed as previously described.[7] Complete histological response is defined as a normalisation of the architecture of the small intestinal mucosa, classified as Marsh 0 or I lesion according to the modified Marsh criteria.[27] A decline of >20% in the percentage of aberrant IELs was considered a significant immunological response. In addition, overall survival and the EATL occurrence were evaluated during follow-up.

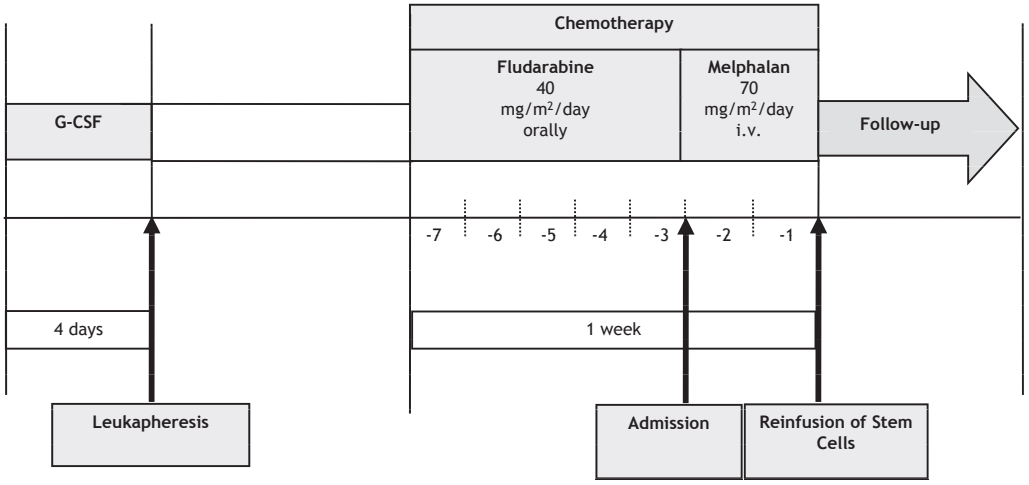
Statistical analysis

Quantitative data were expressed as medians. Kaplan-Meier survival curves were constructed using SPSS software (SPSS Inc. Chicago, Illinois, USA).

Ethics approval and informed consent

Approval of the medical ethics committee was obtained, and all treated patients signed an informed consent in accordance with the Declaration of Helsinki.

Figure 1 | Conditioning regimen before au-SCT.



RESULTS

In total, 18 RCD II patients (Table 1) who were refractory to cladribine therapy were eligible for au-SCT. The median time between cladribine treatment and au-SCT was 6.25 months. All patients entered the treatment protocol, however five patients did not make it to au-SCT (Table 2): two due to unsuccessful leukapheresis and in three patients progression into EATL occurred before stem cells could be collected, as depicted in Figure 2. The patients reaching actual transplant (n=13) had a median follow-up time of >2 years, ranging from 10 up to 67 months.

Survival and disease status

Out of the 18 patients unresponsive to cladribine, eight patients died (44%), five of them due to EATL (28%). If au-SCT was not reached for any reason, mortality in the non-transplanted cohort was high; all patients died within a median follow-up of 5.5 months (range 1-12.5). In the transplanted group, however, 23% (3/13) of the patients died. There was one transplant-related death due to septicaemia with subsequent meningitis. The other two patients died of chronic encephalitis and EATL, complications associated with CD rather than transplantation. Interestingly, in the latter patient EATL occurred four years after transplantation (Figure 2). All five patients in whom EATL occurred, died, with a mean survival of 2 months (range 0-8) after diagnosis. The overall 3- and 4-year survival after undergoing au-SCT in case of unresponsiveness to cladribine therapy is 80% and 66% respectively (Figure 3).

Overall clinical, histological and immunological outcome is depicted in Table 3. All transplanted patients reached follow-up of almost one year to assess remission status. Within one year after au-SCT, the majority of patients (11/13) showed impressive clinical improvement with normalisation of stool frequency, disappearance of gastro-intestinal

symptoms, and normal levels of or improvement of ≥1 point in BMI, albumin and/or Hb. All patients had a WHO performance status of 0 at the end of follow-up. In addition, improvement of BMI was documented from a median level of 20,1 kg/m^2 at baseline to 22.5 kg/m^2 after au-SCT. The median serum albumin level increased from 36 to 42g/L (Table 3).

In total, 38% (5/13) had a complete histological response, defined as Marsh 0 or I. Two of them achieved this response within three months, two within six months and one after two years. One patient had a partial response from a Marsh IIIB to Marsh II lesion and two patients already had a Marsh 0 and I lesion before au-SCT. The remaining two patients showed stable and progressive histological abnormalities after au-SCT. The median percentage of immunophenotypically aberrant IELs was 45% before transplantation and 54% at the end of follow-up. Only one patient showed a decrease in the percentage of aberrant T-lymphocytes to within the normal value. The patient showed a clinical and complete histological response as well.

Figure 2 | Flow chart of cladribine-resistant RCD II patients.

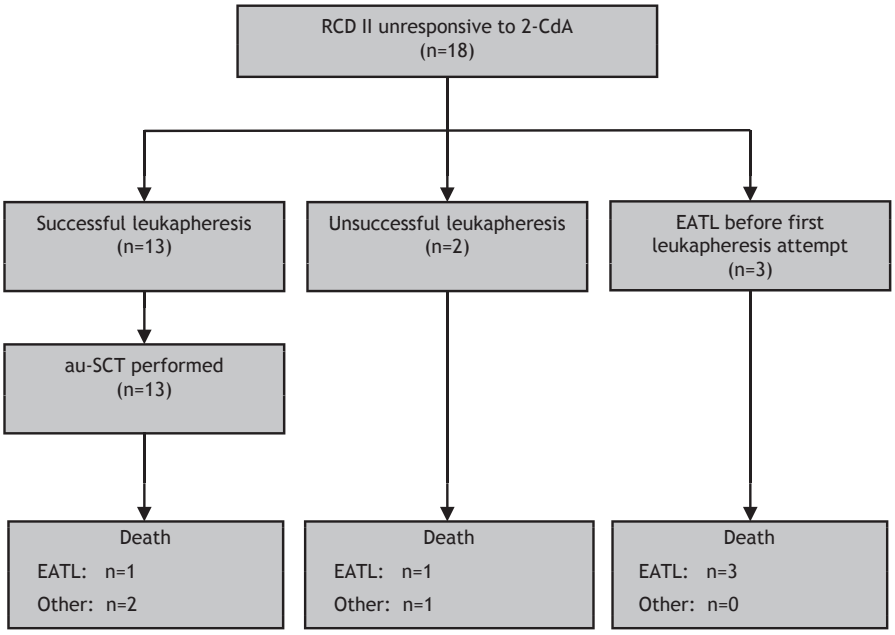


Table 1 | Patient characteristics.

	Transplanted n=1		Not transplanted n=5	
Gender				
Male	7		3	
Female	6		2	
Median age at CD diagnosis (yr), range	50	(37-68)	63	(45-66)
Median age at RCD II diagnosis (yr), range	58	(42-68)	64	(47-70)
Median age at (intention to) au-SCT (yr), range	59	(43-68)	65	(52-70)
Treatment prior to au-SCT				
Cladribine	13		5	
Azathioprine / Prednisone	3		2	
Median time between cladribine and au-SCT (mo), range [1]	6.25	(3-30)		
Median follow-up time (mo), range	26	(10-67)	5.5	(1-12.5)
HLA-DQ status				
DQ2 heterozygous	4		2	
DQ2 homozygous	8		2	
DQ2 and DQ8	1		1	
TCR-γ rearrangement				
Monoclonal	8		4	
Polyclonal	5		1	

Abbreviations: CD, coeliac disease; RCD, refractory coeliac disease.
[1] Due to change in treatment strategy two patients were transplanted two years after cladribine therapy.

Table 2 | Transplant characteristics.

Patients	Duration admission (days)	Re-infusion CD34+ cells ($x10^6$/kg)	Time to neutrophil recovery[1] (days)	Time to platelet recovery[2] (days)	TPN	Platelet transfusion	Red blood cells transfusion
1	19	2.09	12	11	Yes	1x	3x
2	17	2.21	12	11	Yes	2x	6x
3	14	2.06	12	12	Yes	1x	No
4	24	2.00	17	27	No	No	No
5	16	2.26	7	7	No	1x	6x
6	29	2.21	18	17	Yes	No	3x
7	23	2.47	19	17	Yes	No	1x
8	24	2.17	13	14	No	1x	6x
9	19	2.96	16	15	No	No	No
10	25	2.40	20	22	No	1x	2x
11	30	3.60	13	8	Yes	No	2x
12	28	3.26	12	9	Yes	No	No
13	19	2.27	14	12	No	1x	No
Median (range)	**23 (14-30)**	**2.26 (2-3.6)**	**13 (7-20)**	**12 (7-27)**			

Abbreviation: TPN, total parenteral nutrition.
[1] Neutrophil recovery: time to achieve a neutrophil count above $0.5x10^9$/L.
[2] Platelet recovery: time to platelet count above $20x10^9$.

Table 3 | Clinical, histological and immunological results before and at the end of follow-up.

Patient	Alive	Clonality	Performance status WHO		BMI (kg/m2)			Albumin (g/L)			Hb (mmol/L)			Marsh score			Aberrant IEL (%)		
			before	after	before	<1 y	>1 y[3]	before	<1 y	>1 y[3]	before	<1 y	>1 y[3]	before	<1 y	>1 y[3]	before	<1 y	>1 y[3]
1	Yes	Oligoclonal	0	0	20.0	25.4	26.7	34	43	40	7.5	7.6	7.4	IIIA	0	0	41	62	65
2	No[1]	Monoclonal	0	0	19.9	24.5	25.5	37	40	25	8.0	8.8	7.4	IIIB	IIIA	I	92	86	37
3	Yes	Monoclonal	0	0	20.8	22.5	22.5	28	34	42	6.3	7.7	8.6	I	0	0	92	91	91
4	Yes	Oligoclonal	0	0	24.5	25.2	29.1	36	29	40	7.1	8.9	9.6	II	0	0	45	6	6
5	Yes	Oligoclonal	1	0	20.1	20.1	20.4	39	42	42	6.6	7.1	7.2	II	0	0	30	28	40
6	Yes	Monoclonal	0	0	19.1	22.7	22.9	29	46	48	7.5	7.8	7.2	IIIB	IIIA	II	55	47	65
7	No[2]	Monoclonal	1	0	21.2	19.1	20.0	41	41	43	6.2	7.4	5.2	0	IIIA	I	1	4	ND
8	Yes	Monoclonal	1	0	21.2	22.3	22.7	27	41	43	6.4	7.4	7.1	0	I	I	20	48	53
9	No	Monoclonal	0	0	23.5	23.5	-	32	44	-	7.9	8.4	-	IIIB	IIIA	-	81	78	-
10	Yes	Oligoclonal	1	0	19.2	18.4	18.4	36	41	44	8.9	7.6	8.9	IIIB	IIIA	IIIA	86	ND	ND
11	Yes	Monoclonal	2	0	17.9	16.8	-	36	36	-	5.2	6.9	-	IIIC	0	-	60	78	-
12	Yes	Oligoclonal	1	0	18.8	20.3	-	26	40	-	6.9	8.2	-	0	0	-	40	48	-
13	Yes	Monoclonal	0	0	20.1	20.1	-	40	47	-	8.6	8.7	-	0	I	-	40	56	-

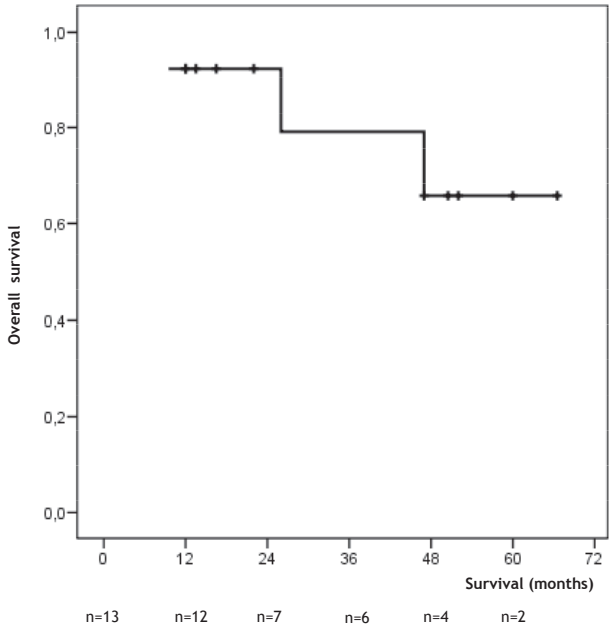
Abbreviations: BMI, body mass index; ND, not determined; WHO, world health organisation.

[1] In this patient progression into EATL occurred.

[2] This patient had persisting ulcerative jejunitis, which is characterised by a low percentage of aberrant IELs detected by flow cytometric analysis.

[3] Results at the end of follow-up, but after at least 1 year.

Figure 3 | Survival curve of RCD II patients unresponsive to cladribine therapy who were treated with high dose chemotherapy followed by au-SCT.



DISCUSSION

Till now there is no standardised treatment strategy for RCD II.[8] The high risk of subsequent progression to EATL with dismal prognosis highlights the need for new treatment strategies. Although treatment with conventional immunosuppressive drugs, including azathioprine and prednisone, may lead to clinical response in several RCD II patients[11, 28, 29], most of them do not have complete histological improvement, do not have a beneficial survival due to progression into EATL[11, 30] and/or become steroid-dependent.[28] Therefore, in our medical centre cladribine rather than azathioprine is prescribed, showing similar clinical improvement, but with better histological response rates without any steroid dependency and need for long-term medication.[31] However, approximately half of the patients is still clinically and histologically unresponsive to this therapy. In our analysis this specific group of RCD II patients refractory to cladribine therapy was scheduled for treatment with high dose chemotherapy followed by au-SCT.

In agreement with our pilot study[16], we report in this larger cohort of RCD II patients that the treatment approach we described is feasible and no serious transplant-related short-term adverse events were noted. Moreover, during extended follow-up no secondary

malignancies and myelodysplastic disorders were observed so far. Treatment-related mortality was not increased compared to other indications for au-SCT.

The 5-year survival reported for RCD II, irrespective of treatment is currently 44-58%.[9, 28, 29] In these series, patients were initially treated with different types of medication, mainly conventional immunosuppressive drugs. In our analysis however, only RCD II patients unresponsive to these treatment options were scheduled for au-SCT, having a 4-year survival of 66% if they manage to proceed transplantation. This survival rate seems promising so far, as this specific group of unresponsive patients has a worse prognosis. In addition, this is supported by the high mortality of the five refractory patients who could not be transplanted.

Four patients were already diagnosed as having overt EATL before first leukapheresis attempt, reflecting the quick development of this condition. The EATL rate observed in the transplanted group (1/13) is much better than that reported in published RCD II series (60-80%)[32], however, in a small series. Even if we include the non-transplanted patients who developed EATL, the EATL rate is less than reported (30%). As one transplanted patient developed an overt EATL only after four years of follow-up, chemotherapy followed by au-SCT might possibly delay the development of this type of lymphoma. Whether progression into EATL is prevented or delayed must be elucidated by more prolonged follow-up.

In addition, in two patients transplantation could not be performed because of failure of stem cell collection, most likely as a consequence of previous administration of cladribine therapy. Both cladribine and fludarabine are purine nucleoside analogues. Although fludarabine-containing regimens are well known for impairing stem cell mobilisation, less is known about the influence of cladribine on mobilisation efficacy.[33] However, it seems from our data that cladribine might indeed influence stem cell collection. A solution might be mobilisation after single-dose cyclophosphamide together with G-CSF and/or implementing plerixafor.[34] This new approach should be evaluated in this patient group.

Our results showed an impressive clinical improvement and enhanced quality of life in almost all patients after transplantation. Approximately half of the patients had a significant recovery of the architectural abnormalities of the small intestinal mucosa. It is intriguing that a high percentage of aberrant IELs persists after au-SCT, particularly in view of the improved Marsh score. These aberrant T-cells reside in the intraepithelial as well as the laminia propria layer.[35] Although this percentage is crucial in the diagnostic work-up for distinguishing RCD types I and II,[7] our data suggest that the percentage of aberrant IELs is not suitable for monitoring therapy and predicting prognosis, at least in this cohort and time of follow-up. In fact, the percentage of aberrant T-cells is not the same as the overall depletion of T-cells after au-SCT. Whether the absolute aberrant T-cell load, which might be quantified by flow cytometric analysis or by quantitative PET scanning, instead of the percentage of aberrant T-cells is more suitable to predict prognosis is not clear yet. If histopathology or another undefined parameter should be used to quantify mass needs to

be investigated as well. Furthermore, clonal expansion of these aberrant IELs is considered to be responsible for progression into an EATL, however in our series the persisting high percentage of aberrant IELs after au-SCT was not reflected in increased EATL development. In addition, the TCR-γ rearrangement performed after au-SCT could not detect a clonal peak with current available technology (data not shown). So far, it is still generally accepted that aberrant T-cells are the factor responsible in EATL development. Thus, further reduction of T-cells or T-cell mass by intensifying conditioning pre-au-SCT might improve outcome. This could be achieved by a higher dose of fludarabine/cladribine combined with anti-CD52 (alemtuzumab), anti-thymocyte globulin or new specific anti-T-cell agents.

Randomised clinical trials comparing conservative follow-up to au-SCT, if unresponsive to cladribine therapy, are not available so far. In the future, multicentre randomised trials or trials with historical control groups will be needed to explore these new treatment strategies.

In conclusion, high dose chemotherapy followed by au-SCT in RCD II patients unresponsive to cladribine therapy is well tolerated and no clinically relevant long-term complications were found so far. Moreover, if the patients manage to proceed au-SCT, an impressive clinical and histological improvement is obtained, and survival rates so far are promising probably because of less progression to EATL.

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CHAPTER 10

Disappointing outcome of allogeneic haematopoietic stem-cell transplantation in two EATL patients

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Enteropathy associated T-cell lymphoma (EATL) is associated with refractory coeliac disease (RCD).[1, 2] Approximately 50% of patients with coeliac disease (CD) is diagnosed at adulthood, of them 2-5% develops RCD. RCD patients comprise two groups: one with immunophenotypically aberrant intraepithelial lymphocytes (RCD II) and one without these cells (RCD I), the cut-off being 20%.[2] Aberrant IELs are characterised by lack of surface expression of CD3, CD4 and CD8. 60-80% of RCD II patients develops EATL within five years.[3] EATL is almost inevitable lethal, although high-dose chemotherapy followed by autologous stem-cell transplantation (au-SCT) might improve survival.[3] Experience with myeloablative or reduced-intensity conditioned (RIC) allogeneic stem-cell transplantation (allo-SCT) in T-cell non-Hodgkin's lymphoma is limited, but promising.[4] We report the first two coeliac patients with EATL in whom we performed allo-RIC-SCT with a HLA-identical sibling donor.

CASE I

A 66 year old man, recently diagnosed with CD, presented with weight loss and night sweats. Abdominal CT-scan showed increased wall thickness of the jejunum and mesenterial lymphadenopathy, suggestive of EATL.[5] Double balloon enteroscopy revealed ulcerative jejunitis and stenosis due to multiple masses. A diagnosis of EATL was confirmed by biopsies. Bone marrow investigation revealed 15% aberrant T-cells (CD3+, CD7-).[2] He underwent a resection of the involved small bowel segment, with primary anastomosis. After surgery there were no signs of a short-bowel syndrome and a strict gluten-free diet (GFD) was started. Post-surgical FDG-PET evaluation showed multiple lymph nodes suspicious of residual EATL. Treatment with six cycles of CHOP chemotherapy was initiated and subsequent FDG-PET showed complete response afterwards.

HLA-typing identified two siblings as fully matched donors. One of them was diagnosed with CD during pre-transplant screening. The other sibling, with a negative coeliac serotype, was therefore selected. At six weeks after the last chemotherapy, allo-RIC-SCT was performed following conditioning with fludarabine (25 mg/m^2) and cyclophosphamide (500 mg/m^2; flu-cy) for five consecutive days. A total of 4.8x10^6 CD34+ cells/kg were infused after conditioning. Donorchimerisms after one and two months were 83% and 90%, respectively.

At two months after allo-SCT the patient developed a relapse of EATL. Rapid deterioration of his clinical condition precluded further treatment and the patient died three months after allo-SCT.

CASE II

A 61 year old man was admitted with perforation of the jejunum. He underwent partial surgical resection of the jejunum. Pathological examination revealed perforation due to EATL (Marsh IIIA). Bone marrow investigation revealed 0.01% aberrant T-cells.

After surgery there were no signs of a short-bowel syndrome and a strict GFD was initiated. Post-surgical FDG-PET evaluation showed intense uptake in the left upper part of the abdomen. There were no other signs of EATL. After six cycles of CHOP chemotherapy FDG-PET showed clinical remission.

The patient was subsequently transplanted with a HLA compatible matched sibling two months after finishing chemotherapy. A total of 8.3 x 10^6 CD34+ cells/kg were infused after conditioning. At one month after transplantation, donorchimerisms were 23% in peripheral blood and 19% in bone marrow.

At six weeks after transplantation the patient developed non-specific abdominal complaints and a rise of the lactate dehydrogenase. As relapse was suspected, immunosuppressive medication was discontinued. After two weeks, relapse of EATL was confirmed. His condition deteriorated quickly and nine weeks after allo-SCT the patient died.

DISCUSSION

To date, treatment of EATL is rather disappointing. Recently, several studies with high-dose chemotherapy and au-SCT have been published. These regimens showed improving results, but only in selected patients. Bishton and Haynes[3] illustrated the beneficial effect of high-dose chemotherapy in six patients. In total, four patients are still alive after two till four years follow-up. Other reports, however, showed less favourable results.[6-8] New strategies are therefore urgently needed.

Theoretically, allo-SCT might be a treatment option for patients diagnosed with EATL. In a prospective, multicentre phase II trial, a total of 170 elderly (≥45 years) patients with relapsed or refractory lymphomas of all types (including 14% aggressive T-cell non-Hodgkin's lymphoma) received allo-RIC-SCT from HLA-identical sibling donors. This procedure was found to be feasible and effective. A trend towards a higher relapse rate was observed in patients with aggressive lymphoma in partial response or with refractory disease before transplantation. Withdrawal of cyclosporine contributed to a clinical response. Median time to progression was six months.[9] In an earlier study a poor outcome of allo-RIC-SCT in patients with chemoresistant and high-grade lymphomas (including T-cell non-Hodgkin's lymphoma) was reported.[10] Although this retrospective study failed to show an effect of intensity of conditioning, it was hypothesised that these patients benefited less from allo-SCT, as the beneficial effect of graft versus lymphoma (GvL) takes a few months to develop.

Donor lymphocyte infusion was successful, illustrating a GvL effect.[10]

We decided to offer allo-RIC-SCT to chemosensitive patients in clinical response using our flu-cy conditioning scheme. As HLA-DQ2 and DQ8 are associated with CD, the donor search needed special consideration to exclude donors with CD by serological screening.

The two cases presented are the first EATL patients treated with allo-RIC-SCT. The experimental setting led to the selection of HLA-identical siblings only. Although both patients were chemosensitive and in clinical response before transplantation, they developed relapse of EATL within a few weeks after transplantation. As a GvL effect could not have occurred, the conditioning probably was not sufficiently potent to buy time.

Possibly more intensive consolidation, for instance the Bishton schedule, is necessary to improve current results in this highly aggressive lymphoma.[3] Moreover, it may be that introducing GvL in an earlier stage, established by more rapid exclusion of immunosuppressive medication, might improve results.

In conclusion, this report of allo-RIC-SCT in patients with EATL and CD illustrates its feasibility, but also disappointing results in two patients in clinical response in excellent condition pre-transplant. It is possible that more intensive consolidation is needed to improve results.

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PART IV

GENERAL DISCUSSION & DUTCH SUMMARY

CHAPTER 11

General discussion and future perspectives

TOEKOMSTPERSPECTIEF

Een beeld schetsen
van de wereld om je heen
kan niet zonder
(gebruik te maken van)
perspectief.

Dat leidt tot (denk)beelden
die naast elkaar blijven bestaan
zonder kans op convergeren.

In toekomstperspectief gezien,
kan dit tot niets anders leiden
dan zonder inzicht te (ver)dwalen.

Pas na divergeren
is er weer (uit)zicht.
Een beter en breder
toekomstperspectief!

Jan Tack

GENERAL DISCUSSION

Growing insight into the clinical presentation of coeliac disease (CD) has resulted in novel diagnostic, prognostic and therapeutic dilemmas. **Chapter one** provides an overview of the latest trends in epidemiology, clinical presentation, diagnosis, complications and treatment with respect to the spectrum of CD. Since the recognition of its broad clinical spectrum, especially adult-onset CD and complications of CD are a matter of debate, in particular refractory coeliac disease (RCD) and enteropathy associated T-cell lymphoma (EATL), which are recognised as relevant clinical entities since more than two decades. As a subset of these patients has a poor prognosis, it is of utmost importance to accurately differentiate between uncomplicated and complicated forms of CD in order to enable early intervention. Despite increased understanding during recent years, the underlying pathophysiology is not completely understood, some aspects of the diagnostic work-up are still under debate, a standardised treatment protocol is lacking and parameters for predicting and monitoring these complicated stages of CD are not yet available. This thesis provides novel insight in the diagnostic aspects and therapeutic options in CD and its complicated stages.

PART I: DIAGNOSTIC ASPECTS

Serum parameters beyond standard antibody testing

To date, small intestinal biopsies obtained by gastro-duodenal endoscopy are still required in the diagnostic work-up and follow-up of uncomplicated as well as complicated CD. The histopathological observation of villous atrophy in florid CD is well appreciated. To date, small intestinal biopsies obtained by gastro-duodenal endoscopy are still required in the diagnostic work-up and follow-up of uncomplicated as well as complicated CD in adults. The recently published revised ESPGHAN (European Society for Paediatric Gastroenterology Hepatology and Nutrition) guidelines for the diagnosis of coeliac disease in children mention the option for omitting duodenal biopsies and histology if certain conditions are fulfilled.[1] But, the guidelines for adult patients have not been revised yet. The histopathological observation of villous atrophy in florid CD is well appreciated. However, the behaviour of the enterocyte itself in adult-onset CD has not yet been investigated. As mucosal villous atrophy is the hallmark of both active CD and refractory CD, histopathological classification according to the modified Marsh criteria[2] is of no use to distinguish between these stages of CD. Although CD associated antibodies are valuable for diagnosing active CD and monitoring response to a gluten-free diet (GFD), these antibodies are within their reference range in complicated CD and therefore of no use in predicting as well as monitoring RCD and EATL.

Chapter two shows that enterocyte destruction determined by serum intestinal fatty-acid binding protein (I-FABP) levels is evidently increased in adult CD patients at time of diagnosis as compared to healthy controls. I-FABP levels correlate with both villous atrophy and IgA-tTG titres and decline after initiation of a GFD. In contrast to serum I-FABP levels in

childhood CD that rapidly decrease after initiation of a GFD[3], low grade enterocyte damage, detected by serum I-FABP levels, persists in adults upon long-term adherence to a GFD, despite the lack of villous atrophy and detectable IgA-tTGA levels in the majority of cases. These findings are in keeping with the slow and incomplete mucosal recovery observed in a substantial number of adult-onset CD patients upon a GFD with subsequent normalised serology and disappearance of symptoms.[4-6] Moreover, serum I-FABP levels correlate clearly with the degree of villous atrophy observed in patients adhering to a GFD. So far, however, it is unknown whether the observed persisting enterocyte damage in adults may indicate non-compliance or unintentional gluten intake, despite evaluation by a specialised dietitian, or a manifestation of the autoimmune disease itself. Taken together, our findings support the currently changing opinion towards the clinical aspects of adult CD. Although the diagnosis of CD in adult patients proceeds along the same lines as that of younger patients, it appears to be more complex due to its initial presentation with less prominent symptoms of impaired intestinal absorption, with signs of micronutrient deficiencies, other autoimmune diseases or even with malignancies.[7] Furthermore, as mucosal recovery may be slow in adult patients diagnosed with CD, closely monitoring clinical response and possible complications, including implications of long-lasting malabsorption, development of autoimmune diseases, refractory CD and (intestinal) malignancies, appears to be important. I-FABP clearly represents a potential marker for monitoring a GFD to detect ongoing mucosal abnormalities without the need to perform a gastro-duodenal endoscopy. However, larger prospective studies are mandatory to elucidate the specific value of serum I-FABP in diagnosis and follow-up. Currently, serum I-FABP levels in the complicated forms of CD are under investigation. Preliminary data show no differences in the severity of enterocyte damage between active CD and RCD I, II as well as EATL. In addition, it might be of interest to investigate the presence of IgA-tTG deposits in the small intestine and other (serum) markers indicating intestinal integrity in complicated CD.

Chapter three provides further insight in the ability of several immunological and biochemical markers in the peripheral blood to distinguish the various CD subsets at time of diagnosis. The gluten-induced inflammation in active CD and the gluten-independent inflammation in RCD I/II show resemblance based on the serum levels of IL-8, IL-17, IL-22 and sCD25 as well as the biochemical inflammatory parameters CRP, ESR and leukocyte count. RCD II differentiates itself by elevated serum IL-6 and granzyme-B concentrations. Furthermore, the clearly elevated levels of IL-8 and sCD25 found in both RCD subsets over the GFD group, suggest that these parameters might be helpful in monitoring the inflammatory disease status and in differentiating between these groups in ongoing research and daily clinical practice. In addition, serum IL-6 and albumin levels might be potentially useful to identify patients with complicated CD, as elevated IL-6 levels are a distinctive feature of both RCD II and EATL, and lower levels of albumin were observed in RCD I/II and EATL.

Whether RCD type I and II are two related disease entities or two independent conditions is still under debate. Our results show that both types share similar inflammatory characteristics and T-cell activation status. In addition, progression of RCD I into both RCD II[8] and EATL[9] has been observed, although only sporadically. However, a substantial aberrant IEL population[10] displaying a cytotoxic profile is the specific hallmark of RCD II. Increased serum levels of soluble granzyme-B and the high expression of intracellular granzyme-B in aberrant IELs (*chapter four*) were observed. Although group-wise trends can be appreciated, at least at the single patient level, so far no serum parameter appears sufficiently sensitive and specific for differentiating RCD type I and II or predicting transition into EATL, including those analysed in the present thesis. Hence, it follows that this is an area that needs further study.

Origin and immunophenotype of aberrant IELs in RCD II

The aberrant IEL population observed in RCD II lacks expression of the T-cell lineage-specific surface CD3-TCR-complex, but expresses the cytoplasmatic CD3 antigens and displays T-cell receptor rearrangements[10, 11] indicative of T-cell lineage commitment. These cells are considered a premalignant cell population from which aggressive EATL evolves in half of the cases.[11, 12] However, currently the cellular origin of these cells is unclear and there are no histopathological or immunophenotypic features identified that have a prognostic value in the evolution of aberrant IELs into an EATL. It has been hypothesised that they derive from mature TCR+ IELs, in particular gamma-delta T-lymphocytes[11, 13, 14], or from CD3-CD7+ NK/T-cell precursor cells found in the small intestine of healthy individuals possibly in consequence of the ongoing extrathymic maturation of T-cells in the intestinal mucosa throughout life.[15, 16] Following this, **chapter four** reveals a heterogeneous TCR-gene rearrangement pattern and a lack of expression of the early T-cell development-associated markers TdT, CD1alpha and CD34 in RCD II duodenal biopsies. This strongly suggests that the aberrant IEL population originates from a monoclonal expansion of partly matured T-cells that have deranged in their development before reaching full maturity. In contrast, Schmitz et al.[17] showed that three RCD II cell lines expressed several activation receptors and NK-associated genes, including KIRs, granzyme-H and NKG2D. Interestingly, in our study only those patients harbouring the most mature aberrant IEL population, based on an almost completed TCR rearrangement, developed an EATL. It is tempting to speculate that the presence of aberrant IEL populations with different levels of maturity is, at least in part, an explanation for the clinical observation that only half of the RCD II patients eventually develops an EATL. The observed decreased PCNA expression in aberrant IELs might indicate an impaired DNA-mismatch repair system[18, 19] and may consequently facilitate further transformation of these aberrant cells. Further research addressing the cause and consequences of this abnormal IEL development is definitely warranted.

As some RCD II patients display polyclonal TCR-gamma, yet clonal TCR-beta or TCR-delta rearrangements, we suggest TCR-beta gene rearrangement analysis as the most valuable of available rearrangement analyses in the diagnostic work-up of RCD. This recommendation is supported by a recent publication by Perfetti et al.[20] In addition, these results confirmed our previous findings that clonality analysis of only the TCR-gamma gene rearrangement misses monoclonal populations at risk for EATL. In contrast to immunophenotypical identification of aberrant populations.[10] Furthermore, as aberrant IELs are not strictly confined to the small intestine but are also observed in other, extra-intestinal organs[21], it is of interest to clarify their dissemination pattern in order to find a lead for novel therapeutic options.

Phenotypic and genomic characteristics of EATL

Currently, the immunophenotype of EATL determined by immunohistochemistry plays a pivotal role in its diagnostic work-up, showing expression of CD3, CD7 and CD103, but lack of expression of CD4, CD5 and CD8 in the majority of cases. Furthermore, type 1 EATL usually exhibits a cytotoxic immunophenotype, characterised by expression of CD30, TIA-1, perforin and granzyme-B.[22-24] **Chapter five** reports on an in-depth flow cytometric analysis of the immunophenotype and analysis of genomic alterations of EATL cells primarily presenting as leukemic ascites. The immunophenotype of this EATL is substantially different from the corresponding aberrant T-lymphocytes found in RCD II. First, the absence of the T-cell lineage associated markers CD2, CD7, CD52 and CD103, indicating further transformation. Second, the lack of CD103 and LFA-1 expression that may have contributed to migration of these cells outside the gastro-intestinal tract. Importantly, lack of CD7 and CD103 expression on EATL has not been reported so far and is not included in the current WHO classification of haematological malignancies.[24] Third, compared to aberrant IELs in RCDII *(chapter four)* an extensive proliferative activity indicated by high expression levels of Ki-67 and PCNA was found. Furthermore, the observed expression of CD25 on the EATL cells was in agreement with previous work indicating that sCD25 is a sensitive marker for tumour burden in some lymphomas.[25] However this could not be confirmed by the previously described study in *chapter three* (the serum levels of sCD25 did not significantly differ between EATL and RCD). Moreover, in keeping with the evidently increased serum levels of IL-6 in EATL as compared RCD I/II *(chapter three)*, IL-6 was clearly detected in the leukemic ascites, illustrative for the local pro-inflammatory environment.

In agreement with limited studies on the karyotype of aberrant IELs in RCD, we observed a gain in chromosome 9q and trisomy of chromosome 1, previously suggested as an early and late event of EATL transformation, respectively.[26] The current WHO classification postulates a 9q gain or a 16q loss responsible for inducing transition from RCD into EATL, and gains of 1q/5q and 8q distinguishing between most cases of type 1 and 2 EATL, respectively.[24] In contrast, we found both 1q and 8q gains, suggesting an increased level of chromosomal instability. Further insight into the mechanisms responsible for the development of EATL

may open new doors for early recognition and treatment of EATL. Extended genetic analysis including karyotyping and array CGH as well as functional and expression profiling of small intestinal aberrant IELs as compared to their normal counter parts, could be supportive in the search for new, more sensitive markers to predict the occurrence of EATL in an early stage.

PART II: THERAPEUTIC OPTIONS

Coeliac disease: AN-PEP

The only currently available treatment for CD consists of life-long dietary exclusion of gluten[27], perceived as a substantial burden particularly due to high costs, dietary restriction, reduced social activity, and increased health worries.[28] Alternative treatment modalities that reduce the need of dieting focus on modification of dietary components, enzymatic degradation of gluten, inhibition of intestinal permeability and modulation of the immune response.[29] In line with this, **chapter six** of this thesis describes a randomised double-blind placebo-controlled pilot-study to the gluten-degrading *Aspergillus niger-derived prolyl endoprotease* (AN-PEP) in CD. Although AN-PEP appears to be well tolerated in the majority of coeliac patients, a two week gluten challenge (with placebo) seems to be insufficient to induce a clear clinical response, based on Marsh grade and CD associated antibodies. Consequently, no treatment effects of AN-PEP could be detected. Therefore, further studies with modified study design including a high dose of gluten consumption for an extended period of time with many more patients are warranted to support the potential of AN-PEP in degrading gluten. It might also be interesting to perform a study designed the other way round: small amounts of gluten with AN-PEP or placebo for a much longer period of time. Moreover, in addition to a dietitian inquiry and the CD associated antibodies IgA-tTG, IgA-EMA, and IgA/G-DGA, it might be of interest to search for more sensitive makers to evaluate diet compliance and/or unintentional gluten intake. Recently, Biagi et al[30] presented a simple and rapid questionnaire that verifies compliance to a GFD, however, education by an experienced dietitian remains very important. As suggested previously *(chapter two)*, serum I-FABP levels and IgA-tTG deposits in the small intestine might be of potential use.

RCD I: Tioguanine

Although the prognosis of RCD I is much more favourable than that of RCD II, as reflected in 5-year survival rates of around 90% and 44-58%, respectively[9, 12, 31], treatment is believed to be important in preventing complications of longstanding malabsorption. Apart from a GFD and nutritional support, corticosteroids are the mainstay of treatment in RCD I. Unfortunately there are also snags attached to this therapy, including systemic side effects and corticosteroid dependency in the majority of cases.[9] To reduce corticosteroid dependency and in case of corticosteroid refractoriness, azathioprine has been advocated

with good clinical response rates.[32] Currently, treatment is largely empiric based on small series, showing good clinical response rates, yet histological response is lagging behind. **Chapter seven** reports on the non-conventional thiopurine derivative tioguanine in RCD I showing a good safety profile and clinical and histological response in the majority of cases. Therefore, tioguanine might be a suitable alternative treatment option for RCD I, either as first-line treatment, second-line treatment in patients unresponsive to corticosteroids and/or azathioprine, or as a steroid-sparing drug. In addition, further research is also warranted to establish the optimal treatment duration of tioguanine. In consequence of its rare incidence large multicentre international randomised trials including azathioprine, tioguanine, budesonide and placebo should be performed to establish the validity of each and any treatment option in RCD I.

RCD II: Cladribine and au-SCT

In the past decade several conventional and more experimental therapies have been evaluated in small series of RCD II[9, 31-39], yet, a validated standard treatment of RCD II is lacking. The high risk of progression to EATL with dismal prognosis highlights the need for new treatment strategies. Since 2005 cladribine is the drug of first choice in our medical centre, but patients not responding to cladribine receive high dose chemotherapy with subsequent autologous haematopoietic stem-cell transplantation (au-SCT). **Chapter eight** and **nine** show that our suggested treatment protocol seems to hold promise. Both cladribine therapy and high dose chemotherapy followed by au-SCT were well tolerated. Approximately half of the patients responded well to cladribine, having a significantly higher survival rate compared to those who were unresponsive. The observed 80% clinical response rate was consistent with previous studies[9, 31], hence, the potential advantage of cladribine seemed to be a better histological response, besides its steroid-sparing effect. The median overall survival and 2-year histological response rate of patients treated with up-front cladribine appeared to be superior to patients unresponsive to thiopurines and/or prednisone and subsequently treated with cladribine. Unfortunately progression into EATL could not be fully prevented, but, compared to previous reports showing EATL in 33–52% within 5 years[9, 12], cladribine therapy appeared to result in a lower rate of lymphomagenesis (17%), yet approximately half of the patients had a 5-year follow-up.

Although the overall clinical response rate of cladribine was actually good, with regard to our response criteria (defined as clinical, and complete histological and/or immunological response), for unknown reasons half of RCD II patients were unresponsive. Possibly a higher dose and/or a prolonged treatment schedule might result in a higher response rate. Therefore dose-finding studies need to be conducted. This specific group of unresponsive patients has a worse prognosis. If they manage to proceed high dose chemotherapy followed by au-SCT the observed 4-year survival of 66% seems promising. This is supported by an impressive clinical improvement and enhanced quality of life in almost all patients after transplantation. Approximately half of them showed a significant recovery

of the architectural abnormalities of the small intestinal mucosa. As EATL was observed only after four years of follow-up in one transplanted patient, chemotherapy followed by au-SCT might possibly delay the development of this type of lymphoma. Whether progression into EATL is prevented or delayed must be elucidated by more prolonged follow-up.

Overall, multicentre randomised trials comparing thiopurines and cladribine, and if unresponsive followed by au-SCT, to further explore these treatment strategies is warranted. Ongoing efforts to investigate other new treatment options such as IL-15 blocking antibodies (as IL-15 has a key role in the pathogenesis of RCD) are recommended in order to further decrease morbidity and mortality in this patient group.

EATL: Allo-SCT

EATL is a virtually lethal condition with an overall 5-year survival of less than 20%.[12, 40] To date, treatment of EATL is rather disappointing with a 2-year survival of 28% upon the classic CHOP (cyclophosphamide, doxorubine, vincristine and prednisone) protocol[41] and an estimated median survival of 7.5 months upon CHOP-like or other new chemotherapy regimens.[40, 42, 43] Approximately half of the cases is eligible for chemotherapy due to an advantaged stage at initial presentation, a poor performance status, multifocal involvement of the small intestine and/or complications. In addition, only half of them completes their scheduled chemotherapy.[41, 44] In small series dealing with au-SCT preceded by different induction and conditioning chemotherapy regimens, variable results were reported.[44-47] Patients with an early stage of disease and use of a more aggressive conditioning regimen seemed to benefit most. Following a prospective study of 14 patients, a single course of CHOP with subsequent aggressive regimen IVE/MTX (ifosfamide, etoposide, epirubicin/ methotrexate) followed by au-SCT seemed promising, with a 5-year survival of 60%.[44] An alternative treatment option evaluated in case-reports is Alemtuzumab (anti-CD52) plus chemotherapy, however, so far relapse could not be prevented[48, 49]. We are awaiting the results of a randomised phase III study in The Netherlands to evaluate the effectiveness of Alemtuzumab with 2-weekly CHOP versus 2-weekly CHOP alone and consolidated by au-SCT in patients <60 years of age (www.hovon.nl). As previous reports indicated lower relapse rates in allogenic haematopoietic stem-cell transplantation (allo-SCT) as compared to au-SCT in patients with indolent and high-grade non-Hodgkin lymphomas[50, 51], **chapter ten** reports the first two patients with a type 1 EATL in whom allo-RIC-SCT with a HLA-identical sibling donor was performed. Although both patients were chemosensitive and in clinical remission before transplantation, a relapse of EATL occurred within a few weeks after transplantation. As a graft versus lymphoma effect could not have occurred, the conditioning (fludarabine, cyclophosphamide) probably was not sufficiently potent to buy time. Possibly more intensive consolidation is necessary to improve current results in this highly aggressive lymphoma. Introducing graft versus lymphoma in an earlier stage, established by more rapid exclusion of immunosuppressive medication, might improve results.

FUTURE PERSPECTIVES

This thesis "On the work-up of (refractory) coeliac disease" reveals novel insight into clinical aspects and treatment of the spectrum of CD, yet many areas require further study.

First, the majority of coeliac patients respond well to a GFD, however it still has to be unravelled for what reason some, in particular adult patients, are refractory to a GFD ensuing RCD and/or EATL. In addition, the controversial role of strict adherence to a GFD in the development of RCD and EATL[52-54] has to be further addressed in ongoing research. Following this, further identification of novel serological, immunophenotypic, genetic and/or molecular prognostic parameters for these complicated forms of CD is of utmost importance to decrease morbidity and mortality. To differentiate between RCD II patients who will develop an EATL and those who will not, is the ultimate target. Apart from serum I-FABP levels, other parameters representing intestinal permeability, such as antibodies against food antigens and zonulin levels are included in current investigation. In addition to IL-6, IL-8 and granzyme-B levels *(chapter three)*, cytokine production profiles in the peripheral blood or by intestinal aberrant IELs may be topic of further research. Ongoing immunophenotypic analysis of aberrant IELs as compared to their normal counter parts *(chapter four and five)* involving additional markers representing the NK- or T-cell lineage, dendritic cells, DNA mitotic cell cycle profile, homing receptors and apoptosis are recommended. At the molecular level, TCR-beta gene rearrangement analysis is strongly suggested as an additional marker in the current diagnostic work-up of RCD (*chapter four*), hence larger series are mandatory to validate these data. Investigation of TCR-alpha chain rearrangements may provide further information on the origin of aberrant IELs. Furthermore, it is likely that in addition to MYO9B[55], HLA-DQ2 homozygosity[56] and chromosomal gains of 1q, 9q and 5q[26] *(chapter 5)* or deletion in 16q[24], which are not routinely detected in the diagnostic work-up, other genes are associated with the development of RCD and EATL. International genome-wide association studies, and functional and expression profiling of small intestinal aberrant IELs in complicated CD may shed new lights on this matter.

Second, RCD II is a commonly accepted clinical disease entity with specific characteristics, whereas the role of RCD I within the spectrum of CD sill remains under debate. Do RCD I and II refer to a sliding scale within the CD spectrum, or does RCD I resemble florid CD in consequence of long-term unintentional gluten intake which could not be detected by currently available methods? Or does it represent an independent disease entity showing great similarity to CD?

Third, as aberrant IEls are not strictly confined to the small intestine[21] and can even be found outside the gastro-intestinal tract at initial presentation *(chapter five)*, analysis of homing receptors and genetic profiling will largely contribute to our understanding of their dissemination pattern and may provide a lead for novel therapeutic options. In addition, it might be interesting to investigate aberrant IELs in the stomach and colon as well.

Fourth, a GFD remains the only effective treatment for CD despite alternative treatment modalities, including enzymatic degradation of gluten by AN-PEP *(chapter six)*, therefore new clinical trials are warranted. Several therapeutic options for RCD and EATL have been investigated. However treatment remains challenging due to the fact that EATL development could not be fully prevented in RCD II *(chapter eight and nine)* and EATL itself still has a dismal prognosis despite treatment *(chapter ten)*. In addition to novel treatment options such as blocking IL-15 or CD30 based on their involvement in the pathogenesis of RCD and EATL, respectively, evaluation of the most commonly used therapies in these complicated forms of CD in large randomised trials is mandatory (i.e. azathioprine, budesonide and tioguanine in RCD I; azathioprine and cladribine in RCD II; CHOP with alemtuzumab in EATL). As the low incidence of RCD and EATL hampers the development of such trials, improvement of therapeutic strategies requires international collaboration with consensus about diagnostic criteria, and clinical and histologic response definition during follow-up.

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CHAPTER 12

Nederlandse samenvatting
(Dutch summary)

TOEWERKEN NAAR DOELTREFFENDER DIAGNOSE EN BEHANDELING VAN (REFRACTAIRE) COELIAKIE

In **hoofdstuk 1** van dit proefschrift wordt een algemene beschrijving van coeliakie gegeven, inclusief epidemiologie, diagnose, behandeling en complicaties.

COELIAKIE

Coeliakie is een chronische darmaandoening gekenmerkt door glutenintolerantie bij mensen met een bepaalde genetische aanleg. Gluten is een eiwit dat in tarwe, rogge en gerst voorkomt. De aandoening wordt veroorzaakt door een reactie van het afweersysteem met vorming van antistoffen tegen gluten waardoor de darm ontstoken raakt en de slijmlaagcellen (enterocyten) afsterven (vlokatrofie) met als gevolg een verminderde absorptie van voedingsstoffen. Naar schatting treft deze auto-immuun ziekte een op de honderd mensen in de westerse wereld, de meesten zonder diagnose. Van oudsher is coeliakie een kinderziekte met klassieke maag-darmklachten: diarree, vette plakkerige ontlasting en gewichtsverlies. De laatste twee decennia wordt coeliakie in toenemende mate vastgesteld bij adolescenten en volwassenen met een uiterst divers klachtenpatroon, variërend van bovengenoemde symptomen tot atypische verschijnselen zoals bloedarmoede, botontkalking, blaasjesvormende huidaandoening (dermatitis herpetiformis) en gedragsveranderingen. Voor het stellen van de diagnose is een maag-darm onderzoek met afname van weefsel uit de dunne darm tot op heden de gouden standaard. Het aantonen van antistoffen in het bloed ondersteunt de diagnose.

Alhoewel er steeds meer bekend wordt over de ontstaanswijze van coeliakie, is er nog weinig onderzoek verricht naar schade van de enterocyten zelf in het ziekteverloop. In **hoofdstuk 2** van dit proefschrift wordt dit bij volwassenen met coeliakie onderzocht met behulp van I-FABP (intestinal fatty-acid binding protein) concentraties in het bloed. Zoals verwacht worden er bij het stellen van de diagnose coeliakie hogere I-FABP waarden gevonden dan in het bloed van mensen zonder deze diagnose. Van grotere betekenis is de correlatie tussen de gemeten I-FABP waarden enerzijds en de antistof concentraties in het bloed en de mate van vlokatrofie in weefsel uit de dunne darm anderzijds. Na behandeling met een glutenvrij dieet dalen de I-FABP waarden sterk. Opvallend genoeg niet tot de waarden bij niet-coeliaken, ondanks normalisatie van antistoffen en het verdwijnen van vlokatrofie bij de meerderheid van de patiënten. Ook tijdens langdurige follow-up lijken de I-FABP waarden te correleren met de mate van vlokatrofie. Het is onduidelijk of deze persisterende enterocytschade het gevolg is van ‘therapieontrouw’, onbedoelde gluten-inname of de ziekte coeliakie zelf. Mogelijk kan I-FABP in de toekomst gebruikt worden voor de evaluatie van een glutenvrij dieet. Grote prospectieve studies zijn nodig om dit vast te stellen.

De enige doeltreffende behandeling van coeliakie is het verwijderen van gluten uit de voeding, maar een dergelijk dieet is lastig vol te houden door hoge kosten, gluten contaminatie van voedingsmiddelen, sociaal-culturele aspecten en beperkte beschikbaarheid van alternatieve glutenvrije producten. Wetenschappelijk onderzoek richt zich derhalve op alternatieven voor een glutenvrij dieet, zoals vaccinatie, neutraliseren van gluten in producten door middel van genetisch gemodificeerde tarwe en orale enzymen die gluten afbreken voordat deze de dunne darm bereiken. In **hoofdstuk 6** wordt een eerste placebo-gecontroleerde studie beschreven naar een dergelijk enzym, namelijk *Aspergillus niger-derived* prolyl endoprotease (AN-PEP). Volwassen coeliaken werd gevraagd gedurende twee weken gluten bevattende crackers met AN-PEP gelei als ontbijt in te nemen, gevolgd door een normaal glutenvrij dieet gedurende de daarop volgende twee weken. In de aansluitende twee weken werden patiënten gerandomiseerd voor inname van gluten bevattende crackers, met een gelei van ANPEP of placebo. AN-PEP werd goed verdragen door de meerderheid van de patiënten. Echter, een periode van twee weken blijkt te kort om symptomen en darmschade (bloedonderzoek en darmbiopten) bij patiënten uit de placebogroep aan te tonen. Derhalve kunnen er geen conclusies over de werking van AN-PEP worden getrokken. Vervolgstudies zijn gewenst, met een langere periode van gluten inname en met patiënten die recent met een glutenvrij dieet zijn gestart.

Bij de meeste jonge patiënten herstelt de darmschade en verdwijnen de klachten met als gevolg een betere kwaliteit van leven. Daarentegen is bij een aanzienlijk percentage van de nieuw gediagnosticeerde volwassen coeliaken het herstel van het darmepitheel traag en onvolledig, ondanks normalisatie van de antistoffen en het verdwijnen van de klachten.

RCD EN EATL

Het op volwassen leeftijd stellen van de diagnose coeliakie, met name boven het 50^{e} levensjaar, is sterk geassocieerd met het ontwikkelen van de complicaties refractaire coeliakie (RCD) en enteropathie geassocieerd T-cel lymfoom (EATL). In tegenstelling tot de groep patiënten met een traag herstel van het darmepitheel, wordt RCD gekenmerkt door persisterende of recidiverende symptomen en voor coeliakie karakteristieke darmschade, ondanks een streng glutenvrij dieet gedurende 12 maanden en normalisatie van de antistoffen. Refractaire coeliakie wordt onderverdeeld in twee typen; op basis van het aankleuren van verschillende ‘celoppervlakte eiwitten’ op specifieke cellen in de dunne darm. Type I met minder dan 20% en type II met meer dan 20% afwijkende cellen. Deze zogenaamde aberrante cellen worden beschouwd als voorstadium van EATL, kanker met een slechte prognose. Bij ongeveer de helft van de RCD type II patiënten ontstaat een EATL.

Alhoewel bepaalde genetische kenmerken zijn geassocieerd met RCD en EATL, zijn er tot nu toe geen testen die de ontwikkeling van deze gecompliceerde vormen van coeliakie kunnen voorspellen. In **hoofdstuk 3** van dit proefschrift worden daarom nieuwe

(immunologische en/of biochemische) parameters in het bloed onderzocht, die kunnen differentiëren tussen de ongecompliceerde en gecompliceerde vormen van coeliakie op het moment van diagnose. Uit dit onderzoek blijkt dat de gluten-geïnduceerde ontsteking in actieve coeliakie veel overeenkomsten heeft met de gluten-onafhankelijke ontstekingsreactie in RCD I/II. Anderzijds onderscheiden RCD II en EATL zich door verhoogde waarden van de ontstekingsparameter IL-6 en worden bij RCD II patiënten hogere waarden van de cytoxische parameter granzyme-B gevonden. Verder worden bij de gecompliceerde vormen van coeliakie evident lagere albumine waarden gevonden ten opzichte van de ongecompliceerde vormen van coeliakie. Er worden geen parameters gevonden die RCD I en RCD II kunnen differentiëren. Vooralsnog blijft voor dit onderscheid het percentage aberrante cellen in dunne darmweefsel van essentieel belang.

Naast nieuwe serologische parameters, kan meer inzicht in de oorspong en karakteristieken van deze aberrante cellen mogelijk richting geven aan het opstellen van een risicoprofiel voor de ontwikkeling van een EATL. In **hoofdstuk 4** wordt in een grote groep RCD II patiënten, middels DNA afkomstig uit dunne darmweefsel en oppervlakte kenmerken van aberrante cellen, de origine van dergelijke cellen onderzocht. Deze aberrante cellen zijn zeer waarschijnlijk door oorsprong T-cellen (cellen betrokken bij de afweer) die ergens in hun ontwikkeling stagneren. Echter, er wordt een grote diversiteit in de mate van rijping van deze cellen tussen de verschillende patiënten waargenomen. Opvallend is dat juist de patiënten met de meest rijpe aberrante populatie een EATL hebben ontwikkeld. Op basis van onze resultaten is het aannemelijk om het standaard (moleculair) onderzoek, tegenwoordig gebruikt in de diagnostiek van RCD, uit te breiden. Op dit moment wordt in de diagnostiek naar RCD alleen de zogenaamde T-cel receptor-gamma bepaald, terwijl ons onderzoek aangeeft dat er ook bij een normale TCR-gamma (niet clonaal) toch een verhoogd risico op een EATL lijkt te bestaan. Deze uitbreiding (TCR-beta) kan er voor zorgen dat een geselecteerde patiëntengroep met een verhoogd risico op een EATL niet wordt gemist.

Ondanks de goede 5-jaars overleving van RCD I (ongeveer 90%), is behandeling uitermate belangrijk om de lange termijn gevolgen van absorptiestoornissen van nutriënten in de darm te voorkomen. Naast een glutenvrij dieet zijn middelen die de ontstekingsreactie in de darm onderdrukken (corticosteroïden, azathioprine) een belangrijk onderdeel in de behandeling. Corticosteroïden hebben bij langdurig gebruik bijwerkingen en de meerderheid van de patiënten blijft afhankelijk van deze medicijnen. Behandeling met azathioprine heeft een goede klinische respons maar volledig herstel van het darmepitheel is er slechts bij een minderheid van de patiënten. **Hoofdstuk 7** evalueert een eerste onderzoek naar de behandeling van tioguanine (ontstekingsremmer) bij 12 RCD I patiënten. Tioguanine werd goed verdragen zonder ernstige bijwerkingen. Bijna alle patiënten vertoonden een klinische respons en bij meer dan de helft herstel van het darmepitheel. Mogelijk is dit medicijn een alternatief voor de behandeling van RCD I

In tegenstelling tot RCD I, is de 5-jaars overleving van RCD II slechts 44% tot 58%. Op dit moment is er geen eenduidige behandeling voor RCD II. In de afgelopen jaren zijn verscheidene behandelingen geëvalueerd, echter een EATL kan (nog) niet worden voorkomen. Anders dan bij RCD I, is behandeling met azathioprine en corticosteroïden niet effectief gebleken voor RCD II. De korte termijn resultaten van cladribine en chemotherapie gevolgd door autologe stamcel transplantatie waren veelbelovend bij kleine aantallen patiënten. **Hoofdstuk 8 en 9** beschrijven de lange termijn resultaten van het vaste behandelschema van RCD II dat sinds 2005 in het VU medisch centrum te Amsterdam wordt gehanteerd: eerst cladribine en bij onvoldoende of het ontbreken van een respons chemotherapie en autologe stamcel transplantatie. Net als in de eerder gepubliceerde korte termijn studies met een beperkt aantal patiënten lijkt deze behandelstrategie veilig. In totaal werden 32 patiënten behandeld met cladribine. Een klinische respons van 80% is vergelijkbaar met eerdere studies waarin verschillende medicamenten werden geëvalueerd. Echter onze resultaten laten een beter herstel van het darmepitheel (ongeveer 50% van de patiënten) zien. Patiënten met een goede respons hadden een significant betere overlevingskans ten opzichte van de patiënten zonder respons. Tevens zijn er aanwijzingen dat patiënten die na diagnose direct met cladribine zijn behandeld een betere overleving hebben dan patienten die niet respondeerden op azathioprine en vervolgens met cladribine zijn behandeld. Alhoewel ongeveer de helft van de patiënten een follow-up duur van 5 jaar heeft, lijkt cladribine behandeling de ontwikkeling van een EATL te voorkomen dan wel uit te stellen. EATL werd gediagnosticeerd bij slechts 17% van de patiënten in vergelijking met 30-50% beschreven in eerdere studies. Achttien patiënten met onvoldoende herstel na cladribine behandeling kwamen in aanmerking voor hoge doses chemotherapie gevolgd door transplantatie. Vijf patiënten konden niet worden getransplanteerd en hadden een mediane overleving van slechts 5.5 maand. De overige dertien getransplanteerde patiënten met een follow-up van ruim twee jaar hadden een 4-jaars overleving van 66%. Klinische respons met verbetering van de kwaliteit van leven werd gezien bij de meerderheid van de patiënten en herstel van het darmepitheel bij iets minder dan de helft van de patiënten. Slechts één patiënt ontwikkelde een EATL, en wel vier jaar na transplantatie. Gezien het ontbreken van grote gerandomiseerde onderzoeken, mede door de kleine aantallen patiënten met RCD, zijn internationale studies wenselijk om de verschillende bestaande behandelopties onderling te vergelijken.

Tot op heden is er geen doeltreffende behandeling voor EATL, waarbij de 5-jaar overlevingskans minder dan 20% is. Meer inzicht in de mechanismen die ten grondslag liggen aan de ontwikkeling van een EATL zal waarschijnlijk leiden tot vroege herkenning van deze aandoening en mogelijk tot de ontwikkeling van nieuwe behandelopties. **Hoofdstuk 5** beschrijft een bijzondere presentatie van een patiënt met een EATL, waarbij de tumorcellen in het buikvocht uitgebreid geanalyseerd zijn voor wat betreft oppervlakte kenmerken en genetische veranderingen. De oppervlakte kenmerken van deze EATL verschillen van

aberrante cellen bij RCD II patienten. De gevonden chromosomale veranderingen komen grotendeels overeen met de huidige classificatie van dit type kanker, aangevuld met enkele tot dusver onbekende veranderingen. Verder onderzoek naar de functionele betekenis van deze en andere genetische veranderingen is noodzakelijk.

Wetenschappelijk onderzoek richt zich verder op nieuwe behandelopties van EATL. **Hoofdstuk 10** beschrijft twee EATL patiënten behandeld met chemotherapie gevolgd door allogene stamcel transplantatie. Hoewel beide patiënten een goede respons op de chemotherapie lieten zien, werd een recidief EATL binnen een paar weken na transplantatie waargenomen. Mogelijk dat intensievere behandeling met chemotherapie en/of immunotherapie voorafgaand aan transplantatie tot betere resultaten zal leiden. Vooralsnog is er geen doelmatige behandeling voor EATL en lijkt het vroegtijdig onderkennen van RCD II van essentieel belang. Daarnaast zal voortschrijdend inzicht in de ontstaanswijze van RCD en EATL in de toekomst aanleiding kunnen zijn tot het ontwikkelen van nieuwe alternatieve behandelopties.

TOEKOMST PERSPECTIEF

Hoewel in dit proefschrift nieuwe aspecten met betrekking tot de diagnostiek en behandeling van het spectrum van coeliakie zijn weergegeven, blijft verder onderzoek noodzakelijk. Het is vooralsnog onduidelijk waarom slechts een deel van de coeliakie patiënten RCD en/of een EATL ontwikkelt. Bovendien is de rol van een strikt glutenvrij dieet hierin controversieel. Nieuwe parameters die een voorspellende waarde hebben bij het identificeren van deze gecompliceerde vormen van coeliakie resulteren mogelijk in afname van morbiditeit en mortaliteit. In tegenstelling tot RCD type II dat een erkend ziektebeeld met specifieke kenmerken is, staat RCD type I binnen het spectrum van coeliakie ter discussie. Een beperkt aantal RCD I patiënten ontwikkelt een RCD type II, dat een nauwe relatie met het spectrum van coeliakie doet vermoeden. Aan de andere kant wordt in de literatuur gesuggereerd dat vanwege de overeenkomsten met actieve coeliakie, RCD I het gevolg is van langdurig onbewust gluten inname, hoewel dit met de huidige testen niet aan te tonen is. Het percentage aberrante cellen heeft een belangrijke plaats in de huidige diagnostiek van RCD II, echter vervolgonderzoek naar de karakteristieken van deze cellen is essentieel voor het verkrijgen van meer inzicht in de ontstaanswijze van RCD II en EATL. Aangezien deze aberrante cellen ook buiten de dunne darm kunnen voorkomen, kan verder onderzoek naar kenmerken van een dergelijke verspreiding een aanzet zijn tot het ontwikkelen van nieuwe behandelopties. Helaas zijn er nog te weinig grote internationale studies die het onderlinge effect van de verschillende behandelopties van RCD I en RCD II vergelijken en bovenal is er een grote vraag naar alternatieve middelen die de ontwikkeling van RCD II naar EATL (kunnen) voorkomen. Vooralsnog blijft een glutenvrij dieet de enige bewezen doeltreffende behandeling van ongecompliceerde coeliakie, maar veelbelovende alternatieve behandelingen zijn in ontwikkeling.

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DANKWOORD

‘It syl heve!’ … ‘It giet oan!’

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LIST OF PUBLICATIONS & CONFERENCE PROCEEDINGS

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Tack GJ, van Wanrooij RL, von Blomberg BM, Amini H, Coupe VM, Bonnet P, Mulder CJ, Schreurs MW.
Serological parameters in the spectrum of coeliac disease: beyond standard antibody testing.
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Poster, International Coeliac Disease Symposium,
Amsterdam, The Netherlands 2009
Allogeneic hematopoietic stem-cell transplantation in two EATL patients

Oral presentation, Dutch Society of gastroenterology (NVGE),
Veldhoven, The Netherlands 2009
Chemotherapy and stem-cell transplantation in refractory coeliac disease type II

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Chicago, USA 2009
Effectiveness of cladribine / au-SCT therapy in refractory coeliac disease type II

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UEGW, London, Great-Britain 2009
Immunophenotyping of small intestinal biopsy specimens by flowcytometry in RCD

Oral presentation, UEGW,
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AN-PEP: a potential new therapeutic for coeliac disease

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New Orleans, USA 2010
Autologous stem-cell transplantation in refractory coeliac disease type II patients unresponsive to cladribine therapy

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6-Tioguanine in refractory coeliac disease type I

Oral presentation, Annual Meeting Dutch Society of Internal Medicine (NIV),
Maastricht, The Netherlands 2011
Adult T-cell leukemia lymphoma

COLOUR FIGURES

Figure 1 | Morphology and immunohistochemical analysis of the ascitic fluid EATL cells.

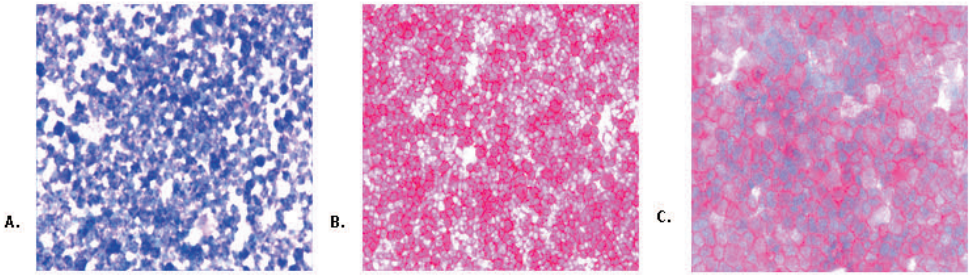
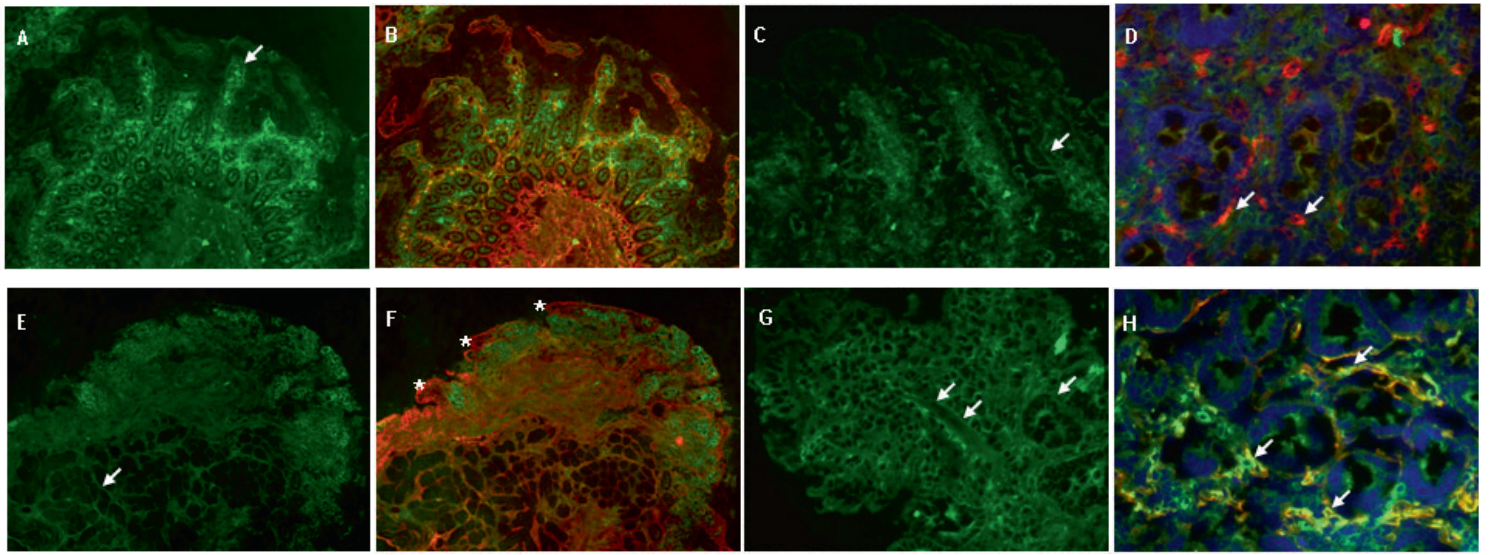


Figure 2 | Small intestinal tissue transglutaminase IgA antibody deposits in two patients; in one patient at baseline (A, B) and after randomisation to AN-PEP (C, D) and in one patient at baseline (E, F) and after randomisation to placebo (G, H).



Baseline evaluation (A, B and E, F) showed preserved villous architecture (asterisks) in both cases. Intense, grade 3 IgA deposition (green) subepithelially and around crypts (A) merge to yellow indicating co-localisation with tTG shown in red (B). IgA deposits diminished after two weeks AN-PEP treatment to grade 1, when only faint and patchy antibody deposition was seen (C, arrow). In the patient on placebo at baseline, faint, grade 1 IgA depositions were seen in the deep mucosal layer around Brunner glands (E, arrow) which were not sufficient to obtain a yellow colour at merging with tTG shown in red (F). IgA deposition increased to grade 2 subepithelially (G, arrows) and to grade 3 in the crypt region (H, arrows) after two weeks placebo. The IgA deposition after a 2-week placebo-treatment (H) co-localised with tTG to intense yellow (arrows) while tTG appeared in red in the AN-PEP-treated patient (D, arrows) in the absence of IgA deposition. The cell nuclei were stained with DAPI in D and H (blue).