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Diagnosing Coeliac Disease

Beyond the Conventional Histology

■

ACADEMIC DISSERTATION
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”We can do anything we want to if we stick to it long enough”

Helen Keller

To my husband Kimmo

ABSTRACT

According to the current criteria the diagnosis of coeliac disease should be based on the presence of small bowel mucosal villous atrophy and crypt hyperplasia; serology has a supportive role. However, the diagnosis is often complicated, since small bowel specimens may be of poor quality and villous atrophy patchy. Moreover, atrophy can also be present in other disorders, and early developing coeliac disease without villous atrophy is particularly difficult to diagnose, since early changes are unspecific. This study focused on improving the quality of coeliac disease diagnosis. The aim was to investigate the diagnostic significance of the determination of villous height-crypt depth ratio (Vh/CrD), the densities of CD3+, αβ+, γδ+ and villous tip intraepithelial lymphocytes (IELs) and serum and intestinal coeliac antibodies in untreated coeliac disease with villous atrophy, in early developing coeliac disease and in treated disease.

In studies **I-IV** altogether 223 patients had untreated and 241 treated coeliac disease or dermatitis herpetiformis. Further, 66 patients were considered to suffer from early developing coeliac disease, and in 608 patients coeliac disease was suspected but excluded because of normal small bowel mucosal villous architecture. Altogether 138 individuals served as non-coeliac controls. In untreated coeliac disease with villous atrophy CD3+ IELs had a 85% sensitivity and 69% specificity for the condition, the corresponding percentages for αβ+ IELs being 80% and 59% and for γδ+ IELs 92% and 81%, respectively. The villous tip IELs determined from haematoxylin- and eosin-stained samples cannot be investigated in untreated coeliac disease cases where total or subtotal villous atrophy is present; but in severe partial villous atrophy, this method nonetheless proved to be more accurate than the determination of CD3+ and αβ+ cells and at least as good as the determination of γδ+ cells, which requires frozen sections. In early developing coeliac disease the sensitivities of all IELs were lower than in coeliac disease with villous atrophy, but those of villous tip and γδ+ IELs (85% and 82%) proved superior to CD3+ and αβ+ IELs (61% and 58%, respectively).

Serum immunoglobulin (Ig) A-class reticulin (ARA) and endomysial (EmA) antibodies proved reliable in disclosing coeliac disease with villous atrophy; the sensitivity of coeliac autoantibodies for the condition was 84%; specificity was 100%. In the second study (**II**), focusing on seronegative coeliac disease, altogether 22 IgA-competent untreated coeliac disease patients out of 177 were found to be serum EmA-negative. The majority of EmA-negative patients were male, and they were found to be on average older than EmA-positive patients. EmA-negative patients suffered from abdominal symptoms more often than EmA-positive, and three EmA-negative subjects were diagnosed with enteropathy-associated T-cell lymphoma at the time when coeliac disease diagnosis was established. The severity of histological lesion was similar in

EmA-negative and –positive patients, but the densities of γδ+ IELs were statistically significantly higher in EmA-positive patients.

The follow-up study (**IV**) focusing on early developing coeliac disease showed that the majority of patients with positive IgA-class coeliac autoantibodies in the serum (ARA or EmA) having normal villous architecture subsequently develop coeliac disease; the sensitivity of coeliac autoantibodies in early developing coeliac disease was shown to be 70%. Thus patients having "false-positive" coeliac autoantibodies in the serum are at risk of developing overt coeliac disease and should be followed up.

Coeliac autoantibodies are produced in the small bowel mucosa, and in this study autoantibody deposits were also investigated from the intestine. All untreated coeliac disease patients (n = 35) with villous atrophy, both EmA-negative and –positive, had intestinal coeliac-type IgA deposits, and these were shown to be gluten-dependent. None of the non-coeliac controls were found to have these intestinal IgA deposits. In the follow-up study (**IV**) focusing on coeliac disease without villous atrophy small bowel mucosal IgA deposits reached 93% sensitivity for early developing coeliac disease, and this method proved superior to earlier utilized approaches such as determination of IELs. IgA deposits detected in the intestine of untreated coeliac disease patients were found in co-localization with transglutaminse 2 (TG2) and the TG2-target specificity of the deposits was ascertained by showing that they had the ability to bind externally added recombinant human TG2.

It emerged that the diagnostic criteria for coeliac disease need to be revised. In other words, it was shown that Vh/CrD determination as the gold standard in the diagnosis is nowadays inadequate; this method failed to recognize 66 (23%) out of 289 untreated coeliac disease patients. These 66 patients were suffering from early developing coeliac disease, where minor mucosal abnormalities may be present in the absence of villous atrophy. However, such changes are difficult and subjective in interpretation. Determination of IELs, especially of γδ+ IELs, is helpful in obscure cases, but these cells are not invariably increased in untreated coeliac disease with villous atrophy. In severe partial villous atrophy the determination of villous tip IELs is at least as valuable as the determination of γδ+ IELs. If early developing coeliac disease is suspected, patients evincing an increased density of γδ+ or villous tip IELs in their small bowel mucosa should be followed up to detect forthcoming coeliac disease, but an increased density of CD3+ or αβ+ IELs alone does not require routine surveillance. Serum coeliac autoantibodies (ARA/EmA) are highly specific for coeliac disease, and IgA-class serum coeliac autoantibodies with normal histology indicate early developing coeliac disease. However, the single most sensitive and specific means of establishing untreated coeliac disease with and without villous atrophy is the investigation of intestinal TG2-specific IgA deposits. The investigation of intestinal IgA deposits seems to be of special value in obscure cases when the serology does not support the diagnosis or conventional histology is ambiguous.

TIIVISTELMÄ

Nykyisten kriteerien mukaan keliakiadiagnoosin tulisi perustua ohutsuolikoepalassa todettuun villusatrofiaan sekä kryptahyperplasiaan; vasta-aineet tukevat diagnoosia. Diagnostiikka on kuitenkin usein ongelmallista, sillä ohutsuolinäytteet voivat olla huonolaatuisia ja villusatrofia läiskittäistä. Lisäksi villusatrofiaa tavataan muidenkin tautien yhteydessä, ja alkavan keliakian muutokset ilman villusatrofiaa ovat erityisen vaikeita diagnosoida, sillä ne ovat epäspesifejä. Tämä tutkimus keskittyi keliakian diagnostiikan parantamiseen. Tavoitteena oli tutkia villuskrypta-suhteen, CD3+, αβ+, γδ+ ja villusten kärkien intraepiteliaalisten lymfosyyttien tiheyden ja seerumin sekä ohutsuolen keliakiavasta-aineiden määrittämisen diagnostista merkitystä hoitamattomassa keliakiassa, jossa on villusatrofia, sekä alkavassa keliakiassa ja hoidetussa taudissa.

Osatöissä **I-IV** yhteensä 223 potilaalla oli hoitamaton ja 241 potilaalla hoidettu keliakia tai dermatitis herpetiformis. Lisäksi 66 potilaalla oli alkava keliakia, ja 608 potilaalla keliakia poissuljettiin epäilyistä huolimatta, sillä ohutsuolikoepaloissa todettiin normaali villusrakenne. Yhteensä 138 potilasta toimi ei-keliakiakontrollina. Hoitamattomassa perinteisessä keliakiassa CD3+ soluilla todettiin 85% sensitiivisyys ja 69% spesifisyys tautiin, ja vastaavat luvut αβ+ soluille olivat 80% ja 59% ja γδ+ soluille 92% ja 81%. Villusten kärkien lymfosyyttitiheys lasketaan hematoksyliinillä ja eosiinilla värjätyistä ohutsuolikoepaloista, ja määritystä ei voida tehdä hoitamattomassa keliakiassa, jossa on todettavissa totaali tai subtotaali villusatrofia. Hoitamattomassa keliakiassa, jossa ohutsuolessa on vaikea partiaalinen villusatrofia tämä metodi todettiin kuitenkin paremmaksi kuin CD3+ ja αβ+ solumääritys ja vähintään yhtä hyväksi kuin γδ+ solumääritys, joka vaatii jääleikkeen. Alkavassa keliakiassa kaikkien intraepiteliaalisten lymfosyyttien sensitiivisyydet olivat matalammat kuin perinteisessä keliakiassa, mutta villusten kärkien lymfosyyttitiheyden ja γδ+ solujen sensitiivisyydet (85% ja 82%) olivat paremmat kuin CD3+ ja αβ+ solujen sensitiivisyydet (61% ja 58%).

Seerumin immunoglobuliini (Ig) A-luokan retikuliini- (ARA) ja endomysiumvasta-aineet (EmA) todettiin luotettaviksi perinteisen keliakian diagnostiikassa; näiden vasta-aineiden sensitiivisyys taudille oli 84% ja spesifisyys 100%. Toisessa osatyössä (**II**), jossa tutkittiin seronegatiivista keliakiaa, yhteensä 22 potilaalla 177 potilaasta seerumin EmA todettiin negatiiviseksi. Suurin osa EmA-negatiivisista keliaakikoista oli miehiä, ja he olivat keskimäärin vanhempia kuin EmA-positiiviset potilaat. EmA-negatiiviset potilaat kärsivät vatsavaivoista useammin kuin EmA-positiiviset, ja kolmella EmA-negatiivisella potilaalla todettiin ohutsuolen lymfooma samaan aikaan kun keliakia diagnosoitiin. Ohutsuolen histologinen vaurio todettiin vaikeusasteeltaan samanlaiseksi EmA-negatiivisilla ja –positiivisilla potilailla, mutta γδ+ solut olivat tilastollisesti merkitsevästi korkeammat EmA-positiivisessa ryhmässä.

Alkavan keliakian seurantatutkimus (**IV**) osoitti, että suurimmalle osalle potilaista, joilla IgA-luokan keliakiavasta-aineet ovat seerumissa koholla (ARA tai EmA) mutta ohutsuolen villusrakenne on normaali, kehittyy myöhemmin keliakia ja villusatrofia; keliakiavasta-aineiden sensitiivisyys alkavassa keliakiassa oli 70%. Näin ollen potilailla, joilla on "vääriä positiivisia" tuloksia seerumin keliakiavasta-aineissa on suurentunut riski keliakian myöhempään kehittymiseen ja näitä potilaita tulisi seurata.

Keliakiavasta-aineet muodostuvat ohutsuolessa, ja tässä tutkimuksessa vasta-ainekertymiä tutkittiin myös ohutsuolesta. Kaikilla hoitamattomilla keliaakikoilla (n = 35), joilla oli villusatrofia, sekä EmA-negatiivisilla että -positiivisilla, oli keliakialle tyypilliset IgA-kertymät todettavissa ohutsuolessa. Lisäksi näiden IgA-kertymien osoitettiin olevan riippuvaisia ravinnon gluteenipitoisuudesta. Yhdelläkään ei-keliakiakontrollilla vastaavanlaisia IgA-kertymiä ei todettu. Alkavan keliakian seurantatutkimuksessa (**IV**) ohutsuolen IgA-kertymien tutkimiselle todettiin 93% sensitiivisyys keliakiassa ilman villusatrofiaa, ja tämä metodi todettiin paremmaksi kuin aikaisemmin käytetyt metodit kuten intraepiteliaalisten lymfosyyttien määrittäminen. IgA-kertymät hoitamattomien keliaakikoiden ohutsuolessa sijaitsivat samalla alueella kuin transglutaminaasi 2 (TG2), ja IgA-kertymien TG2 kohdespesifisyys varmistettiin todistamalla, että kertymät pystyivät sitomaan ulkopuolelta lisättyä ihmisen rekombinantti TG2:ta.

Tämä tutkimus osoitti, että keliakian diagnostiset kriteerit vaativat uudistamista. Tulosten perusteella villuskrypta-suhteen analysointi keliakiadiagnoosin kulmakivenä on nykyään riittämätön; tämä metodi ei tunnistanut 66 (23%) hoitamatonta keliaakikkoa 289:stä. Näillä 66 potilaalla oli alkava keliakia, jossa saattaa olla todettavissa lieviä villusrakenteen muutoksia, vaikka varsinainen villusatrofia ei olekaan kehittynyt. Tällaisia lieviä muutoksia on kuitenkin vaikeita todeta ja lisäksi niiden tulkinta on subjektiivista. Intraepiteliaalisten lymfosyyttien, erityisesti γδ+ solujen, määrittäminen on hyödyllistä epäselvissä tilanteissa, mutta nämä solut eivät ole välttämättä koholla hoitamattomassa perinteisessä keliakiassa. Kun ohutsuolessa on todettavissa vaikea partiaalinen villusatrofia villusten kärkien lymfosyyttitiheyden määrittäminen on vähintään yhtä hyödyllistä kuin γδ+ solujen määrittäminen. Alkavaa keliakiaa epäiltäessä potilaita, joilla on ohutsuolessa normaali villusrakenne, mutta γδ+ tai villusten kärkien lymfosyyttitiheydet ovat koholla, tulisi seurata, jotta myöhemmin kehittyvä keliakia todettaisiin. Sen sijaan CD3+ ja αβ+ solujen lisääntynyt tiheys ainoana löydöksenä ei vaadi rutiiniseurantaa. Seerumin keliakiavasta-aineet (ARA tai EmA) ovat hyvin spesifejä taudille, ja IgA-luokan keliakiavasta-aineiden löytyminen seerumista silloin, kun ohutsuolen histologia on normaali, viittaa alkavaan keliakiaan. Tämän tutkimuksen tulosten perusteella kuitenkin kaikkein sensitiivisin ja spesifisin markkeri hoitamattomasta keliakiasta, ennen ja jälkeen villusatrofian kehittymisen, on ohutsuolen TG2-spesifeiden IgA-kertymien tutkiminen. Näiden ohutsuolen IgA-kertymien tutkiminen vaikuttaisi olevan erityisen luotettava ja käyttökelpoinen metodi epävarmoissa diagnostisissa tilanteissa, kun serologia ei tue diagnoosia tai histologinen löydös on epäselvä.

CONTENTS

ABBREVIATIONS

AGA	gliadin antibodies
APC	antigen presenting cell
ARA	reticulin antibodies
CD	coeliac disease
CI	confidence interval
DH	dermatitis herpetiformis
EATL	enteropathy-associated T cell lymphoma
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibodies
ESPGAN	European Society for Paediatric Gastroenterology and Nutrition
GFD	gluten-free diet
GST	glutathione S-transferase-tagged
HE	haematoxylin and eosin
HLA	human leukocyte antigen
ICAM-1	intracellular adhesion molecule 1
IF	immunofluorescence
IFNγ	interferon γ
IEL	intraepithelial lymphocyte
Ig	immunoglobulin
IL	interleukin
KSCN	potassium thiocyanate
MHC	major histocompatibility complex
MICA	MHC class I chain-related gene A
MMP	matrix metalloproteinase
NK	natural killer
OCT	optimal cutting temperature
PBS	phosphate-buffered saline
TCR	T cell receptor
TG2	transglutaminase 2 or tissue transglutaminase
TGFβ	transforming growth factor-β
Th	helper T-lymphocyte
TNFα	tumour necrosis factor α
U	unit value
Vh/CrD	villous height-crypt depth ratio

LIST OF ORIGINAL PUBLICATIONS

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

I Järvinen TT, Kaukinen K, Laurila K, Kyrönpalo S, Rasmussen M, Mäki M, Korhonen H, Reunala T and Collin P (2003): Intraepithelial lymphocytes in celiac disease. Am J Gastroenterol 98:1332-1337. (Reprinted with permission of Blackwell Publishing)

II Salmi TT, Collin P, Korponay-Szabo IR, Laurila K, Partanen J, Huhtala H, Kiraly R, Lorand L, Reunala T, Mäki M and Kaukinen K (2006): Endomysial antibody-negative coeliac disease: clinical characteristics and intestinal autoantibody deposits. Gut. In press. (Reprinted with permission of the BMJ Publishing Group)

III Järvinen TT, Collin P, Rasmussen M, Kyrönpalo S, Mäki M, Partanen J, Reunala T and Kaukinen K (2004): Villous tip intraepithelial lymphocytes as markers of early-stage coeliac disease. Scand J Gastroenterol 39:428-433. (Reprinted with permission of Taylor & Francis, www.tandf.no/gastro)

IV Salmi TT, Collin P, Järvinen O, Haimila K, Partanen J, Laurila K, Korponay-Szabo IR, Huhtala H, Reunala T, Mäki M and Kaukinen K (2006): Immunoglobulin A autoantibodies against transglutaminase 2 in the small intestinal mucosa predict forthcoming coeliac disease. Aliment Pharmacol Ther 24:541-552. (Reprinted with permission of Blackwell Publishing)

INTRODUCTION

Coeliac disease is a common intestinal disease characterized by permanent intolerance to gluten. Susceptibility to the condition has been shown to be strongly genetic, since about 90% of coeliac disease patients have human leukocyte antigen (HLA) DQ2 haplotype, and most of those remaining HLA DQ8 haplotype (Sollid et al. 1989, Polvi et al. 1996). However, approximately one third of the non-coeliac population in general have DQ2 or DQ8 (Sollid et al. 1989, Polvi et al. 1996). Untreated coeliac disease patients may suffer from various clinical symptoms, but the most common clinical reason for disease suspicion is abdominal complaints (Lo et al. 2003, Zipser et al. 2003). Even though severe malabsorption syndrome and growth failures have constituted typical symptoms in untreated coeliac disease patients in the past (Visakorpi et al. 1967, Young and Pringle 1971, Cooke and Holmes 1984), nowadays many patients suffer from only mild symptoms or even remain asymptomatic (Volta et al. 2001, Mäki et al. 2003). Asymptomatic coeliac disease patients are frequently encountered when screening at-risk groups such as patients with autoimmune thyroid disease (Collin et al. 1994b, Sategna-Guidetti et al. 1998, Larizza et al. 2001), Sjögren's syndrome (Iltanen et al. 1999a, Szodoray et al. 2004), insulin-dependent diabetes mellitus (Collin et al. 2002) or relatives of coeliac disease patients (Mäki et al. 1991b, Hervonen et al. 2002). Serology is an important tool when screening coeliac disease at-risk groups, serum immunoglobulin (Ig) A-class gliadin (AGA), reticulin (ARA), endomysial (EmA) or transglutaminase 2 (TG2) antibodies being elevated in 85-100% of patients in the untreated stage of the disease (McMillan et al. 1991, Ferreira et al. 1992, Ladinser et al. 1994, Sulkanen et al. 1998b).

According to the revised criteria of the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) the diagnosis of coeliac disease should be based on the presence of small bowel mucosal villous atrophy and crypt hyperplasia. Further, a clear-cut clinical, or histological recovery in asymptomatic patients, on a gluten-free diet is required; serology has a supportive role in the diagnosis (Walker-Smith et al. 1990). It has been clearly shown that some patients consuming a normal, gluten-containing diet and having normal villous architecture may later develop manifest mucosal lesion compatible with coeliac disease and thus suffer from latent coeliac disease (Weinstein 1974). It is now recognized that villous atrophy is only the end stage in coeliac disease; the development is initiateted from mucosal inflammation advancing to hypertrophy of the crypts and finally, overt villous atrophy (Marsh 1992). Ferguson and Murray (1971) showed as far back as 1971 that besides

villous atrophy another typical feature of untreated coeliac disease was an increased density of intraepithelial lymphocytes (IELs). It was subsequently shown that even though most IELs are αβ+, a prominent feature in coeliac disease, albeit not pathognomic, is an increased density of γδ+ IELs (Halstensen et al. 1989, Spencer et al. 1989). An increased density of γδ+ IELs was further demonstrated occasionally to precede the development of villous atrophy, and thus to be a marker of latent coeliac disease (Mäki et al. 1991a, Iltanen et al. 1999c).

The only current treatment for coeliac disease is a life-long gluten-free diet where wheat, rye and barley have to be totally excluded. Treatment for coeliac disease is justified, since untreated coeliac disease patients run an increased risk of developing malignancies such as small bowel lymphoma (Cooper et al. 1982, Askling et al. 2002, Green et al. 2003). Also, the majority of untreated coeliac disease patients have reduced bone mineral density (Collin et al. 2002), and the quality of life is decreased (Mustalahti et al. 2002). Histological recovery of the small bowel mucosal lesion, disappearance of clinical symptoms and improvement in bone mineral density (Valdimarsson et al. 1996, Mustalahti et al. 1999) are usually detected in coeliac disease patients adhering to a strict gluten-free diet, and further, the risk of malignancies seems to decrease (Holmes et al. 1989).

The coeliac disease diagnosis faces many challenges today. Small bowel biopsy has its limitations; the diagnosis should be made from well-oriented high-quality samples, while in clinical practice approximately 10% of specimens have in fact proved to be of poor quality (Collin et al. 2005), increasing the risk of false-positive and -negative diagnosis. Occasionally villous atrophy is patchy and seen only in certain parts of the small bowel mucosa (Scott and Losowsky 1976); this type of atrophy can easily be overlooked. Furthermore, it is currently known that patients evincing only minor abnormalities in the small bowel mucosa may already be suffering from clinical symptoms and even complications of coeliac disease (Kaukinen et al. 2001, Tursi and Brandimarte 2003, Paparo et al. 2005). This early developing coeliac disease remains a challenge for clinicians, since minor mucosal changes are unspecific and no reliable markers of this condition have been available.

The purpose of this study was to improve the quality of coeliac disease diagnosis. The special focus was on establishing whether the determination of CD3+, αβ+, γδ+ or villous tip IELs or serum IgA-class antibodies or intestinal IgA deposits is helpful in the diagnosis of untreated disease in borderline cases, where the histology is equivocal, differential diagnostic difficulties arise or early developing coeliac disease is suspected. Further experiments were also carried out to investigate the target specificity of intestinal IgA deposits detected in untreated coeliac disease.

REVIEW OF THE LITERATURE

1. HISTORY

The first detailed clinical description of coeliac disease dates back to 1888, when Gee (1888) published his often quoted article "On the Coeliac affection". The condition was described as a chronic indigestion, affecting mainly children, and the clinical signs were severe steatorrhoea, cachexia and failure to thrive. The author proposed that the only cure, if available, was by means of diet, but like many others afterwards, he failed to recognize the dietary factor behind this chronic condition. It was not until the late 1940s, when the link between coeliac disease and wheat ingestion was established by Dicke (1950), and a wheat-free diet as treatment for the disease was thus discovered. A few years later Dicke, together with van de Kamer and Weyers, showed that the alcohol-soluble gliadin was the toxic component in wheat-gluten and the dietary factor behind the manifestation of coeliac disease (van de Kamer et al. 1953).

The small bowel mucosal abnormalities typical of coeliac disease were first described by Paulley (1954). He studied surgical small bowel biopsy specimens taken from coeliac disease patients undergoing laparotomy and noted broad villi and chronic inflammatory cell infiltrate in the intestinal mucosa. A few years later peroral intestinal biopsy equipment was developed, facilitating the histological diagnosis of coeliac disease based on typical small bowel mucosal villous atrophy and crypt hyperplasia (Shiner 1957).

2. CLASSICAL SYMPTOMS

Even in the 1970s diarrhoea, steatorrhoea, weight loss and malabsorption syndrome were common manifestations of coeliac disease in all age groups (Visakorpi et al. 1967, Young and Pringle 1971, Cooke and Holmes 1984), and in addition, children often suffered from failure of growth (Young and Pringle 1971). In the 1980s some paediatricians reported that the incidence of coeliac disease in children was decreasing (Challacombe and Bayliss 1980, Stevens et al. 1987), but at the same time other investigators noted a shift toward milder symptoms and older age at diagnosis (Mäki et al. 1988, Mäki and Holm 1990). In adults, the typical malabsorption syndrome became rare (Logan et al. 1983) while the proportion of patients recognized due to milder symptoms significantly

increased (Corazza et al. 1993). It was eventually concluded that the incidence of coeliac disease was increasing rather than decreasing (Logan et al. 1983, Pare et al. 1988, Corazza et al. 1993).

Typical presentations of coeliac disease today continue comprise various abdominal complaints (Lo et al. 2003, Zipser et al. 2003), but the number of coeliac disease patients suffering from severe diarrhoea has decreased (Lo et al. 2003). Sometimes indeed the presenting symptom can even be constipation (Bode and Gudmand-Hoyer 1996, Zipser et al. 2003). Anaemia is fairly frequently detected (Bode and Gudmand-Hoyer 1996, Hin et al. 1999, Dickey 2002, Zipser et al. 2003), its aetiology being for example isolated malabsorption of iron, folic acid, or vitamin B12 (Bode and Gudmand-Hoyer 1996, Dickey 2002). Severe malabsorption syndrome is currently rare, while in contrast, some coeliac disease patients are overweight at the time of the diagnosis (Dickey and Bodkin 1998, Zipser et al. 2003).

Dermatitis herpetiformis (DH) is one classical manifestation of coeliac disease. This itching, blistering skin disease was first described by Louis Duhring (1884), but the association between DH and enteropathy was demonstrated much later, in 1966 (Marks et al. 1966). The diagnosis is based on the demonstration by direct immunofluorescence (IF) examination of granular IgA deposits in the dermal papillae in the unaffected part of the skin (van der Meer 1969). Abdominal symptoms are less common in DH than in coeliac disease patients (Reunala et al. 1984), although the majority of DH patients have villous atrophy and crypt hyperplasia in their small bowel mucosa (Reunala et al. 1984, Garioch et al. 1994). A gluten-free diet is the treatment for both, the skin condition and enteropathy, but oral medication with dapsone is often necessary to alleviate the troublesome skin symptoms in the beginning of the dietary treatment (Reunala et al. 1984, Garioch et al. 1994).

3. ATYPICAL SYMPTOMS AND COMPLICATIONS

The conception of the clinical spectrum of coeliac disease has widened, largely due to the introduction of serological screening methods. Nowadays there is a vast body of evidence demonstrating that the manifestations of the disease are not always intestinal; atypical or extraintestinal symptoms most often associated with coeliac disease are presented in Table 1. It is not always obvious whether these associations are true or coincidental findings, and further, occasionally the distinction between atypical symptoms and complications of coeliac disease is ambiguous.

Bone mineral density is often decreased in untreated coeliac disease patients (Collin et al. 2002) and according to recent studies 2.4-3.4% of patients with osteoporosis are affected with coeliac disease (Nuti et al. 2001, Stenson et al. 2005). Reduced bone mineral density increases the risk of fractures (Vazquez et

al. 2000, West et al. 2003), and the majority of such events seem to occur before the diagnosis of coeliac disease or in noncompliant patients (Vazquez et al. 2000). Reduced bone mineralization may also be detected in asymptomatic coeliac disease patients (Mazure et al. 1994, Mustalahti et al. 1999), possibly even preceding the development of villous atrophy occurring in early developing coeliac disease patients (Kaukinen et al. 2001). The mechanisms underlying the disturbances in bone metabolism are still poorly understood, but reduced calcium intake and impaired calcium absorption and secondary hyperparathyroidism, increasing bone turnover, are probably at least partly responsible for impaired bone mineral density (Corazza et al. 1995b, Bernstein and Leslie 2003). Serum calcium levels in untreated coeliac disease patients may be low (Corazza et al. 1995b), but this is not invariably the case even when bone mineral density is reduced (Mazure et al. 1994, Stenson et al. 2005).

The most severe and often fatal complication of coeliac disease is the development of malignancy. Especially the risk of malignant small bowel T-cell lymphoma is increased in untreated coeliac disease (Cooper et al. 1982, Askling et al. 2002, Green et al. 2003); this complication is often associated with delayed diagnosis of coeliac disease or poor dietary compliance. Fortunately nowadays the survival rate among treated coeliac disease patients does not differ from that in the general population (Collin et al. 1994a, Collin et al. 1996a), probably due to the better recognition of the disease, early treatment and good adherence to the gluten-free diet.

Refractory sprue and ulcerative jejunoileitis are non-malignant complications of coeliac disease (Daum et al. 2005). The former is a rare condition characterized by the lack of a histological and clinical response to a gluten-free diet. Patients with ulcerative jejunoileitis have mucosal ulcerations mostly in the jejunum and the condition is likewise characterized by unresponsiveness to a gluten-free diet. Patients with refractory sprue or ulcerative jejunoileitis have a high risk of subsequent small bowel lymphoma, causing increased mortality rates (Daum et al. 2005).

Table 1. *Atypical symptoms of coeliac disease (CD) in adult or adolescent patients*

References	Symptom	Results
(Corazza et al. 1995a, Volta et al. 1997)	Alopecia areata	0.9-1.2% of patients with alopecia areata have CD
(Hadjivassiliou et al. 1996, Luostarinen et al. 2001a)	Ataxia	Approximately 16% of patients with idiopathic ataxia have CD
(Collin et al. 1991, Luostarinen et al. 1999)	Dementia	Case reports of untreated CD patients with early-onset dementia
(Aine et al. 1990, Ballinger et al. 1994)	Dental enamel defects	10-83% of CD patients have systemic dental enamel defects
(Hallert and Derefeldt 1982, Ciacci et al. 1998, Pynnönen et al. 2004)	Depression	19-32% of CD patients suffer from depression or neurotic disorders
(Cronin et al. 1998, Luostarinen et al. 2001b)	Epilepsy	2.3-2.5% of patients with epilepsy have CD
(Regan and DiMagno 1980, Carroccio et al. 1997)	Exocrine pancreatic insufficiency	Exocrine pancreatic impairment can be associated with untreated CD
(O'Grady et al. 1984, Corazza et al. 1999)	Hyposplenism	33-76% of untreated CD patients evince signs of splenic hypofunction
(Volta et al. 1998a, Bardella et al. 1999, Kaukinen et al. 2002)	Liver disorders	Approximately 9% of patients with hypertransaminasaemia of unknown origin have CD. Untreated CD can also be associated with severe liver failure.
(Bourne et al. 1985, Collin et al. 1992)	Mono- or polyarthritis	Nonspecific arthritis can be the prominent symptom of CD
(Hadjivassiliou et al. 1996, Luostarinen et al. 1999)	Polyneuropathy	1.5-5% of patients with peripheral neuropathy have CD
(Ferguson et al. 1976, Ferguson et al. 1980)	Recurrent aphthous ulcerations	4-24% of patients suffering from recurrent aphthous ulcerations have CD
(Farthing et al. 1982, Collin et al. 1996b, Sher and Mayberry 1996, Meloni et al. 1999b)	Reproductive difficulties	4-8% of women suffering from unexplained infertility have CD; untreated CD in females is also associated with unfavourable outcome of pregnancy and in males gonadal function may be reduced

4. SILENT COELIAC DISEASE AND COELIAC DISEASE AT-RISK GROUPS

Silent coeliac disease is characterized by manifest small bowel mucosal lesion in the absence of evident clinical symptoms related to the disease (Ferguson et al. 1993). Nowadays at least one third of untreated coeliac disease patients are virtually asymptomatic (Volta et al. 2001, Mäki et al. 2003). Silent cases have been detected when screening at-risk groups such as first-degree relatives of coeliac disease patients, where approximately 6-11% have the disease (Mäki et al. 1991b, Hervonen et al. 2002).

Particularly close associations have been reported to obtain between coeliac disease and various autoimmune disorders. The prevalence of coeliac disease in insulin-dependent diabetes mellitus has been approximately 4% in several studies (Collin et al. 2002); in autoimmune thyroid disease the prevalence figure for coeliac disease has been 3.3-4.8% in adults (Collin et al. 1994b, Sategna-Guidetti et al. 1998) and 7.8% in children (Larizza et al. 2001). In Sjögren's syndrome the prevalence of coeliac disease has varied from 4.5% (Szodoray et al. 2004) to 14.7% (Iltanen et al. 1999a) and in Addison's disease between 7.9-12.2% (O'Leary et al. 2002, Myhre et al. 2003). Coeliac disease can further be associated with autoimmune hepatitis (Volta et al. 1998b), primary biliary cirrhosis (Floreani et al. 2001) and autoimmune cholestatic disorders (Volta et al. 2002).

Coeliac disease has been shown to co-exist with Down's (Carlsson et al. 1998 , Bonamico et al. 2001a) and Turner's syndrome (Ivarsson et al. 1999b, Bonamico et al. 2002). Further, it has been reported that 1.7-2.6% of coeliac disease patients have selective serum IgA deficiency (Cataldo et al. 1997, Cataldo et al. 1998) and conversely, the prevalence of coeliac disease in IgA-deficient children has been 7.7% (Meini et al. 1996). This association is important to realize, since patients with IgA deficiency remain negative for serum IgA-class coeliac disease antibodies.

5. DIAGNOSTIC CRITERIA

The first diagnostic criteria for coeliac disease were defined at the Interlaken meeting in 1969 by the members of ESPGAN (Meeuwisse 1970). Their statement was based on an international enquiry with 33 respondents and was published in 1970. The permanence of gluten intolerance was introduced for the first time and the diagnosis of the disease should comprise altogether three small bowel biopsies. The first requirement was the finding of subtotal villous atrophy in the small bowel mucosa in a patient consuming a normal gluten-containing diet. Further, clinical and also histological improvement on a gluten-free diet and

finally a recurrence of the typical mucosal lesion after gluten challenge had to be demonstrated.

In 1977, 53 members of ESPGAN completed a questionnaire concerning the practical value of the above-mentioned Interlaken criteria, and answers indicated that occasionally mucosal lesions milder than "flat" were detected at the time of the diagnosis (McNeish et al. 1979). Furthermore, the required deterioration in the histological lesion upon gluten challenge was a matter of debate. The need for a third biopsy was questioned in 1989, and it was suggested that the gluten challenge would be necessary only in atypical or uncertain cases (Guandalini et al. 1989). The importance of coeliac antibodies, discovered a few years earlier, was also addressed. In 1990 the revised criteria for the diagnosis of coeliac disease were introduced (Walker-Smith et al. 1990). It was concluded that the diagnosis can be based on the typical initial finding in the small bowel biopsy specimen together with full clinical and possibly also histological remission, at least in asymptomatic patients, after withdrawal of gluten from the diet. The finding of coeliac antibodies in the serum at time of diagnosis and their disappearance on a gluten-free diet were regarded as supportive in the diagnosis. Further, gluten challenge was encouraged when there were doubts as to the initial diagnosis or the adequacy of the clinical response to a gluten-free diet.

Since the introduction of the revised criteria there has been an ongoing discussion regarding their accuracy. In 1996, at the Seventh International Symposium on Coeliac Disease in Tampere, Finland, the suggested number of small bowel biopsies needed for the diagnosis ranged from zero to three (Walker-Smith 1997). However, it was concluded that two biopsies, before and after a gluten-free diet are ideal, even though the second biopsy confirming the histological recovery was no longer mandatory, in confirming the diagnosis. Regardless of the current approaches in coeliac disease diagnostic methods the diagnostic criteria have not been re-evaluated since 1990.

6. SEROLOGICAL TESTS

The presence of circulating coeliac antibodies during a gluten-containing diet and their disappearance upon adoption of a gluten-free diet strongly supports the diagnosis of coeliac disease (Walker-Smith et al. 1990). Coeliac antibodies are also of great significance when screening for coeliac disease in at-risk groups or in patients with only slight clinical suspicion of the disease. Due to developments in serological methods the awareness of different clinical manifestations and associated disorders of coeliac disease has evolved and antibodies have enabled for example prevalence studies verifying the commonness of this disorder. A wide range of serological approaches are available at the moment: AGA, ARA, EmA and TG2 antibody tests. Serological tests are non-invasive and highly valuable in coeliac diagnostics, but according to the revised ESPGAN criteria the diagnosis cannot be based solely on positive serum coeliac antibodies.

6.1. Gliadin antibodies

Gliadin is the ethanol-soluble fraction of gluten and a toxic factor in wheat protein in coeliac disease. Both IgA- and IgG-class AGA are often found in patients with untreated coeliac disease. Various methods have been introduced to determine these antibodies: IF (Stern et al. 1979), enzyme-linked immunosorbent assay (ELISA) (Vainio et al. 1983), diffusion-in-gel ELISA (Kilander et al. 1983), solid-phase radio-immunoassay (Ciclitira et al. 1983) and a rapid strip ELISA (Not et al. 1993). AGA usually disappears from the circulation during a gluten-free diet and reoccurs after gluten challenge, but sensitivity as well as specificity values of AGA tests have been variable, as shown in Table 2. AGA is found not only in the serum in untreated coeliac disease but also in patients suffering from other gastrointestinal diseases (Stern et al. 1979, Burgin-Wolff et al. 1983) and in healthy controls, among whom positivity for AGA seems to increase with age (Uibo et al. 1993).

Coeliac disease is currently regarded as an autoimmune disorder, and autoantibodies directed against the patient's own tissue material have been identified and are considered highly coeliac disease-specific. Recently the more accurate coeliac autoantibody tests have almost entirely replaced the AGA test.

Table 2. *Sensitivity and specificity values of serum IgA- and IgG-class serum gliadin antibodies (AGA) in untreated coeliac disease*

References	Study populations		IgA-class AGA		IgG-class AGA	
	Patients	Controls	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
(McMillan et al. 1991)	28 adults	68 adults	100	100	57	87
(Ferreira et al. 1992)	21 adults	160 adults	91	85	76	88
(Lerner et al. 1994)	28 children	41 children	52	94	88	92
(Vogelsang et al. 1995)	49 adults	53 adults	82	83	73	74
(Sategna-Guidetti et al. 1995)	100 adults	109 adults	55	100	78	82
(Ascher et al. 1996)	40 children, 15 adults	41 children, 24 adults	91	99	96	69
(Bottaro et al. 1997)	50 children	25 children	92	68	100	36
(Sulkanen et al. 1998a)	92 adults	95 adults	80	86	35	97
(Sulkanen et al. 1998b)	136 children and adults	207 children and adults	85	82	69	73
(Lock et al. 1999)	27 adults	65 adults	93	95	77	91
(Dahele et al. 2001)	53 adults	65 adults	64	85	83	78
(Wolters et al. 2002)	52 children	49 children	83	86	83	80
(Tesei et al. 2003)	250 adults	176 adults	64	92	84	86

6.2. Reticulin, endomysial and transglutaminase 2 antibodies

The serum ARA test, aimed to detect untreated coeliac disease, was introduced in 1971 (Seah et al. 1971). The antigen was detected by a standard indirect IF method using unfixed cryostat sections of rat kidney, liver and stomach as antigens. Five different IF patterns were distinguished (Rizzetto and Doniah 1973), and the R1-type ARA was regarded as specific for coeliac disease (Eade et al. 1977). The sensitivity and specificity values of this test have been variable and its reliability has been questioned; the values in different studies are demonstrated in Table 3. However, it seems that the IgA-class ARA test is reliable provided that experienced and skilful laboratory personnel are used (Hällström 1989).

Coeliac disease patient serum has been shown to react not only with rodent tissues but also with human and other primate tissues. Chorzelski and associates (1984) used monkey oesophagus to test for tissue antibodies in patients with coeliac disease, and named this the EmA test. Later a more feasible and less expensive means of examining EmA using human umbilical cord as substrate was introduced by Ladinser and colleagues (1994). The authors observed that serum samples from untreated coeliac disease patients showed a honeycomb-like fluorescence along the peritubular muscle layers of vessels on umbilical cord staining of the extracellular connective tissue (Ladinser et al. 1994). The human small intestine has also been applied in testing for tissue antibodies, and Karpati and associates (1990) called this test the jejunal antibody test. The correlation between the IgA-class EmA test and the older IgA-class R1-type ARA test has proved to be good in experienced hands (Hällström 1989, Mäki 1995) and the EmA test has proved highly accurate in distinguishing untreated coeliac disease patients from controls, reaching almost 100% specificity in most studies conducted (Table 4). However, some untreated coeliac disease patients remain negative for serum EmA, and it has been proposed that EmA negativity is associated with milder small bowel mucosal lesions that EmA positivity (Rostami et al. 1999, Tursi et al. 2001, Abrams et al. 2004). On the other hand, several studies have indicated that coeliac autoantibodies might be a marker of early developing coeliac disease, appearing in the serum before the development of villous atrophy (Collin et al. 1993, Kaukinen et al. 1998, Iltanen et al. 1999b).

In 1997 TG2 was identified by Dieterich and colleagues (1997) as the autoantigen of EmA. TG2 is expressed in many different tissues and organs, and is found intracellularly as well as extracellularly. In the extracellular environment TG2 has a role in extracellular matrix assembly, cell adhesion and wound healing. The calcium-dependent TG2 catalyzes selective crosslinking or deamidation of protein-bound glutamine residues (Reif and Lerner 2004). Dieterich's group (1997) demonstrated that TG2 is the autoantigen of EmA by pretreating coeliac disease patient sera with TG2, the result being almost complete disappearance of endomysial IF. In the same study the investigators further established an ELISA-based method for detecting IgA-class TG2 antibodies in the sera of untreated coeliac disease patients, and this showed

100% specificity. Since then ELISA TG2 antibody tests have been used with great success, first using guinea pig liver as antigen and later with an improved version using human recombinant tissue. The sensitivity and specificity of this test have been similar to the EmA test (Table 4), but the interpretation of the ELISA-based TG2 antibody test is considered to be less subjective and less laborious. Usually specialized laboratories perform coeliac autoantibody tests to ensure accurate and reliable results, but an onsite self-TG2-based rapid whole blood test was recently introduced; this simple rapid test showed high sensitivity and specificity values for untreated coeliac disease and seems feasible in point-of-care testing settings (Korponay-Szabo et al. 2005).

After EmA was shown to react with TG2 (Dieterich et al. 1997), Korponay-Szabo and colleagues (2000) studied whether the tissue distribution of TG2 is also compatible with reticulin binding patterns. It was demonstrated with a double-staining method and indirect IF that both ARA and EmA detected TG2 in rodent as well as in primate tissues (Korponay-Szabo et al. 2000). It was later further demonstrated using TG2 knockout mice tissues that EmA and ARA binding patterns in serum samples from coeliac disease patients were exclusively TG2-dependent (Korponay-Szabo et al. 2003). These studies demonstrated that ARA, EmA and TG2 antibodies all target the same autoantigen, indicating that they are virtually identical.

Table 3. *Sensitivity and specificity values of serum IgA-class reticulin antibodies (ARA) in untreated coeliac disease*

References	Study populations		IgA-class ARA	
	Patients	Controls	Sensitivity (%)	Specificity (%)
(Mäki et al. 1984)	29 children	245 children	97	98
(Hällström 1989)	32 adults, 18 children	45 adults	94	100
(Volta et al. 1991)	29 children, 41 adults	20 children, 20 adults	47	100
(Ferreira et al. 1992)	21 adults	160 adults	91	99
(Lerner et al. 1994)	28 children	41 children	65	100
(Ascher et al. 1996)	40 children, 15 adults	41 children, 24 adults	89	72
(Sacchetti et al. 1996)	32 children	42 children	94	100
(Bottaro et al. 1997)	50 children	25 children	74	100
(Kolho and Savilahti 1997)	53 children	114 children	96	92
(Sulkanen et al. 1998a)	92 adults	95 adults	78	100
(Sulkanen et al. 1998b)	136 children and adults	207 children and adults	92	96
(Lock et al. 1999)	27 adults	65 adults	59	100
(Mankai et al. 2005)	97 children, 46 adults	64 children, 10 adults	92	100

Table 4. *Sensitivity and specificity values of serum IgA-class endomysial antibodies (EmA) and transglutaminase 2 antibodies (TG2-ab) using human recombinant (hr) or guinea pig liver (gp) as antigen in untreated coeliac disease*

References	Study populations		IgA-class EmA		IgA-class TG2-ab	
	Patients	Controls	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
(Sulkanen et al. 1998b)	136 children and adults	207 children and adults	93	99.5	95 (gp)	94 (gp)
(Lock et al. 1999)	27 adults	65 adults	100	100	85 (gp)	97 (gp)
(Biagi et al. 1999)	39 adults	61 adults	100	100	95 (gp)	90 (gp)
(Sblattero et al. 2000)	65 children and adults	20 children and adults	93	100	84 (gp), 92 (hr)	100 (gp), 100 (hr)
(Bardella et al. 2001)	40 adults	110 adults	100	97	100 (gp)	98 (gp)
(Dahele et al. 2001)	53 adults	65 adults	75	100	66 (gp)	95 (gp)
(Biagi et al. 2001)	56 children and adults	52 adults	95	100	98 (gp)	85 (gp)
(Bonamico et al. 2001b)	62 children	56 children	95	98	90 (gp)	100 (gp)
(Salmaso et al. 2001)	59 children, 23 adults	48 children, 58 adults	96	100	93 (gp)	98 (gp)
(Wolters et al. 2002)	52 children	49 children	92	90	96 (gp), 96 (hr)	92 (gp), 100 (hr)
(Carroccio et al. 2002)	24 adults	183 adults	100	100	100 (gp), 100 (hr)	92 (gp), 97 (hr)
(Scoglio et al. 2003)	100 children, 34 adults	31 children, 16 adults	96	87	99 (gp)	74 (gp)
(Tesei et al. 2003)	250 adults	176 adults	86	100	90 (hr)	95 (hr)
(Mankai et al. 2005)	97 children, 46 adults	64 children, 10 adults	96	100	86 (gp)	96 (gp)
(Collin et al. 2005)	126 children and adults	106 children and adults	89	98	94 (hr)	99 (hr)

6.3. New aspects in coeliac autoantibody detection

Mucosal surfaces are the primary sites where the body encounters foreign antigens. It is thus only logical that 80% of all immunoglobulin-producing plasma cells in humans are situated in the intestinal mucosa. The majority of these cells produce the dimers of IgA. It was shown already decades ago that untreated coeliac disease patients have deposited IgA in their small bowel mucosa along epithelial basement membranes and around blood vessels (Shiner and Ballard 1972, Jos et al. 1979, Rantala et al. 1985, Karpati et al. 1988). However, the target of this intestinal IgA deposition remained until very recently unsolved.

Coeliac antibodies are typically detected in the serum in the untreated stage of the disease. Serum coeliac autoantibodies have been shown to bind to small intestinal tissues (Karpati et al. 1990) and in vitro studies have demonstrated that the target of these autoantibodies in both intestinal and extraintestinal tissues is TG2 (Korponay-Szabo et al. 2000, Korponay-Szabo et al. 2003). Evidence suggests that coeliac antibodies are produced in the small bowel mucosa (Marzari et al. 2001, Sblattero et al. 2006); IgA and IgM antibodies to gliadin and anti-reticulin antibodies have been detected in the jejunal secretion of coeliac disease patients and of those with a suspicion of coeliac disease (Mawhinney and Love 1975, Arranz and Ferguson 1993). Furthermore, EmA has been detected in duodenal biopsy organ culture supernatants from untreated coeliac disease patients and after in vitro gliadin challenge from treated coeliac disease patients (Picarelli et al. 1996a). Reinforcing the local production of coeliac autoantibodies, phage antibody libraries from the peripheral and intestinal lymphocytes of coeliac disease patients have shown that the humoral response against TG2 occurs at the local level in the intestinal mucosa, but not peripherally (Marzari et al. 2001). A phage display library method to detect local TG2 antibody synthesis was developed by Sblattero and colleagues (2004) in 2004. In the same year a group under Korponay-Szabo (2004) showed in vivo, using direct IF, that untreated coeliac disease patients have IgA deposits below the basement membrane along the villous and crypt epithelium and around mucosal vessels. It was further shown that small bowel mucosal coeliac-type IgA deposits were targeted against TG2. A simple staining method based on direct IF was provided in detecting TG2-specific IgA deposits in small bowel samples, and preliminary results indicated that intestinal coeliac autoantibody deposits might be detectable even before the development of villous atrophy (Korponay-Szabo et al. 2004 , Kaukinen et al. 2005).

7. SMALL BOWEL MUCOSAL MORPHOLOGY AND IMMUNOLOGY

7.1. Morphology

In untreated coeliac disease the small bowel mucosal damage is usually present in the proximal part of the small intestine (MacDonald et al. 1964), but may also be detectable in the whole small intestine. When coeliac disease is suspected, small bowel biopsies are obtained from the distal part of the duodenum or proximal jejunum during upper gastrointestinal endoscopy or using a Watson capsule. For histological examination, the specimens are formalin-fixed, stained with haematoxylin-eosin (HE) and studied under light microscopy. Villous height-crypt depth ratios (Vh/CrD) should be determined from well orientated and high quality samples to investigate objectively the small bowel mucosal architecture. The degree of intestinal inflammation is also estimated. In normal small bowel mucosa the villi are long and finger-like and covered with columnar epithelial cells. Vh/CrD usually ranges from 2.6 to 5 (Shidrawi et al. 1994, Lähdeaho et al. 2005). The density of IELs calculated from the surface epithelium is usually under 40 IELs per 100 epithelial cells in normal mucosa (Ferguson and Murray 1971).

In coeliac disease the small bowel mucosal damage develops gradually when the patient is consuming gluten-containing diet. This development of the mucosal lesion, according to the Marsh classification, is depicted in Figure 1 (Marsh 1992). The first change is an increased density of IELs in otherwise normal mucosa, indicating an infiltrative lesion (Marsh I); the density of IELs exceeds 40 cells per 100 villous epithelial cells (Ferguson and Murray 1971). Subsequently a hyperplastic lesion develops (Marsh II), similar to the Marsh I lesion but with the addition of enlarged crypts. Finally, the condition progresses to a destructive lesion (Marsh III), which is considered diagnostic for untreated coeliac disease. Here, the villous architecture is destroyed and severe partial, subtotal, or even total villous atrophy is present, together with crypt hyperplasia. In untreated coeliac disease Vh/CrD is < 2, the density of IELs is increased and the surface epithelial cells are flattened. Furthermore, there is an increased rate of cell division in crypts together with increased enterocyte apoptosis (Maiuri et al. 2001a), which might be at least partly responsible for the villous atrophy and crypt hyperplasia detected in untreated coeliac disease.

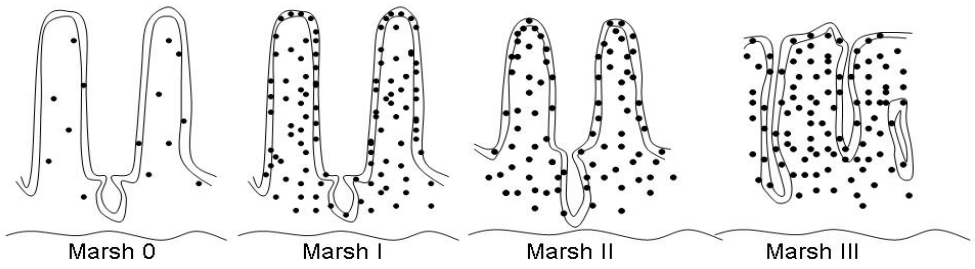

Figure 1. *The development of coeliac small bowel mucosal lesion and classification according to Marsh (1992)*

7.2. Lymphocytes

7.2.1. Intraepithelial compartment

Small bowel mucosal IELs are located between the columnar epithelial cells covering the villi, in close proximity to the basement membrane. As stated above, an increase in the number of IELs is considered the first detectable histological feature of coeliac disease (Fry et al. 1972, Marsh 1992). Furthermore, previous evidence based on small patient series has suggested that in untreated coeliac disease IELs are distributed evenly along the villi, even in the early stages of coeliac disease development, compared to the normal descrendo pattern where more IELs are detected at the base than at the tip of the villi (Goldstein and Underhill 2001). However, in untreated coeliac disease with total or subtotal villous atrophy an even villous tip IEL pattern cannot be demonstrated, since small bowel mucosal villous structures have been destroyed.

In coeliac disease small bowel mucosal IELs are almost exclusively CD3+ T cells; over 90% of these are CD8+ cells, and only a minority of CD4+ cells can be detected. In the small bowel mucosal epithelium B cells are very scarce or absent (Selby et al. 1983, Verkasalo et al. 1990). The immunohistochemical characteristics of IELs in the small bowel mucosal epithelial compartment of coeliac disease patients and in non-coeliac controls are shown in Table 5. The majority of CD3+ IELs express αβ+ T cell receptors (TCR). In untreated coeliac

disease the density of αβ+ T cells is typically increased; these cells have been shown to be gluten-dependent and thus their density decreases on a gluten-free diet (Savilahti et al. 1990, Savilahti et al. 1992, Kutlu et al. 1993). However, a striking feature of coeliac disease is an increased density of γδ+ IELs; these cells are typically increased in the active phase of the disease (Halstensen et al. 1989, Spencer et al. 1989, Spencer et al. 1991) and even though their density decreases on a gluten-free diet, it remains elevated long after gluten has been withdrawn (Savilahti et al. 1990, Savilahti et al. 1992, Kutlu et al. 1993). Previous studies suggest that the increased density of these cells precedes the development of villous atrophy (Mäki et al. 1991a, Iltanen et al. 1999c). Even though an increased density of γδ+ IELs has previously been shown to be highly indicative of coeliac disease in small patient series (Camarero et al. 2000), these cells have also been elevated in other disorders such as cow's milk intolerance, food allergy and postenteritis syndrome and in patients with other autoimmune diseases such as Hashimoto's thyroiditis or insulin-dependent diabetes mellitus (Spencer et al. 1991, Chan et al. 1993, Kaukinen et al. 2000, Kokkonen et al. 2000, Valentino et al. 2002, Auricchio et al. 2004). Further, an increased density of γδ+ IELs is not exclusively restricted to coeliac-type genetics, i.e. HLA DQ2 and DQ8 (Chan et al. 1993, Iltanen et al. 1999c, Kaukinen et al. 2000).

Table 5. *Characteristics of intraepithelial lymphocytes (IELs) in the small bowel mucosa of coeliac disease (CD) patients, healthy controls and patients with other diseases causing villous atrophy*

	CD patients	Non-CD controls	Patients with other causes of villous atrophy
Density of IELs/100 epithelial cells	>40	<40	< or ≥40
Characteristics of IELs			
CD8+	62-90%	>90%	>90%
CD4+	<10%	<10%	<10%
αβ+	50-80%	>90%	>90%
γδ+	20-50%	<10%	<10%

Adapted from Arato et al. (1998)

The role of IELs in coeliac disease has remained an unsolved question. It has been suggested that γδ+ IELs might have a role in surveillance and in repair (Boismenu and Havran 1994) or in the elimination (Schesinger 1994) of

damaged epithelial cells. Some evidence also shows that both αβ+ and γδ+ IELs have a cytolytic potential (Lundqvist et al. 1996, Oberhuber et al. 1996), but until recently it was not known what controlled their cytotoxicity. IELs have been shown to express natural killer (NK) receptors (Jabri et al. 2000, Roberts et al. 2001) such as NKG2D on their cell surface (Roberts et al. 2001). NKG2D expressed at the surface of CD8 αβ+ and γδ+ T cells is the receptor of major histocompatibility complex (MHC) class I chain-related gene A (MICA) proteins, and it was recently shown that MICA is strongly expressed at the small intestinal epithelial surface in untreated coeliac disease patients (Hue et al. 2004). It has been suggested that NKG2D and MICA interaction may cause IEL-mediated damage to epithelial cells (Hue et al. 2004, Meresse et al. 2004). Interleukin (IL)-15 seems to have an important role in this cytotoxicity, since it has been shown to be involved in the upregulation of enterocyte MICA molecules (Hue et al. 2004) and in increased expression of NKG2D (Roberts et al. 2001). Furthermore, IL-15 causes proliferation of IELs (Ebert 1998, Maiuri et al. 2001b).

An abnormal IEL population can be observed in the majority of patients with refractory sprue. Small bowel mucosal samples from these patients typically show complete or partial disappearance of normal subsets of IELs, which may be replaced by phenotypically abnormal lymphocytes. These cells express intracytoplasmic CD3e but not surface CD3e, TCR, CD4 or CD8, and also evince restricted rearrangements of the TCRγ gene (Cellier et al. 1998, Farstad et al. 2002). Furthermore, the expression of CD30+ by IELs has been suggested to be associated with a poorer prognosis, increasing the risk of subsequent small bowel lymphoma (Farstad et al. 2002).

7.2.2. Lamina propria

The lamina propria is the area between the epithelium and the muscularis mucosa populated by large numbers of plasma cells, macrophages, granulocytes, fibroblasts, smooth muscle cells and both T and B lymphocytes. In this area T cells account for 25-40% of all leukocytes, and the CD4+ T cell subset predominates. T cells are almost exclusively of the αβ+ T cell type, though occasionally also γδ+ T cells can be detected.

In small bowel specimens from patients with untreated coeliac disease, the volume of the lamina propria is two to three times greater than that in the normal intestine (Risdon and Keeling 1974). The density of plasma cells and IgA-, IgG- and IgM-containing cells is increased in untreated coeliac disease (Savilahti 1972, Lancaster-Smith et al. 1976). These cells might be of critical significance in the pathogenesis of coeliac disease, since they are thought to locally produce coeliac autoantibodies (Picarelli et al. 1996a, Vogelsang et al. 1999). A similar increase in T cells densities is not usually detected: the densities of CD3+, CD4+ and CD8+ IELs have been shown to be unchanged in untreated coeliac disease patients compared to controls (Verkasalo et al. 1990). Eosinophilic and

neutrophilic granulocytes as well as mast cells may be found in varying amounts. However, none of the changes in the lamina propria are specific or diagnostic for coeliac disease.

7.2.3. Immunologic activation markers

In patients with a suspicion of coeliac disease and in those carrying an increased risk of coeliac disease, markers suggesting activation of mucosal T cell-mediated immunity have been detected even in the absence of villous atrophy. In these subjects the density of lamina propria mononuclear cells expressing the IL-2 receptor (CD25) has been increased, as well as the expression of intracellular adhesion molecule 1 (ICAM-1) (Picarelli et al. 1996b, Auricchio et al. 2004, Paparo et al. 2005). Furthermore, in coeliac disease patients HLA-DR expression can also be detected in the crypts, not only at the top of the villi as in non-coeliac controls (Arnaud-Battandier et al. 1986, Ciclitira et al. 1986). However, these markers of immunologic activation are not restricted to coeliac-type HLA and not exclusively specific for coeliac disease (Arnaud-Battandier et al. 1986, Picarelli et al. 1996b).

8. LATENT AND EARLY DEVELOPING COELIAC DISEASE

8.1. Latent coeliac disease

Small bowel mucosal damage progresses gradually in coeliac disease (Marsh 1992), and manifest intestinal lesion can develop at any age in susceptible individuals. The designation latent coeliac disease usually refers to patients yielding normal intestinal biopsy findings while on a normal gluten-containing diet, who are subsequently shown to have villous atrophy and crypt hyperplasia in the small bowel mucosa which recovers on a gluten-free diet (Ferguson et al. 1993). The condition was first described in 1974 by Weinstein (1974), who also showed that the latent form of coeliac disease can be unmasked by adding extra gluten to the diet. The diagnosis of latent coeliac disease is always retrospective, and the time interval between normal mucosa and manifest mucosal lesion is not known but most likely varies between individuals.

Previous studies conducted on latent coeliac disease, involving relatively small patient series, are shown in Table 6. It is recognized and also apparent in the Table that many coeliac disease patients have clinical symptoms before the development of overt villous atrophy (Collin et al. 1993, Troncone 1995). Evidence further suggests that coeliac disease complications such as osteoporosis can precede manifest mucosal lesion (Kaukinen et al. 2001). It is not yet

established whether coeliac patients without villous atrophy also carry an increased risk of malignancies, but a case report on two patients with small bowel malignancy prior to the development of coeliac disease has been published (MacGowan et al. 1996).

These above-mentioned considerations suggest that the recognition of coeliac disease without villous atrophy is important, though challenging. Serum coeliac antibodies are often detected before the development of coeliac mucosal lesion (Collin et al. 1993, Kaukinen et al. 1998, Iltanen et al. 1999b), even though contradictory evidence exists; some studies suggest that coeliac antibodies are usually detected only when villous atrophy is present (Rostami et al. 1999, Tursi et al. 2001). Coeliac autoantibodies can now also be detected from their production site, the small bowel mucosa, and preliminary evidence suggests that the presence of these intestinal autoantibodies might precede the development of overt villous atrophy (Korponay-Szabo et al. 2004, Kaukinen et al. 2005).

Histologically intraepithelial lymphocytosis is typically the first finding in the progression of the coeliac mucosal lesion (Marsh 1992), but its specificity has not been confirmed. Counting the density of IELs from the villous tips might be more indicative of early coeliac disease development (Goldstein and Underhill 2001), but currently an increased density of γδ+ IELs is considered the most reliable marker of coeliac disease without villous atrophy (Kaukinen et al. 1998, Iltanen et al. 1999b, Iltanen et al. 1999c).

8.2. Early developing coeliac disease

It is relatively rare that the criteria for latent coeliac disease are fulfiled, that is, a patient has normal small bowel biopsy finding available prior to the development of villous atrophy. It is more common that patients are considered to suffer from coeliac disease based on some histological or serological findings even though typical villous atrophy and crypt hyperplasia cannot be demonstrated in their small bowel mucosa while they are consuming a normal gluten-containing diet. In the literature these patients have been referred to early developing or potential coeliac disease patients. However, the terminology regarding these cases has been variable, and occasionally also latent coeliac disease is confused with this condition. DH can be considered a model for early developing coeliac disease, since even though the majority of DH patients have villous atrophy in their small bowel specimens, in approximately 10% of these patients the small bowel mucosa can be normal or only an increased density of IELs or minor villous alterations can be detected (Reunala et al. 1984).

In early developing coeliac disease the diagnosis is usually based on several markers suggesting coeliac disease, for example clinical symptoms, serum coeliac antibodies and increased density of CD3+ or γδ+ IELs in the small bowel mucosa. Occasionally also HLA DQ2 or DQ8 is required to ascertain genetic gluten intolerance. If these patients are left without dietary treatment villous atrophy may develop, confirming latent coeliac disease, which as noted, is

always diagnosed retrospectively. However, nowadays patients suspected of suffering from early developing coeliac disease are occasionally advised to adhere to a gluten-free diet, even though they do not fulfil the traditional ESPGAN diagnostic criteria for coeliac disease. A clinical and histological response (decrease in inflammation) to a gluten-free diet supports the diagnosis of coeliac disease in these cases, but occasionally a gluten challenge revealing villous atrophy is needed in ascertaining the diagnosis (Wahab et al. 2001, Kaukinen et al. 2005). There are a limited number of studies conducted on early developing coeliac disease, and the documentation of the clinical, serological and histological findings of these patients is variable; Table 7 shows studies demonstrating early developing coeliac disease patients treated with a gluten-free diet.

Table 6. *Studies focusing on latent coeliac disease (CD) patients with normal small bowel biopsy finding at baseline subsequently developing villous atrophy and crypt hyperplasia without intervention. Baseline clinical, serological and histological findings are shown.*

Reference	Patients	Primary reason for CD suspicion	Positive CD antibodies in the serum	Small bowel mucosal histological abnormalities	Time interval until the demonstration of villous atrophy
(Egan-Mitchell et al. 1981)	1 child	1 failure to grow	ND	None	1.2 years
(Marsh 1989)	2 adults	1 malabsorption, 1 anaemia	ND	2 increased density of IELs and crypt hypertrophy	2.7-5 years
(Mäki et al. 1990)	3 children, 1 adult	2 malabsorption, 1 skin symptoms, 1 family history of CD	1 AGA positive	None	2.6-9 years
(Mäki et al. 1991a)	1 adult	1 family history of CD	1 ARA positive	1 increased density of CD3, αβ+ and γδ+ IELs	2 years
(Mäki et al. 1991b)	3 patients	3 family history of CD	3 ARA positive	None	3 years
(Collin et al. 1993)	7 adults	7 abdominal symptoms	5 ARA positive, 5 AGA positive	1 increased density of IELs	1-5 years
(Mäki et al. 1995)	1 child	1 IDDM	1 ARA positive	None	1 year
(Troncone 1995)	14 children	4 failure to grow, 3 diarrhoea, 2 anaemia, 1 arthralgia, 1 skin symptoms, 3 IDDM	3 AGA positive, 2 AGA and EmA positive	1 increased density of IELs	0.8-10 years
(Corazza et al. 1996)	3 adults	1 epigastric pain, 1 malabsorption, 1 family history of CD	1 AGA and EmA positive	None	0.2-13 years
(Kaukinen et al. 1998)	5 adults	3 abdominal symptoms, 1 weight	2 ARA positive, 3 AGA	4 increased density of CD3+ IELs,	0.3-1.5 years

		loss, 1 anaemia	positive	3 increased density of αβ+ IELs, 5 increased density of γδ+ IELs	
(Feighery et al. 1998)	2 adults	ND	ND	None	ND
(Iltanen et al. 1999c)	4 children	ND	4 ARA positive	4 increased density of γδ+ IELs	1.5-4.5 years
(Iltanen et al. 1999b)	9 children	5 abdominal symptoms, 3 failure to grow, 1 autoimmune thyroiditis	8 ARA and EmA positive, 4 AGA positive	1 increased density of αβ+ IELs, 6 increased density of γδ+ IELs	0.8-4.5 years
(Niveloni et al. 2000)	2 adults	2 family history of CD	2 AGA positive	None	8.2-8.5 years
(Goldstein and Underhill 2001)	6 adults	Abdominal symptoms, anaemia	ND	Mean villous tip IEL density increased	ND
(Valletta et al. 2002)	1 child	1 loose stools and abdominal pain	1 EmA positive	None	4 years
(Tursi and Brandimarte 2003)	1 adult	1 abdominal pain	1 AGA, EmA, and TG2-ab negative	1 increased density of IELs	~1 year
(Sbarbati et al. 2003)	2 children	1 anaemia, 1 screening	2 EmA positive, 1 AGA positive	None	1 year
(Lähdeaho et al. 2005)	6 children	1 family history of CD, 2 abdominal symptoms, 1 loose stools, 1 arthralgia, 1 failure to grow	1 EmA positive, 1 AGA positive	2 increased density of IELs	ND
(Paparo et al. 2005)	2 children	ND	2 EmA positive	ND	0.5-6 years
(Bister et al. 2005)	2 children	ND	2 EmA and TG2-ab positive	2 increased density of CD3+ and γδ+ IELs	ND
(Dickey et al. 2005)	6 adults	ND	6 EmA positive	2 increased density of IELs	1-6 years

ND=No data
IDDM=insulin-dependent diabetes mellitus
ab=antibodies

Table 7. *Studies focusing on early developing coeliac disease (CD) involving patients with normal villous architecture in the small bowel specimens advised to adhere to gluten-free diet (GFD) and the number of patients considered gluten-sensitive (GS) in each study*

Reference	Patients	Clinical symptoms		Serum CD antibodies		Small bowel histological findings		Final diagnosis
		Normal diet	GFD	Normal diet	GFD	Normal diet	GFD	
(Cooper et al. 1980)	17 adults	17 diarrhoea	9 improved	ND	ND	ND	8 decrease in inflammation	9 GS
(Arranz and Ferguson 1993)	9 adults	8 diarrhoea, 1 mouth ulcers	6 improved	8 CIA-positive*	ND	5 increased density of IELs	3 decrease in inflammation, 2 no change, 4 ND	6 GS
(Picarelli et al. 1996b)	10 adults	6 diarrhoea, 4 atypical symptoms	10 improved	10 EmA positive, 7 AGA positive	10 normalization of EmA	4 increased density of IELs	4 decrease in inflammation	10 GS
(Feighery et al. 1998)	1 patient	ND	ND	ND	ND	1 increased density of IELs	1 decrease in inflammation	1 GS
(Kaukinen et al. 2001)	10 adults	7 abdominal symptoms, 2 malabsorption, 1 atypical symptoms	8 improved	9 EmA and/or TG2-ab positive, 7 AGA positive	8 normalization of EmA and TG2-ab, 6 normalization of AGA	10 increased density of γδ+ IELs, 10 Marsh I or Marsh II	7 decrease in inflammation or normalization of mucosa, 3 ND	10 GS
(Wahab et al. 2001)	10 adults	ND	3 improved	ND	ND	10 increased density of IELs	2 decrease in inflammation, 8 no change	ND
(Goldstein and Underhill 2001)	10 adults	ND	10 improved	8 EmA positive, 5 AGA positive	ND	Mean villous tip IEL density increased	ND	10 GS

(Wahnschaffe et al. 2001)	26 adults	26 diarrhoea	Improved stool frequency in general	8 intestinal TG2-ab or AGA	Decrease in antibody titers in general	8 increased density of IELs	ND	ND
(Mahadeva et al. 2002)	2 adults	ND	2 improved	1 EmA positive	ND	2 increased density of IELs	2 no change	ND
(Kakar et al. 2003)	4 adults	3 diarrhoea, 1 anaemia	4 improved	3 EmA positive	ND	4 increased density of IELs	ND	4 GS
(Tursi and Brandimarte 2003)	23 adults	11 abdominal symptoms, 1 anaemia, 11 atypical symptoms	23 improved	6 EmA and/or TG2-ab positive, 2 AGA positive	5 EmA/TG2-ab normalized, 2 AGA normalized	16 crypt hypertrophy, 7 increased density of IELs	19 decrease in inflammation or crypt improvement, 4 no change	23 GS
(Kaukinen et al. 2005)	20 adults	18 abdominal symptoms, 1 anaemia, 1 screening	ND	5 TG2-ab positive, 2 EmA positive	ND	20 increased density of γδ+ IELs	ND	6 GS
(Paparo et al. 2005)	6 adults	ND	6 improved	6 EmA positive	6 EmA normalized	ND	ND	ND
(Bister et al. 2005)	8 children	ND	ND	8 EmA and/or TG2-ab positive	ND	5 increased density of CD3+, 5 increased density of γδ+ IELs	ND	8 GS
(Dickey et al. 2005)	27 adults	ND	26 improved	27 EmA positive	27 EmA normalized	12 increased density of IELs	ND	26 GS

ND=No data
ab=antibodies
* Coeliac-like intestinal antibody positive, IgM-AGA in jejunal fluid in all and IgA-AGA in some patients

9. DIFFERENTIAL DIAGNOSTICS

Small bowel mucosal villous atrophy and crypt hyperplasia are characteristic findings in the coeliac mucosal lesion. The diagnosis is nonetheless often challenging. The biopsy samples must be taken from the distal duodenum or proximal jejunum and good quality and orientation are also required in order to facilitate correct diagnosis. Small bowel specimens taken from the proximal duodenum may show shortened villi over regions of Brunner's glands even in healthy individuals, while crypt hyperplasia is not detectable (Freeman 2004). In addition, even in the presence of overt intestinal villous atrophy coeliac disease is not always the underlying condition (Goldstein 2004). In a review article by Freeman (2004) many non-coeliac disorders causing small bowel mucosal villous atrophy are listed; for example cow's milk intolerance, infectious gastroenteritis, parasitic infection, tropical sprue, Crohn's disease and graft-versus-host disease. Also autoimmune enteropathy is a rare condition causing small bowel villous atrophy and crypt hyperplasia (Corazza et al. 1997). Furthermore, case reports have been published on patients receiving immunosuppressant medication and presenting small bowel histological findings usually found in coeliac disease (Ziegler et al. 2003, Kamar et al. 2004). Usually in patients with non-coeliac-related intestinal lesion a response to a gluten-free diet cannot be demonstrated, and further, coeliac autoantibodies in the serum are negative. Hence, in untreated coeliac disease patients evincing villous atrophy and crypt hyperplasia but negative coeliac autoantibodies differential diagnostics should be borne in mind (Korponay-Szabo et al. 1997, Kwiecien et al. 2005) and occasionally in obscure cases a gluten challenge should be considered in addition to a trial of gluten-free diet in such individuals (Korponay-Szabo et al. 1997). Naturally in seronegative patients IgA-deficiency must also be considered and IgG-class antibodies investigated.

Minor histological abnormalities compatible with early developing coeliac disease are even more unspecific than overt villous atrophy. Intraepithelial lymphocytosis, even though typical in the early stages of coeliac development, can also be found in association with several other conditions. This inflammatory lesion has been detected in non-coeliac patients with autoimmune disorders, parasitic infections such as giardiasis lamblia, tropical sprue, cow's milk intolerance and psoriasis, and also in patients using nonsteroidal anti-inflammatory drugs (Ferguson and Murray 1971, Kuitunen et al. 1982, Marsh and Crowe 1995, Kakar et al. 2003, Augustin et al. 2005). An increased density of γδ+ IELs rather than an increased density of total IELs is considered more specific for untreated coeliac disease. However, evidence shows that patients with other autoimmune disorders and healthy controls or children with for example cow's milk intolerance, food allergy or postenteritis syndrome can also

express increased densities of γδ+ IELs in their small bowel mucosa (Spencer et al. 1991, Chan et al. 1993, Iltanen et al. 1999c, Kaukinen et al. 2000, Kokkonen et al. 2000, Valentino et al. 2002, Auricchio et al. 2004, Kaukinen et al. 2005).

10. EPIDEMIOLOGY

The prevalence of coeliac disease has increased dramatically during recent decades from the previously assumed 0.1%, largely due to the recognition of mild and atypical clinical symptoms of the disease and also of silent coeliac disease cases detected by screening at-risk groups. Recent population-based screening studies have shown that 1.2-1.5% of children have positive coeliac autoantibodies (EmA or TG2 antibodies) in the serum and that 0.5-1.1% of children have biopsy-proven coeliac disease (Csizmadia et al. 1999, Meloni et al. 1999a, Mäki et al. 2003, Tommasini et al. 2004). Similarly, evidence shows that the prevalence figures for coeliac autoantibody-positivity in adult materials is 0.5-1.1% and that of biopsy-proven coeliac disease is 0.5-1.2% (Kolho et al. 1998, Ivarsson et al. 1999a, Cook et al. 2000, Volta et al. 2001). Nowadays, even though the prevalence of diagnosed coeliac disease still varies widely, the estimates for combined symptomatic and asymptomatic coeliac disease are fairly similar in most populations (Meloni et al. 1999a, Cook et al. 2000, Mäki et al. 2003, Tommasini et al. 2004). On the other hand, coeliac disease is virtually unknown in populations in the Eastern Asia, who lack coeliac-type HLA, while the highest reported prevalence figures have been in the Saharawi children living in the desert area in Algeria (Catassi et al. 1999).

11. GENETIC FACTORS

Susceptibility to coeliac disease has been shown to be markedly inheritable. There is an unequivocal association between coeliac disease and the HLA gene region on chromosome 6. The HLA DQ2 haplotype encoded by the alleles DQA1*0501 and DQB1*02 is present in over 90% of coeliac disease patients (Sollid et al. 1989, Polvi et al. 1996). The HLA DQ2 alleles can be inherited either in cis, that is on one chromosome (DR3 or DR17 haplotype), or in trans, one DQ allele coming from a chromosome of each parent (DR5/7 or DR11/12 haplotype). Evidence shows that individuals homozygous for DQ2 carry a higher risk of coeliac disease development than those heterozygous for DQ2 (Ploski et al. 1993, Louka et al. 2002), but data has been more controversial as to whether there is a gene dose effect of the DQ2 heterodimer on symptoms of coeliac disease or on the age at diagnosis. Most DQ2-negative coeliac disease patients have HLA DQ8 encoded by DQA1*03 and DQB1*0302, and in addition, a few coeliac patients carry only DQA1*0501 or DQB1*02, that is, half of the DQ2

molecule (Spurkland et al. 1992, Polvi et al. 1998). Coeliac disease patients negative for HLA DQ2 and DQ8 and not carrying half of the DQ2 molecule are extremely rare (Karell et al. 2003).

It would appear, however, that the presence of HLA DQ2 or DQ8 haplotypes is not sufficient for coeliac disease onset. These haplotypes are also associated with several other autoimmune diseases (Dalton et al. 1992) and in addition, approximately one third of the population in general have DQ2 or DQ8 (Sollid et al. 1989, Polvi et al. 1996). Furthermore, the results of a logistic regression analysis in a recent population based twin study suggested that other genes than HLA are involved in the pathogenesis of coeliac disease (Greco et al. 2002). Research has struggled in discovering HLA-unlinked susceptibility genes in coeliac disease, and previous studies have suggested several different gene regions. However, often the results of the studies have been inconsistent. Cumulative evidence suggests that chromosome regions 5q or 5q31-33 (Greco et al. 1998, Liu et al. 2002) and 2q33 (Djilali-Saiah et al. 1998, Holopainen et al. 1999) are strong candidate susceptibility gene regions, and since there appears to be sufficient evidence of the linkage, these regions have been assigned as COELIAC2 and COELIAC3, the HLA region being COELIAC1. Furthermore, chromosome region 19p13.1, COELIAC4, was recently connected to coeliac disease (Van Belzen et al. 2003). Fine-mapping of the COELIAC4 region showed a strong peak of association in MYO9B, which encodes an unconventional myosin molecule which has a role in the actin remodelling of epithelial enterocytes. The risk of coeliac disease was shown to be 2.3-times higher in individuals homozygous for this risk allele (Monsuur et al. 2005).

12. PATHOGENETIC ASPECTS

The pathogenetic aspects of coeliac disease have not yet been fully revealed, even though great advances in understanding the immune response causing mucosal lesion have been made during the last decade. Both environmental and genetic factors contribute to the development of coeliac mucosal lesion. Nevertheless, the only environmental factor thus far identified is gluten.

According to the current understanding functionally different gluten fragments are able to induce mucosal damage in coeliac disease patients. Some of them, for example gliadin peptide 31-43, are toxic, being able to cause harmful effects in the intestinal mucosa when in contact with the intestine in vivo (Marsh 1992, Maiuri et al. 2003, Ciccocioppo et al. 2005), while some fragments are immunogenic (Ciccocioppo et al. 2005) stimulating HLA DQ2 (Sjöström et al. 1998, Arentz-Hansen et al. 2000) or DQ8 (Mazzarella et al. 2003) restricted T cells derived from small intestinal mucosa or circulating blood of coeliac disease patients. Both toxic and immunogenic gliadin fragments can enter the intestinal mucosa, activating both innate and adaptive responses. It is not known whether gliadin is transported through the epithelium via a transcellular route, in vacuoles

(Zimmer et al. 1998) or via a paracellular route, which might be accessible due to the increased permeability of tight junctions caused by increased zonulin expression (Fasano et al. 2000). In addition, MYO9B upregulation affecting tight junction assembly and remodelling of the cytoskeleton might increase epithelial paracellular permeability, which would indicate that intestinal barrier abnormality may be crucial in the pathogenesis of the disease (Monsuur et al. 2005).

After entering the lamina propria, toxic gliadin peptides have been shown to be able to induce an immune response by causing direct upregulation of IL-15 in lamina propria mononuclear and dendritic cells (Maiuri et al. 2003). IL-15 has been found to be involved in the upregulation of enterocyte MICA molecules (Hue et al. 2004), in the proliferation of IELs (Ebert 1998, Maiuri et al. 2001b) and also in increased expression of NKG2D, the receptor of MICA molecules in CD8 αβ+ and γδ+ T cells (Roberts et al. 2001). It has been suggested that MICA and NKG2D interaction is at least partly responsible for the IEL-mediated enterocyte apoptosis and villous atrophy in coeliac disease (Hue et al. 2004).

In genetically susceptible individuals carrying coeliac-type HLA immunogenic gliadin peptides are presented by antigen-presenting cells (APC) via HLA DQ2 or DQ8. Before gliadin presentation, TG2 deamidates glutamic residues present in gliadin peptides to negatively charged glutamic acid in order to facilitate their binding to the peptic groove of HLA DQ2 or DQ8 molecules on the APCs (Molberg et al. 1998). Gliadin presented by APCs stimulates T cells, eliciting T cell activation and proliferation. T helper (Th) 1 cells release interferon γ (IFNγ) and tumour necrosis factor α (TNFα), which causes secretion of matrix metalloproteinases (MMPs) from intestinal fibroblasts. MMPs have been suggested to be responsible for small bowel mucosal matrix breakdown and remodelling, resulting in villous atrophy (Schuppan 2000). Th2 cells activate B cells, causing subsequent production of IgA-class antibodies to gliadin and TG2. It has further been proposed that autoantibodies targeted against TG2 might be at least partly responsible for villous atrophy and crypt hyperplasia by inhibiting the differentiation of the epithelial cells while increasing proliferation via blocking TG2-mediated activation of transforming growth factor-β (TGFβ) (Halttunen and Mäki 1999). Moreover, a further role for TG2 as a regulator of lymphocyte migration and controller of the early non-adaptive phases of coeliac disease was recently introduced in an organ culture in vitro model (Maiuri et al. 2005). However, the involvement of coeliac autoantibodies in the pathogenesis of coeliac mucosal lesion has not yet been proved (Figure 2).

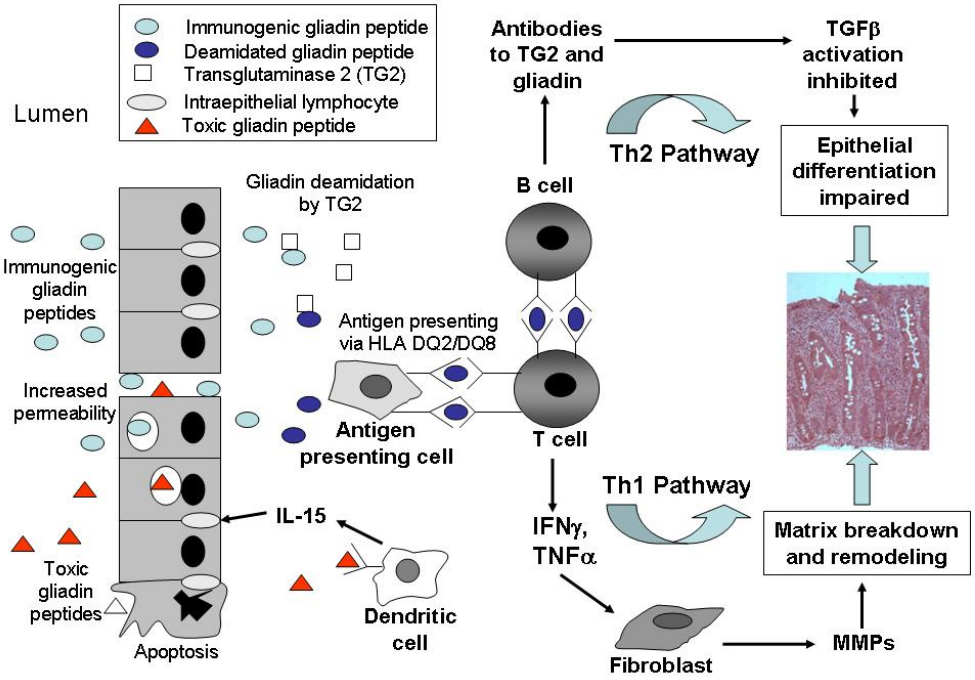

Figure 2. *Current theories of coeliac disease pathogenesis leading to villous atrophy and crypt hyperplasia (HLA=human leukocyte antigen, IL=interleukin, IFNγ=interferon γ, TNFα=tumour necrosis factor α, Th=helper T-lymphocyte, MMP=matrix metalloproteinase, TGFβ=transforming growth factor-β)*

13. TREATMENT

Gluten is the protein fraction of wheat which confers the property of stickiness and enables the baking of bread. Gluten can be fractioned into the ethanol-soluble prolamines, gliadins, and ethanol-insoluble glutenins. The prolamines of rye (secalins) and barley (hordeins) are closely related to gliadins and they all have a high content of glutamine and proline. Oat prolamins avenins have low proline content and oats in general are only distantly related to wheat, rye and barley.

The treatment for coeliac disease is a life-long gluten-free diet. Wheat, rye and barley are to be totally excluded, but the inclusion of oats in a gluten-free diet has been controversial. In 1995 Janatuinen and associates (1995) showed that oats can be safely consumed by the majority of coeliac disease patients, and a few years later also DH patients were shown to tolerate oats in their diet (Hardman et al. 1997, Reunala et al. 1998). Since then accumulating evidence deriving from both short- and long-term studies involving both children and adults have supported the safety of oats (Janatuinen et al. 2002, Storsrud et al. 2003, Högberg et al. 2004). However, the issue remains debatable, since

although oats are probably tolerated by most patients, oats-induced villous atrophy has also been demonstrated (Lundin et al. 2003).

Wheat starch-based gluten-free products are used in some European countries as part of the coeliac diet. In general a food product is considered gluten-free by Codex standards if it contains less than 0.05 g nitrogen per 100 g dry matter. Wheat starch-containing gluten-free products may contain trace amounts of gluten, and can thus in theory be harmful. Chartrand and colleagues (1997) showed in an open challenge study that abdominal symptoms were aggravated during a wheat starch-based gluten-free diet. In contrast, others have reported that clinical and histological recovery during a wheat starch-based gluten-free diet equals that during a natural gluten-free diet (Kaukinen et al. 1999, Peräaho et al. 2003). It seems that occasional dietary lapses are more harmful to the small bowel mucosa than trace amounts of gluten in the diet (Kaukinen et al. 1999).

A gluten-fee diet is not always easy to maintain, and thus a notable proportion of coeliac disease patients have proved non-compliant (Kumar et al. 1988, Mayer et al. 1991). Poor compliance jeopardizes small bowel mucosal recovery, which takes more than one year even on a strict gluten-free diet (Grefte et al. 1988, Kaukinen et al. 1999), especially in severe mucosal lesion. More infrequent reasons for the lack of histological response than non-compliance are refractory coeliac disease, ulcerative jejunoileitis or even small bowel lymphoma (Daum et al. 2005). In addition to a gluten-free diet patients with refractory sprue seem to profit from immunosuppressive treatment (Daum et al. 2005).

A gluten-free diet has been found to prevent the occurrence of coeliac disease complications such as malignancies (Holmes et al. 1989) and osteoporosis (Valdimarsson et al. 1996, Mustalahti et al. 1999). Furthermore, reproductive difficulties such as infertility and miscarriages might be resolved with dietary treatment (Sher and Mayberry 1996), and depressive symptoms may be alleviated (Pynnönen et al. 2005). Also the prevalence of autoimmune disorders has been suggested to be dependent on the duration of gluten exposure (Ventura et al. 1999). The role of gluten-free dietary treatment in asymptomatic patients or symptomatic patients without villous atrophy is less unambiguous. Especially the compliance of asymptomatic coeliac patients has been a matter of debate (Fabiani et al. 2000), but recent evidence has shown that dietary adherence is nowadays excellent regardless of the presence or absence of clinical manifestations of the disease (Viljamaa et al. 2005). Even in silent coeliac disease a gluten-free diet has been shown to improve bone mineral density (Mustalahti et al. 1999) and quality of life (Mustalahti et al. 2002). In early developing coeliac disease patients usually suffer from clinical symptoms, which are alleviated with a gluten-free diet (Kaukinen et al. 2001, Tursi and Brandimarte 2003). Furthermore, a significant proportion of patients with early developing coeliac disease have been shown to have reduced bone mineral density (Kaukinen et al. 2001), which speaks in favour of dietary treatment also in these individuals. However, it has not yet been confirmed whether the risk of malignancies in silent or early developing coeliac disease is increased.

THE PRESENT STUDY

1. PURPOSE

The aim of the present study was to improve accuracy in the diagnosis of untreated and treated coeliac disease and to compare traditional diagnostic methods with new ones. The specific objectives were:

1. To assess the diagnostic significance of CD3+, αβ+, γδ+ and villous tip IELs and serum IgA-class coeliac antibodies (AGA, ARA and EmA) compared to conventional Vh/CrD determination in untreated coeliac disease with villous atrophy, in early developing coeliac disease, and in treated coeliac disease **(I-IV)**

2.1. To investigate the target specificity of IgA deposits detected in the small bowel mucosa of untreated coeliac disease patients (**II**)

2.2. To assess the diagnostic significance of determination of intestinal IgA deposits in untreated coeliac disease with villous atrophy and in early developing coeliac disease and to assess the gluten-dependency of the deposits (**II, IV**)

2.3. To establish whether serum EmA-negative coeliac disease patients have coeliac-type IgA deposits in their small bowel mucosa, and whether seronegativity is associated with a particular coeliac disease phenotype (**II**)

2. PATIENTS, CONTROLS AND STUDY PROTOCOLS

The four original studies (**I-IV**) forming the present thesis involved patients and controls shown in Table 8. The study protocols for each study were approved by the Ethical Committee of Tampere University Hospital and informed consent was obtained from all study subjects.

*2.1. Untreated and treated coeliac disease or dermatitis herpetiformis patients (**I, II, III**)*

Altogether 928 adult patients underwent small intestinal biopsy at the Department of Medicine in Tampere University Hospital between 1995 and 1999 due to coeliac disease suspicion or to control mucosal recovery on a gluten-free diet (**I**). In 138 patients the coeliac disease diagnosis was based on small bowel mucosal villous atrophy and crypt hyperplasia; all patients were subsequently shown to fulfil the ESPGAN diagnostic criteria for coeliac disease. Furthermore, 24 patients on a normal, gluten-containing diet had had a skin biopsy based diagnosis of DH (van der Meer 1969) and were also considered to suffer from untreated coeliac disease. In 198 coeliac patients and in 23 DH patients with previously confirmed diagnosis the investigation was carried out during a gluten-free diet. The densities of CD3+, αβ+ and γδ+ IELs were determined in all study patients and their value in coeliac disease diagnosis was assessed. Furthermore, IELs were also counted from the villous tips in 22 untreated coeliac disease patients with severe partial villous atrophy and in 20 treated coeliac patients (**III**); in this study untreated coeliac disease patients with subtotal or total villous atrophy were not included, since counting the villous tip IELs is impossible from such specimens.

All 138 newly detected coeliac disease patients (**I**) together with patients diagnosed with coeliac disease during the year 2000 had serum EmA results available (**II**). The clinical and histological severity of untreated EmA-negative coeliac disease was compared to EmA-positive disease. For the investigation of intestinal autoantibody deposits one age- and sex-matched EmA-positive control was selected for each EmA-negative coeliac disease patient. Small bowel mucosal TG2-specific IgA deposits were investigated at the time the coeliac disease diagnosis was established and one year (median) after the adoption of a gluten-free diet. The applicability of the detection of intestinal autoantibody deposits targeted against TG2 in the diagnosis of seronegative coeliac disease was investigated (**II**).

Table 8. *Patients in original studies **I-IV** involving coeliac disease (CD) and dermatitis herpetiformis (DH) patients and controls*

	Study group		Non-CD control group	
Study number	Subjects	n (female)	Subjects	n (female)
I	- Untreated CD or DH	162 (110)	- Dyspepsia controls	59 (30)
	- Treated CD or DH	221 (139)		
	- CD suspected but excluded	545 (387)		
II	- Untreated CD patients	177 (117)	- Disease controls	20 (13)
	- Treated CD patients	44 (18)		
III	- Early developing CD		- Disease controls	59 (39)
	CD without atrophy*	20 (15)		
	Latent CD	17 (13)		
	DH	12 (5)		
	- CD patients			
	Untreated CD	22 (15)		
	Treated CD	20 (15)		
	- CD suspected but excluded	33 (24)		
IV	- Patients with a previous suspicion of CD but excluded for the condition			
	Serum CD autoantibodies positive†	25 (19)		
	Marsh 0 mucosal finding‡	25 (19)		
	Marsh 1 mucosal finding‡	25 (19)		

* CD diagnosis was based on several markers of CD such as serum EmA, HLA DQ2 or DQ8, increased density of CD3+ or γδ+ IELs and on clinical gluten-dependency
† IgA-class ARA or EmA
‡ Age- and sex-matched to coeliac autoantibody-positive patients (patients had negative serum ARA/EmA)

*2.2. Patients with coeliac disease suspicion but excluded for the disease (**I, III, IV**)*

Out of the 928 adult patients undergoing endoscopy and small bowel biopsy between 1995 and 1999 altogether 545 were excluded for coeliac disease despite clinical or serological suspicion (**I**); small bowel specimens from each patient showed normal villous architecture. The densities of CD3+, αβ+ and γδ+ IELs were determined in all. Furthermore, villous tip IELs were determined in thirty-three patients with coeliac disease suspicion but excluded for the disease while undergoing endoscopy during the years 1995 and 2001 (**III**). In these subjects coeliac disease was excluded based on normal villous structure, negative EmA and the absence of coeliac-type HLA (DQ2 and DQ8).

Twenty-nine out of the 545 patients excluded for coeliac disease during the years 1995 and 1999 had positive coeliac autoantibodies in the serum (IgA-class ARA or EmA) despite normal villous structure, and 69 seronegative patients had Marsh I lesion in the small bowel mucosa. Altogether 75 patients out of the 545 previously excluded for coeliac disease were selected for the follow-up study (**IV**). Twenty-five of these follow-up patients had positive serum coeliac autoantibodies and Marsh 0 or Marsh 1 findings in the small bowel mucosa, and the remaining age- and sex-matched study patients all had negative coeliac autoantibodies in the serum; 25 had Marsh I and 25 Marsh 0 findings in the small bowel mucosa at baseline when coeliac disease was excluded. In 2004 all patients without a previous coeliac disease diagnosis were invited for a follow-up visit a median of seven years after the baseline investigation and small bowel biopsy was offered. Patients having normal villous architecture in their small bowel specimens were again excluded for coeliac disease. It was ascertained with food-records analysed by a dietitian that all patients were consuming a normal gluten-containing diet at the time of the follow-up visit.

*2.3 Early developing coeliac disease patients (**III, IV**)*

In the follow-up study (**IV**) described above patients with normal small bowel mucosa at the follow-up visit were again excluded for coeliac disease. The remainder developed small bowel histological findings diagnostic for coeliac disease during the study: in 2004 before study enrolment the emergence of coeliac disease during the follow-up was investigated based on patient files and in the remaining patients coeliac disease was diagnosed at the follow-up visit based on small bowel biopsy findings. Patients with initially normal villous architecture progressing to villous atrophy and crypt hyperplasia during the whole follow-up period were considered to suffer from early developing coeliac disease at baseline, when coeliac disease was excluded. In these patients also the diagnosis of latent coeliac disease could have been made retrospectively (Ferguson et al. 1993). Finally, the baseline serological and histological findings

in patients shown to have developed coeliac disease during the follow-up were compared to those in patients again excluded for coeliac disease based on normal villous structure while maintaining a normal gluten-containing diet.

In addition to the patients developing coeliac disease during the follow-up study (**IV**), altogether 49 patients were considered to have early developing coeliac disease between the years 1995 to 2002 (**III**). Seventeen of these subsequently developed small bowel mucosal villous atrophy and crypt hyperplasia, and were hence initially considered to have early developing coeliac disease. Twelve patients had DH with normal villous structure or mild mucosal lesion. In twenty patients the diagnosis of early developing coeliac disease was based on the presence of several indicators of coeliac disease such as clinical symptoms, positive IgA-class EmA, HLA DQ2 or DQ8 and/or increased density of CD3+ or γδ+ IELs, and all patients had clinical response to a gluten-free diet. The density of small bowel mucosal villous tip IELs was calculated and the value of this method in the diagnosis of early developing coeliac disease was evaluated and compared to the method of counting CD3+ and γδ+ IELs from the whole surface epithelium.

*2.4. Non-coeliac control patients undergoing small bowel biopsy (**I, II, III**)*

Altogether 138 patients served as non-coeliac controls in the thesis; 104 of these suffered from dyspepsia (**I, II, III**) and 34 had intestinal diseases other than coeliac disease. These 34 patients suffered from ulcerative colitis, Crohn's disease, collagen colitis, giardiasis lamblia or autoimmune enteropathy (**II, III**). The number of non-coeliac controls in each original study is shown in Table 8. Three non-coeliac patients with autoimmune enteropathy (**II**) had villous atrophy but none was shown to have HLA DQ2 or DQ8; all of the remaining control patients had normal villous architecture.

2.5. New study groups for diagnostic analysis

For this thesis all patients in the four original studies (**I-IV**) described above were combined, and new patient groups were established in order to evaluate the results in different patient groups (Table 9). Altogether 223 patients (**I**, **II**, **III)** were consuming a normal, gluten-containing diet and had biopsy-proven coeliac disease or DH and were thus considered to suffer from untreated disease, and 241 patients were on a gluten-free diet due to prior diagnosis of coeliac disease or DH (**I, III**). Altogether 66 patients were considered to have early developing coeliac disease (**III, IV**). Of these in study **III**, 49 patients were considered to suffer from coeliac disease without villous atrophy, and in the follow-up study (**IV**) 17 patients previously excluded for coeliac disease were shown to have developed coeliac disease during the follow-up. In studies **I**, **III** and **IV** 608

patients underwent investigations due to coeliac disease suspicion, but the disease was excluded in all. Furthermore, altogether 138 patients served as non-coeliac controls (**I**, **II**, **III)**.

Table 9. *New study groups for diagnostic analysis consisting of coeliac disease (CD) and dermatitis herpetiformis (DH) patients and controls from studies **I-IV***

	CD or DH untreated	CD or DH treated	Early developing CD	CD suspected but excluded	Non-CD controls
Study **I**	138 CD and 24 DH patients	198 CD and 23 DH patients	-	545 patients	59 patients
Study **II**	39 CD patients*	-	-	-	20 patients
Study **III**	22 CD patients	20 CD patients	49 patients	33 patients	59 patients
Study **IV**	-	-	17 patients	30 patients	-
Total	223 patients	241 patients	66 patients	608 patients	138 patients

* Study **II** comprised altogether 177 untreated CD patients, 138 of whom were already included in study **I**

3. METHODS

*3.1. Small bowel biopsy (**I-IV**)*

In all study patients (**I-IV**) seven forceps biopsy specimens were taken from the distal part of the duodenum upon upper gastrointestinal endoscopy. Five of the small bowel specimens were processed and stained with HE and two were freshly embedded in optimal cutting temperature compound (OCT, Tissue-Tec, Miles Inc, Elkhart, IN, USA), snap-frozen in liquid nitrogen and stored at -70°C until used. HE-stained sections were used for morphologic examinations: routine histology was carried out by several different pathologists, but determination of Vh/CrD was performed by one investigator. Villous tip IELs were also counted from HE-stained sections, and the frozen sections were used for counting CD3+, αβ+ and γδ+ IELs and examination of intestinal TG2-specific IgA deposits. All specimens were evaluated without prior knowledge of disease history or laboratory findings.

*3.1.1. Morphometrical studies (**I-IV**)*

Morphometrical analysis was carried out on 2-µm-thick formalin-fixed small bowel sections. Sections were HE-stained and studied under light microscopy. Vh/CrD was determined from several biopsy samples from multiple sites in order also to detect patchy forms of villous atrophy, as previously described (Kuitunen et al. 1982) (**I-IV**). Poorly oriented sections were not accepted, and the samples were dissected again when necessary to obtain well-oriented and good-quality samples. Vh/CrD ≥ 2 was considered normal and coeliac disease was excluded, whereas Vh/CrD < 2 was considered compatible with coeliac disease (Marsh III) (**I-IV**). In study **II** Marsh III was further classified into three subgroups; Marsh IIIa indicating severe partial, Marsh IIIb subtotal and Marsh IIIc total villous atrophy with crypt hyperplasia (Marsh 1992).

*3.1.2. Intraepithelial lymphocytes (**I-IV**)*

The villous tip IELs were investigated using light microscopy in HE-stained small bowel samples; the villous tip IEL score per 20 enterocytes was calculated from the mean value of five random villi, as described by Goldstein and Underhill (2001) (**III, IV**). The reference value was set at 4.2 IELs/20 enterocytes in order to obtain optimal sensitivity and specificity values. The correlation coefficients for intraobserver variation for villous tip IELs were 0.89

and for interobserver variation 0.87 in our laboratory. Furthermore, the distribution of villous IELs along the sides of the villi was determined (**III**); the distribution was considered even when the numbers of IELs were similar over the tips and at the base of the villi and a descrendo pattern was present when the numbers of IELs were higher in the basal portions of the villi than at the tip (Goldstein and Underhill 2001). The intra- and interobserver variations for this method were 0.73 and 0.41, respectively.

Immunohistochemical stainings were carried out on 5-µm-thick frozen small bowel sections (**I-IV**). CD3+ IELs were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA, USA), αβ+ IELs with monoclonal antibody αF1 (T Cell Diagnostics, Woburn, MA, USA) and γδ+ IELs with TCR γ antibody (T Cell Diagnostics or Endogen, Woburn, MA, USA). Positive IELs were counted with a x100 flat field light microscope objective throughout the surface epithelium; at least 30 fields of 1.6 mm epithelial length were counted and IEL density was expressed as cells per millimeter of epithelium, as described elsewhere (Arranz et al. 1994). CD3+ IELs correlate well with the total IEL density in the small bowel mucosa (Arranz et al. 1994) and thus in study **IV** a normal density of CD3+ cells indicated a Marsh 0 and increased density a Marsh I finding (Marsh 1992). The reference values were set at 37 cells/mm for CD3+ IELs, 25 for αβ+ IELs and 4.3 for γδ+ IELs again to obtain the best sensitivity and specificity combination. The correlation coefficients for intraobserver variation for CD3+, αβ+ and γδ+ IELs were 0.95, 0.85 and 0.98, and those for interobserver variation 0.92, 0.82 and 0.98 in our laboratory, respectively.

*3.1.3. Detection of small bowel mucosal transglutaminase 2-targeted IgA deposits (**II, IV**)*

Small bowel mucosal TG2-specific IgA deposits were investigated from frozen small bowel sections (**II, IV**). From each patient investigated, altogether six unfixed, 5-µm-thick sections from frozen small bowel specimens were processed, three for investigation of IgA deposits and three for double-colour labelling for both IgA and TG2. IgA was detected by direct IF using fluorescein isothiocyanate-labelled rabbit antibody against human IgA (Dako AS, Glostrup, Denmark) at a dilution of 1:40 in phosphate-buffered saline (PBS), pH 7.4. In coeliac disease a clear subepithelial IgA deposition can be found below the basement membrane along the villous and crypt epithelium and around mucosal vessels; this is in contrast to normal small bowel samples, where IgA is detected only inside the plasma and epithelial cells (Korponay-Szabo et al. 2004, Kaukinen et al. 2005). Coeliac disease-type IgA deposits were graded from 0-3 based on the intensity along basement membranes in the villous-crypt area. The evaluation was carried out blindly without knowledge of disease history or laboratory findings. To confirm that coeliac-type IgA deposits co-localized with TG2, sections were double-stained for human IgA (green, as above) and for TG2 (red) using monoclonal mouse antibodies against TG2 (CUB7402, NeoMarkers,

Fremont, CA, USA) followed by rhodamine-conjugated anti-mouse immunoglobulin antibodies (Dako), both diluted 1:200 in PBS. The correlation coefficient for both intraobserver and interobserver variations for the detection of presence or absence of TG2-specific IgA deposits was 0.98.

*3.1.4. Investigations of target specificity of small bowel mucosal IgA deposits **(II)***

Unfixed frozen duodenum sections from seven serum EmA-negative and six EmA-positive untreated coeliac disease patients (**II**) were washed in PBS, pH 7.4, and incubated for 30 minutes with 0.1 M sodium citrate buffer (pH 5.0) or with 0.5-1 M potassium thiocyanate (KSCN), which as a chaotropic agent dissolves nonspecific protein complexes (Jones et al. 1987). After further washings in PBS, the sections were stained for human IgA and TG2 as described in the previous section.

In further experiments, extracellular TG2 was removed from the sections with 0.25% chloroacetic acid (Fluka Chemie AG, Buchs, Switzerland) in 0.2 M NaCl, pH 2.7, following the KSCN treatment; chloroacetic acid is needed to disrupt the tight binding of TG2 to fibronectin (Radek et al. 1993) and to remove TG2 from the tissues (Korponay-Szabo et al. 2004). The sections were thereafter similarly stained for remaining IgA and TG2.

In order to prove that extracellular IgA deposits in the small bowel of coeliac disease patients were targeted against TG2, it was investigated whether they would bind labelled TG2 added to the tissue. Glutathione S-transferase-tagged full-length human recombinant TG2 (GST-TG2) was expressed in E.coli as previously described (Ambrus et al. 2001). Unfixed frozen small bowel sections from coeliac and control patients were washed in PBS and incubated for 15 minutes at room temperature with GST-TG2 at a concentration of 0.01 mg/ml. After extensive washings, GST-TG2 bound to the tissue was labelled red by goat antibodies against GST (Pharmacia Biotech, Uppsala, Sweden) followed by Alexa Fluor® 594-conjugated chicken antibodies against goat immunoglobulins (Molecular Probes, Leiden, The Netherlands). Human IgA in the tissue was labelled green as previously described. The anti-GST antibody used did not cross-react with natural TG2 in the tissues. In order to block the binding of GST-TG2 to tissue fibronectin, GST-TG2 was also added to the sections together with the 45kD gelatine-binding fragment of human fibronectin (Sigma F-0162, Sigma-Aldrich Co, St. Louis, MO, USA; 0.2 mg/ml) and monoclonal antibodies G92 (0.4 mg/ml) (Trejo-Skalli et al. 1995). These antibodies recognize the blocked N-terminal segment of TG2 with high specificity.

3.2. *Serology (**I-IV**)*

Serum IgA-class EmA (Ladinser et al. 1994) and ARA (Hällström 1989) (**I-IV**) were determined by an indirect IF method. Human umbilical cord (EmA) and rat kidney and liver sections (ARA) were used as antigens, and a serum dilution of 1:≥5 was considered positive in both. In ARA, a typical R1 pattern was required (Eade et al. 1977). Positive and negative controls were included in every test batch. Coeliac autoantibody tests changed during the study; the EmA test replaced ARA in clinical practice during the study period and the autoantibody used was dependent on the time of testing. In our laboratory ARA and EmA tests have proved to be virtually identical (Mäki 1995) and are in this thesis thus referred to coeliac autoantibodies.

Serum AGA was investigated using ELISA (**I**), and the lower limit of positivity for IgA-class AGA was 0.2 ELISA units per millilitre (Vainio et al. 1983). Assessment of serum IgA-class TG2 antibodies was also carried out by ELISA, in study **II** using guinea pig liver TG2 (Sulkanen et al. 1998b) (Inova Diagnostics, San Diego, CA, USA) or human recombinant TG2 (Mäki et al. 2003) (Celikey®, Pharmacia Diagnostics, GmbH, Freiburg, Germany) as antigen, and in study **IV** using only the TG2 antibody test based on human recombinant as antigen. A unit value (U) ≥ 20U was considered positive when guinea pig liver was the antigen and ≥ 5U positive when human recombinant was the antigen employed.

*3.3. HLA typing (**II, III, IV**)*

HLA DQ alleles encoding HLA DQ2 and DQ8 were investigated at the Tissue Typing Laboratory of the Finnish Red Cross Blood Service in Helsinki, Finland. In study **II** allele groups were investigated using the Olerup SSP DQ low resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden) and in study **III** using the Dynal SSP low-resolution DQ typing kit (Dynal AS, Oslo, Norway). In study **IV** the study patients were genotyped for HLA-DQB1*02, DQB1*0302 and DQA1*05 alleles using the DELFIA® Coeliac Disease Hybridization Assay (PerkinElmer Life and Analytic Sciences, Wallac Oy, Turku, Finland).

*3.4. Statistical analysis (**I-IV**)*

$P < 0.05$ was considered statistically significant in all studies. In studies **I** and **III** quantitative data were expressed as means, ranges and 95% confidence intervals (CIs). Chi-square test was used in cross-tabulations (**I**), and the Pearson two-tailed t test was used to determine correlations between tip IELs and CD3+ and γδ+ IELs and intra- and interobserver variations (**III**).

In studies **II** and **IV** quantitative data were expressed as medians and ranges. Statistical differences between study groups were evaluated using the Pearson

Chi-square test, Fisher's exact test or Mann-Whitney U test, as appropriate. In study **IV** the size of the study groups was determined by power calculations. It was estimated that 50% of patients with positive coeliac autoantibodies in the serum and 10% of patients with negative serum autoantibodies having Marsh I or Marsh 0 findings in the mucosa would develop overt coeliac disease during the study period. α was given the value 0.05 and the power was 90%. Thus in order to elicit significant differences between the study groups the number of patients in each group had to be at least 23.

When new patient groups were established for the thesis, the quantitative data were expressed as medians or means, ranges, and 95% CIs. Statistical differences between study groups were evaluated using Fisher's exact test or Mann-Whitney U test, as appropriate.

4. RESULTS

4.1. Untreated coeliac disease or dermatitis herpetiformis

The most common indication for endoscopy and small bowel biopsy among 223 untreated coeliac disease patients was abdominal symptoms, but almost one fifth of the patients had asymptomatic coeliac disease or minor complaints and underwent investigations in the context of screening of coeliac disease at-risk groups (Table 10).

Compared to non-coeliac controls the Vh/CrD was statistically significantly lower and the densities of CD3+, αβ+ and γδ+ IELs higher in untreated coeliac disease and DH patients (Table 11) (**I, II, III**). In untreated coeliac patients with severe partial villous atrophy the densities of villous tip IELs were statistically significantly higher than in non-coeliac controls. Ninety-five per cent of untreated coeliac disease patients showed an even distribution of IELs along villous sides, i.e. the numbers of IELs were similar over the tips and at the base of the villi, compared to 44% detected in non-coeliac controls (**III**).

Altogether 68% of untreated coeliac disease or DH patients were IgA-AGA-positive, and 84% had positive IgA-class coeliac autoantibodies in the serum (ARA or EmA, depending on the time of the testing) (**I, II, III**). All untreated coeliac disease or DH patients with data available had HLA DQ2 or DQ8 (n = 17).

In study **II,** focusing on seronegative coeliac disease, altogether 26 out of 177 (15%) untreated coeliac disease patients had negative serum EmA; 22 IgA-competent EmA-negative coeliac disease patients constituted the study group. Serum TG2 antibody test results were available in altogether 14 out of 22 EmA-negative coeliac disease patients using guinea pig liver or human recombinant as antigen; four were positive and 10 negative for these antibodies. Fifty-nine per cent of the 22 EmA-negative coeliac disease patients were male, and their median age was higher than among EmA-positive subjects (Table 12). Furthermore, EmA-negative coeliac disease patients were suffering from abdominal symptoms more often than EmA-positive subjects (73% vs 48%, p = 0.039), and three EmA-negative patients were diagnosed with enteropathy-associated T cell lymphoma (EATL) at the same time as coeliac disease diagnosis was established. Two of these patients had HLA DQ2; in one there was no data available. All three had proximal small bowel villous atrophy and crypt hyperplasia compatible with coeliac disease while on a gluten-containing diet. Moreover, two of the EATL patients had small bowel biopsies taken earlier, two and six years prior to the diagnosis of coeliac disease and EATL. Even then both showed partial villous atrophy and crypt hyperplasia, but the diagnosis of coeliac disease was overlooked. Six (27%) out of the 22 EmA-negative and six

(4%) out of the 151 EmA-positive coeliac disease patients had died since the diagnosis of coeliac disease. There were no differences between EmA-negative and EmA-positive patients in Marsh classification ($p = 0.769$), Vh/CrD or in the densities of CD3+ IELs, but γδ+ IELs were statistically significantly higher in the EmA-positive group (Table 12). After a median of 13 months on a gluten-free diet there were no differences in small bowel histological recovery between EmA-negative and -positive coeliac disease patients. Histological improvement was observed in all patients who underwent control small bowel biopsy, except in the three affected by EATL. In the remaining three EmA-negative and four EmA-positive individuals clinical recovery on a gluten-free diet was evident; one EmA-negative and one –positive patient was lost to follow-up.

Small bowel mucosal IgA deposits in co-localization with extracellular TG2 were detected in all untreated coeliac disease patients investigated ($n = 35$), both EmA-negative and –positive (Table 11 and Figures 3 and 4) (**II**). The detection of intestinal TG2-targeted IgA deposits had the best sensitivity and specificity combination in detecting untreated coeliac disease (100% in both) (Table 13). The sensitivity of serum coeliac autoantibodies was lower (84%), but their specificity was also 100%. Increased density of villous tip IELs had better sensitivity and specificity values than increased density of CD3+, αβ+ or γδ+ IELs (Table 13).

Table 10. *Demographic data and primary reason for endoscopy and small bowel biopsy in new study groups*

	CD or DH untreated n = 223 (**I, II, III**)	CD or DH treated n = 241 (**I, III**)	Early developing CD n = 66 (**III, IV**)	CD suspected but excluded n = 608 (**I, III, IV**)	Non-CD controls n = 138 (**I, II, III**)
Age; med (range)	42 (16-81)	45 (16-83)	45 (21-74)	42 (15-88)	49 (19-74)
Female; n (%)	147 (66)	154 (64)	46 (70)	432 (71)	82 (59)
Primary reason for endoscopy					
- Abdominal symptoms; %*	35	13	50	54	94
- Malabsorption or anaemia; %	9	4	6	20	3
- Skin symptoms suggesting DH; %	23	3	14	4	0
- Atypical symptoms; %†	14	2	18	16	3
- Silent, screening at-risk groups; %‡	19	0	12	6	0
- To control histological recovery on GFD; %	0	78	0	0	0
First-degree relatives with CD; n (%)	56/145 (39)	40/103 (39)	19/38 (50)	58/197 (29)	6/67(9)

CD=Coeliac disease
DH=Dermatitis herpetiformis
GFD=Gluten-free diet
* Diarrhoea, flatulence, indigestion, abdominal distension, abdominal pain
† Neurological symptoms, dental enamel defects, mouth ulcerations, osteoporosis, infertility, alopecia areata, arthritis, elevated liver enzymes
‡ Insulin-dependent diabetes mellitus, autoimmune thyroid disease, Sjögren's syndrome, family history of coeliac disease

Table 11. *Mean values, 95% confidence intervals (CIs) and number of abnormal values in small bowel mucosal histological findings and positive serum or intestinal coeliac antibody results in study groups*

	CD or DH untreated (**I, II, III**)	CD or DH treated (**I, III**)	Early developing CD (**III, IV**)	CD suspected but excluded (**I, III, IV**)	Non-CD controls (**I, II, III**)
Vh/CrD					
Mean value	0.6*	1.9*	2.9*	2.9*	3.1
95% CI	0.6-0.7	1.8-2.1	2.7-3.1	2.9-3.0	3.0-3.2
CD3+ IELs; cells/mm					
Mean value	67*	39*	50*	26*	31
95% CI	64-71	36-41	42-58	25-28	29-34
Abnormal values; n (%)	189/223 (85)*	111/241 (46)*	40/66 (61)*	124/608 (20)*	43/138 (31)
αβ+ IELs; cells/mm					
Mean value	41*	22	31*	18*	24
95% CI	39-44	21-24	26-36	17-19	22-26
Abnormal values; n (%)	178/223 (80)*	78/241 (32)	38/66 (58)*	117/608 (19)*	56/138 (41)
γδ+ IELs; cells/mm					
Mean value	19.5*	12.1*	13.2*	3.3	3.3
95% CI	17.9-21.2	11.0-13.1	10.5-15.9	2.9-3.7	2.5-4.1
Abnormal values; n (%)	206/223 (92)*	202/241 (84)*	54/66 (82)*	146/608 (24)	26/138 (19)
Villous tip IELs; cells/20 enterocytes					
Mean value	11.6*†	4.7*	6.9*	3.0	2.9
95% CI	9.7-13.6†	3.9-5.6	6.2-7.6	2.6-3.5	2.6-3.2
Abnormal values; n (%)	21/22 (95)* †	12/20 (60)*	55/65 (85)*	12/61 (20)	7/59 (12)
Even distribution of IELs along villous sides; n (%)	21/22 (95)*	14/20 (70)	52/65 (80)*	29/61 (48)	26/59 (44)
IgA-AGA positive; n (%)	113/167 (68)*	37/159 (23)	28/42 (67)*	226/371 (61)*	3/11 (27)
IgA-ARA or EmA positive; n (%)	185/221 (84)*	40/180 (22)*	45/64 (70)*	46/368 (13)*	0/88 (0)
Intestinal TG2-specific IgA deposits present; n (%)	35/35 (100)*	23/28 (82)*	14/15 (93)*	1/14 (7)	0/20 (0)

CD=Coeliac disease
DH=Dermatitis herpetiformis
* Statistically significant difference compared to non-CD controls
† Untreated coeliac disease patients with total or subtotal villous atrophy excluded

Table 12. *Comparisons between untreated IgA-competent endomysial antibody (EmA)-negative and –positive coeliac disease (CD) patients*

	EmA-negative CD patients (n = 22)	EmA-positive CD patients (n = 151)	p-value
Female; n (%)	9 (41)	106 (70)	0.014
Age; median (range), years	55 (20-79)	40 (16-81)	0.001
Vh/CrD; mean (range)	0.5 (0.1-1.5)	0.5 (0.0-1.9)	0.605
CD3+ IELs; mean (range)*	64 (25-111)	70 (23-170)	0.292
γδ+ IELs; mean (range)*	15.6 (1.4-76.5)	20.4 (1.4-63.0)	0.007

* IEL density expressed as cells/mm of epithelium

Table 13. *Sensitivities and specificities of different markers in detecting coeliac disease (CD) with villous atrophy and early developing CD without villous atrophy. Non-CD patients without CD suspicion served as controls.*

	Sensitivity to detect untreated CD with villous atrophy (95% CI)	Sensitivity to detect early developing CD without villous atrophy (95% CI)	Specificity (95% CI)
Intestinal TG2-specific IgA deposits present	1.00 (0.90-1.00)	0.93 (0.70-0.99)	1.00 (0.84-1.00)
HLA DQ2/DQ8 present	1.00 (0.82-1.00)	1.00 (0.90-1.00)	0.73 (0.63-0.82)*
Increased density of villous tip IELs	0.95 (0.78-0.99)†	0.85 (0.74-0.91)	0.88 (0.77-0.94)
Increased density of γδ+ IELs	0.92 (0.88-0.95)	0.82 (0.71-0.89)	0.81 (0.74-0.87)
Increased density of CD3+ IELs	0.85 (0.79-0.89)	0.61 (0.49-0.71)	0.69 (0.61-0.76)
Positive serum IgA-class ARA/EmA	0.84 (0.78-0.88)	0.70 (0.58-0.80)	1.00 (0.96-1.00)
Increased density of αβ+ IELs	0.80 (0.74-0.85)	0.58 (0.46-0.69)	0.59 (0.51-0.67)
Positive serum IgA-class AGA	0.68 (0.60-0.74)	0.67 (0.52-0.79)	0.73 (0.43-0.90)

* Patients with coeliac disease suspicion but excluded for coeliac disease based on normal villous architecture served as controls
† Untreated coeliac disease patients with total or subtotal villous atrophy excluded

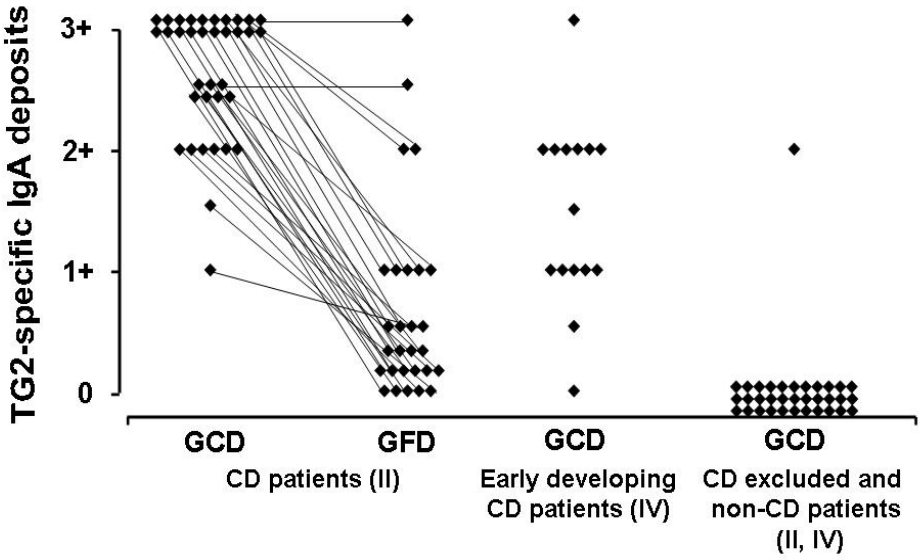

Figure 3. *Transglutaminase 2 (TG2)-specific IgA deposits in the small bowel mucosa of untreated coeliac disease (CD) patients, early developing CD patients and patients excluded for CD and non-CD control patients on a normal gluten-containing diet (GCD). Intestinal autoantibody deposit results are also shown in untreated CD patients after a median of one year on a gluten-free diet (GFD).*

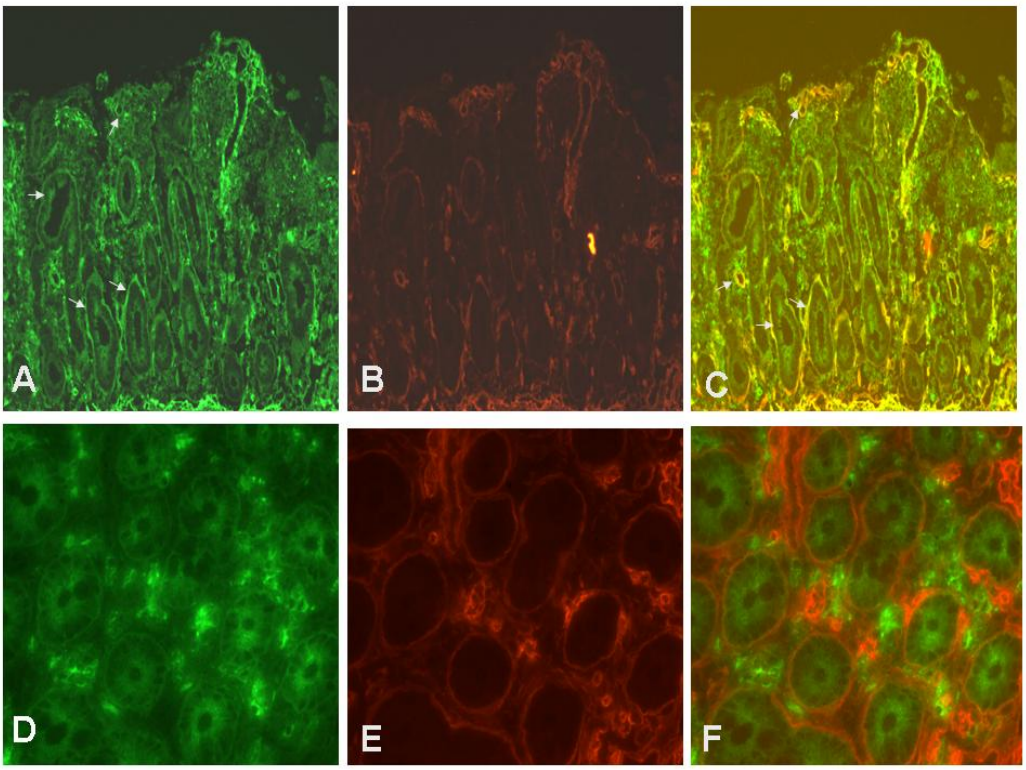

Figure 4. *Subepithelial coeliac-type small bowel mucosal IgA deposits (A, arrows) in untreated coeliac disease patient having villous atrophy and crypt hyperplasia maintaining a normal gluten-containing diet. Yellow colour in composite picture (C, arrows) indicates co-localization of coeliac-type IgA deposits (green) and transglutaminase 2 (TG2, B, red). In non-coeliac control subject IgA deposits are not detected (D) and co-localization of IgA (green) and TG2 (E, red) is not detected (F).*

4.2. Treated coeliac disease or dermatitis herpetiformis

In studies **I** and **III** 241 patients with treated coeliac disease or DH had adhered to a gluten-fee diet a median of 12 months (range 1 month to 32 years). The Vh/CrD was higher than in untreated coeliac disease patients but statistically significantly lower than in non-coeliac control subjects (Table 11) (**I, III**). Similarly, the densities of CD3+, αβ+, γδ+ and villous tip IELs were lower than in untreated coeliac disease patients, but again, CD3+, γδ+ and villous tip IELs remained elevated compared to non-coeliac controls. Twenty-two per cent of the coeliac disease or DH patients remained positive for serum IgA-class coeliac autoantibodies (ARA or EmA) after a median of one year on a gluten-free diet, and 23% were IgA-AGA-positive.

Intestinal TG2-specific IgA deposits were detected in 23 out of 28 treated coeliac disease patients investigated (**II**). Figure 3 shows that the intensity of the

deposits decreased during the dietary treatment, but remained positive in the majority of patients after a median of one year on a gluten-free diet.

4.3. Early developing coeliac disease

In the follow-up study (**IV)**, altogether 17 out of the 75 patients previously suspected but excluded for coeliac disease subsequently developed small bowel mucosal villous atrophy and crypt hyperplasia during the follow-up period (median duration 7 years). Fifteen out the seventeen had been diagnosed with coeliac disease by routine clinical follow-up before study enrolment, and at the follow-up visit two new coeliac disease cases with typical small bowel histological findings were detected. Thirteen out the 17 patients developing coeliac disease were in the autoantibody-positive group, three in Marsh I, and one in Marsh 0 group. In other words, 52% (13 out of 25) of patients with baseline positive autoantibodies in the serum, 12% (3 out of 25) with Marsh I and 4% (1 out of 25) with Marsh 0 at baseline were shown to develop coeliac disease during the follow-up ($p < 0.001$). At the baseline 10 (59%) out of 17 patients with early developing coeliac disease had elevated densities of CD3+ IELs, 13 (76%) out of 17 elevated densities of γδ+ IELs and 14 (88%) out of 16 elevated densities of villous tip IELs. Small bowel mucosal TG2-specific IgA deposits were investigated in 15 early developing coeliac disease patients at the time when the mucosal architecture was interpreted as normal; 14 (93%) out of the 15 were found to have these intestinal IgA deposits before the development of villous atrophy (Table 11, Figure 3).

Of all patient series, altogether 66 patients reported in this thesis suffered from early developing coeliac disease (**III, IV**) and thus did not fulfil the diagnostic criteria for coeliac disease as determined by ESPGAN. The most common indication for endoscopy and small bowel biopsy was abdominal symptoms; half of the patients were investigated because of diarrhoea, flatulence, indigestion, abdominal distension or abdominal pain (Table 10).

The mean value of Vh/CrD was normal in these subjects (Vh/CrD ≥ 2), but statistically significantly lower than in non-coeliac control subjects (Table 11) (**III, IV**). The densities of CD3+, αβ+, γδ+ and villous tip IELs were significantly higher than in non-coeliac controls, and 80% of early developing coeliac disease patients evinced an even distribution of IELs along villous sides (Table 11 and Figure 5). Seventy per cent of the patients had positive IgA-class coeliac autoantibodies in the serum (ARA or EmA) despite normal mucosal architecture, and 67% were IgA-AGA-positive. All 36 early developing coeliac disease patients with available results were HLA DQ2- or DQ8-positive.

The presence of intestinal IgA deposits reached 93% sensitivity and 100% specificity in detecting early developing coeliac disease (Table 13). The percentages for IELs were lower; the sensitivity of CD3+ IELs was 61% and the specificity was 69%, and the corresponding values for γδ+ IELs were 82% and 81%, and for villous tip IELs 85% and 88%, respectively. HLA DQ2/DQ8

reached 100% sensitivity, but the specificity was lower (73%). Also, serum coeliac autoantibodies had 100% specificity but sensitivity was 70%.

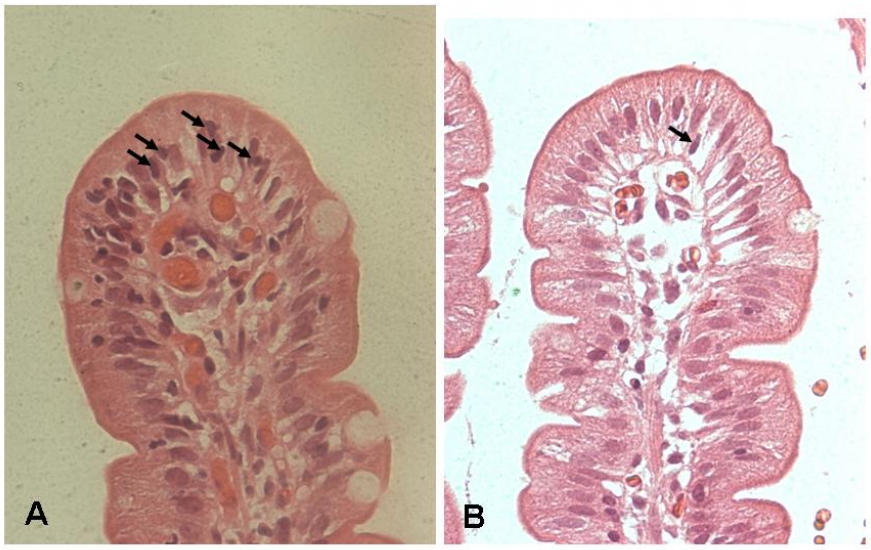

Figure 5. *Increased density of villous tip intraepithelial lymphocytes (IELs) in a haematoxylin and eosin (HE)-stained small bowel biopsy specimen from a patient with early developing coeliac disease (A) compared to the normal density of villous tip IELs shown in a specimen from a non-coeliac control subject (B). Arrows indicate villous tip IELs.*

4.4. Coeliac disease suspected but excluded

In the follow-up study (**IV**) 30 patients consuming a normal gluten-containing diet were again excluded for coeliac disease based on normal small bowel biopsy after the follow-up period (median duration 7 years). All of these patients were EmA and TG2 antibody-negative at the follow-up investigation, and five of these had undergone negative seroconversion, since they were IgA-class coeliac autoantibody-positive (ARA or EmA) at baseline when coeliac disease was first suspected. At baseline 13 out of 30 (43%) patients again excluded for coeliac disease had elevated densities of CD3+ IELs, 12 out of 30 (40%) elevated densities of γδ+ IELs and 8 out of 28 (29%) elevated densities of villous tip IELs. Fourteen patients were investigated for intestinal autoantibody deposits at baseline; one was found to have TG2-specific IgA deposits present in the small bowel mucosa (Table 11 and Figure 3).

In studies **I, III** and **IV** altogether 608 patients were investigated in view of coeliac disease suspicion, but were excluded for the disease based on normal small bowel mucosal villous architecture. Fifty-four per cent underwent small bowel biopsy because of abdominal symptoms, 20% suffered from malabsorption or anaemia, and 16% had atypical symptoms such as neurological symptoms, mouth ulcerations, osteoporosis, infertility, etc (Table 10).

The mean value of Vh/CrD was normal in these subjects (Vh/CrD $\geq$ 2), but still statistically lower than in non-coeliac controls (Table 11) (**I, III, IV**). The densities of $\gamma\delta$+ or villous tip IELs did not differ statistically from those in non-coeliac controls subjects, and the densities of CD3+ and $\alpha\beta$+ IELs were lower than in non-coeliac controls. Thirteen per cent of the patients excluded for coeliac disease had positive coeliac autoantibodies in the serum, and 61% had positive serum IgA-class AGA. Twenty-seven per cent (22 out of 82) were HLA DQ2 or DQ8 positive.

4.5. Target specificity of small bowel mucosal IgA deposits

To ascertain that small bowel mucosal IgA deposits detected in the intestinal mucosa of untreated coeliac disease patients target TG2, further investigations of the target specificity of these deposits were undertaken in seven serum EmA-negative and six EmA-positive coeliac patients (**II**). After citrate buffer and 0.5-1 M KSCN treatments, the small bowel mucosal subepithelial and pericryptal IgA deposits along TG2 in untreated coeliac disease patients remained unchanged. In contrast, the amount of IgA deposits substantially decreased in eight samples and almost completely disappeared in five when the sections were treated additionally with chloroacetic acid, which removes TG2 from its fibronectin binding sites. The amount of detectable TG2 also decreased in parallel, whereas IgA in epithelial cells' brush border remained essentially unchanged.

When the small bowel sections were incubated in vitro with human recombinant GST-TG2, binding of GST-TG2 was observed both to coeliac and to non-coeliac tissue sections along fibronectin. When this nonspecific binding to fibronectin was blocked by preincubating GST-TG2 with the soluble 45 kDa fragment of fibronectin as well as G92 monoclonal anti-TG2 mouse antibodies, GST-TG2 bound only to the coeliac tissue, co-localizing with the IgA deposits. However, it did not bind to duodenum sections from non-coeliac controls without extracellular IgA deposition. Small bowel sections from the serum EmA-negative coeliac patients gave results similar to those from the EmA-positive coeliac samples. These above-mentioned experiments demonstrate that coeliac IgA antibodies were specifically bound in situ to TG2 target antigen in duodenum samples from untreated coeliac disease patients.

5. DISCUSSION

5.1. Challenges in the diagnosis of coeliac disease

According to the criteria determined by ESPGAN the diagnosis of coeliac disease requires the presence of small bowel mucosal villous atrophy and crypt hyperplasia together with clear-cut clinical recovery on a gluten-free diet, and serology has a supportive role (Walker-Smith et al. 1990). However, coeliac disease has no pathognomic histological features (Freeman 2004, Goldstein 2004) and diagnosis can be difficult especially in the presence of borderline histology. The diagnosis should invariably be made from well-oriented high-quality samples (Walker-Smith et al. 1990), but sampling is often compromised (Collin et al. 2005) and false-positive and -negative findings may result. Occasionally villous shortening can be seen only in certain parts of the small bowel mucosa (Scott and Losowsky 1976), and this patchy form of villous atrophy may be easily overlooked. Furthermore, although a positive serum EmA has a close to 100% specific association with coeliac disease (Ladinser et al. 1994), approximately 10-20% of untreated coeliac disease patients remain negative for serum EmA and TG2 antibodies (McMillan et al. 1991, Dickey et al. 2000, Sblattero et al. 2000, Tesei et al. 2003). Negative serology upon coeliac disease suspicion always gives rise to some ambiguity (Korponay-Szabo et al. 1997, Kwiecien et al. 2005) and occasionally, in obscure cases, a histological or clinical response to a gluten-free diet or even gluten challenge is required to ascertain the diagnosis (Korponay-Szabo et al. 1997).

The diagnosis of coeliac disease is rendered even more complicated by the fact that the mucosal deterioration in the condition occurs gradually, villous atrophy (Marsh III) being the end stage in the clinical course of the disease, preceded by infiltration of IELs (Marsh I) and crypt hypertrophy (Marsh II) (Marsh 1992). Before the development of villous atrophy diagnosis of coeliac disease is even more challenging than in classical coeliac disease with villous atrophy, since minor histological abnormalities are unspecific, subjective and difficult to interpret. Nonetheless, it has become evident that the recognition of coeliac disease without villous atrophy is important (Kaukinen et al. 2001, Tursi and Brandimarte 2003, Paparo et al. 2005) especially in that at least some patients might benefit of dietary treatment. However, so far no single marker is considered a reliable indicator of this condition. Marsh I lesion, increased density of γδ+ or villous tip IELs may be indicative of early developing coeliac disease, but the specificities of these findings has not previously been assessed in a large patient material.

Serum IgA-class EmA and TG2 antibodies are thought to enter the circulation only after severe villous atrophy has developed, and thus their value in early developing coeliac disease has been questioned (Rostami et al. 1999, Tursi et al. 2001, Abrams et al. 2004). In contrast, there are also cases where coeliac autoantibodies have appeared in the serum before the development of

villous atrophy, implying that they might be valuable in identifying patients with early developing coeliac disease (Collin et al. 1993, Kaukinen et al. 1998, Iltanen et al. 1999b, Iltanen et al. 1999c). Evidence shows that coeliac autoantibodies are produced in the small bowel mucosa (Picarelli et al. 1996a, Marzari et al. 2001), even though usually measured in the serum. It has been recognized for decades that the small intestinal epithelial basement membrane region contains deposited IgA in untreated coeliac disease (Shiner and Ballard 1972, Jos et al. 1979, Rantala et al. 1985, Karpati et al. 1988), and it was recently demonstrated in vivo that the target of this IgA deposition is TG2 (Korponay-Szabo et al. 2004). Further, preliminary results have suggested that TG2-targeted intestinal autoantibody deposits might be detectable before the development of overt villous atrophy and precede the appearance of serum coeliac autoantibodies (Korponay-Szabo et al. 2004, Kaukinen et al. 2005).

5.2. Morphometrical analysis and determination of intraepithelial lymphocytes in coeliac disease diagnosis and follow-up of dietary treatment

In this study series villous atrophy and crypt hyperplasia was demonstrated in all 223 patients with overt untreated coeliac disease (**I, II, III**); in treated coeliac disease patients adhering to a gluten-free diet a median of 12 months Vh/CrD was higher than in untreated coeliac disease patients, but still lower than in non-coeliac controls (**I, III**) (Table 11). This means that total histological recovery takes a long time, even on a strict diet (Mayer et al. 1991, Kaukinen et al. 1999). It is noteworthy that severe villous atrophy was also demonstrated here in three non-coeliac control patients with autoimmune enteropathy (**II**), which may make for significant diagnostic difficulties in clinical practice. This study further demonstrated that additional 66 patients not fulfiling the diagnostic criteria of ESPGAN suffered from untreated coeliac disease (**III, IV**); these patients were suffering from early developing coeliac disease, where by definition villous atrophy has not yet developed. Thus the sensitivity of the ESPGAN criteria to detect untreated coeliac disease was only 77%. Early developing coeliac disease patients here showed minor histological abnormalities in their small bowel mucosa; their Vh/CrD was statistically significantly lower than in non-coeliac controls, but still within normal range. The interpretation of this sort of minor mucosal abnormalities is particularly difficult and subjective, and most importantly, unreliable by conventional histology.

It was demonstrated as far back as 1971 that the number of intraepithelial lymphocytes is increased in the intestinal mucosa of patients with untreated coeliac disease (Ferguson and Murray 1971). Intraepithelial lymphocytosis is thought to precede manifest mucosal lesion in the development of coeliac disease (Marsh 1992). Small bowel IELs are for the most part CD3+ lymphocytes, and their density corresponds closely to that of IELs counted in ordinary HE-stained biopsy samples (Arranz et al. 1994). This study showed that CD3+ IELs are

usually but not invariably increased in untreated coeliac disease, but this finding proved to be rather unspecific (Table 13). CD3+ IELs were shown to be gluten-dependent, since their density decreased on a gluten-free diet and remained only slightly elevated after a median of 12 months on diet (Table 11). The accuracy of the increased density of CD3+ IELs corresponding to Marsh I lesion in early developing coeliac disease was even poorer than in coeliac disease with villous atrophy; sensitivity being 61% and specificity 69% for the condition (Table 13). This means that the Marsh classification cannot be reliably employed in the detection of early developing coeliac disease, and patients with Marsh I lesion in the small bowel mucosa should not be advised to adhere to a gluten-free diet in the absence of additional evidence. The determination of αβ+ IELs proved even less sensitive and specific in both classical and early developing coeliac disease than the determination of CD3+ IELs (Table 13).

γδ+ IELs determined from frozen sections are considered to be highly sensitive and specific for coeliac disease (Camarero et al. 2000). The current study partially confirmed this conception; in borderline cases, the increase in γδ+ cells further strengthens the probability of coeliac disease. However, γδ+ IELs may be within normal limits in patients with untreated coeliac disease (Table 13). In coeliac disease patients with EATL, the sensitivity of these cells may be even lower; rearrangement in the T-cell receptorγ gene together with low densities of γδ+ IELs has been documented in patients with refractory sprue or EATL (Cellier et al. 1998, Farstad et al. 2002). Also, on the other hand, an increased density of γδ+ IELs is not restricted to HLA DQ2 or DQ8 (Chan et al. 1993, Iltanen et al. 1999c) and hence not a specific finding for coeliac disease, as demonstrated in this study. In treated coeliac disease the density of γδ+ IELs was lower than in untreated disease, but remained elevated compared to non-coeliac controls (Table 11). It was further shown here that increased densities of γδ+ IELs were indicative of early developing coeliac disease, since the sensitivity of these cells to detect coeliac disease without villous atrophy was 82%. However, in the follow-up study (**IV**) 40% of those patients excluded for coeliac disease again on re-evaluation, after a median of 7 year follow-up period, also had had increased densities of these cells at baseline, when coeliac disease was first suspected. Thus apparently false-positive γδ+ cell densities were shown here, as similarly reported elsewhere (Iltanen et al. 1999c, Kaukinen et al. 2005). Altogether the determination of γδ+ IELs may be of value in obscure cases where conventional histology is ambiguous; however, the need for frozen biopsy samples limits its utility.

The clear advantage of the determination of villous tip IELs is that the method is easy and fast and does not require frozen specimens, since villous tip IELs can be studied in routine HE-stained sections. This study demonstrated that the value of villous tip IELs in discovering patients with coeliac disease with severe partial villous atrophy and early developing coeliac disease was superior to Marsh I lesion and at least as good as the determination of γδ+ IELs (Table 13). In contrast, determination of the IEL distribution pattern along villi was not found to be a reliable indicator of early developing coeliac disease in this study.

This was opposite to previous results on small patient series (Goldstein and Underhill 2001), where the method was considered to be a reliable marker of gluten sensitivity. In the present study with a larger patient material, an even distribution was found in most patients with newly detected coeliac disease with severe partial villous atrophy or early developing coeliac disease, but also in almost half of the non-coeliac controls. Further, interpretation is highly observer-dependent, and the density of IELs in villous tips seems clearly to be a more reliable marker of gluten sensitivity than that in other parts of the villi.

5.3. Coeliac antibodies in the serum and intestine in untreated and treated coeliac disease

Serum IgA-class coeliac autoantibodies proved reliable in disclosing coeliac disease with villous atrophy in this study: the sensitivity for the condition was 84% and the specificity was 100%. In contrast, IgA-class AGA was found to be relatively insensitive and unspecific for coeliac disease (Table 13). The coeliac autoantibodies used in the present study were ARA or EmA, depending on the time of testing; these tests have been shown to be virtually identical in experienced hands (Hällström 1989). Collection of data began in 1995, and TG2 was not identified as the main and probably the sole autoantigen for EmA until 1997 (Dieterich et al. 1997). ARA and EmA have both been shown to detect TG2 in rodent as well as in primate tissues (Korponay-Szabo et al. 2000), and it has further been demonstrated that EmA and ARA binding patterns in serum samples from coeliac disease patients are exclusively TG2-dependent (Korponay-Szabo et al. 2000, Korponay-Szabo et al. 2003). EmA and TG2 antibody tests correlate closely (Sulkanen et al. 1998b, Mäki et al. 2003) and neither test has proved to be superior to the other; their sensitivity and specificity values have been equal (Hill 2005), even though some occasional patients remain negative for EmA despite being positive for TG2 antibodies, and vice versa. One explanation for this could be that the EmA and TG2-ELISA test systems expose TG2 antigenic epitopes in different ways.

The prevalence figure (15%) for EmA-negative coeliac disease in study **II** was comparable to that reported elsewhere (McMillan et al. 1991, Collin et al. 2005). Interestingly, the majority of EmA-negative patients were male, and furthermore, EmA-negative patients were older and had more abdominal symptoms than EmA-positive subjects (Table 12). One reason for the more conspicuous clinical symptoms in EmA-negative individuals might be that EmA-positive patients are more rigorously examined for coeliac disease, whereas the disease has remained unrecognized for a long time in EmA-negative individuals. The disappearance of gliadin antibodies from the serum of coeliac patients who had discontinued their gluten-free diet for a long period of time has previously been shown (Burgin-Wolff et al. 1988). Moreover, the lack of the humoral immune response typical of coeliac disease in patients with EATL has also been demonstrated (O'Farrelly et al. 1986). In the present study, negative EmA in

three untreated coeliac disease patients with EATL also supports the conclusion that EmA-negativity is connected with long-lasting, severe disease.

The majority of patients with positive coeliac autoantibodies in the serum but normal villous architecture were shown in this study to develop coeliac disease (**IV**). Hence patients having "false-positive" coeliac autoantibodies in the serum are at risk of developing overt coeliac disease; the sensitivity of IgA-class ARA/EmA in early developing coeliac disease proved to be 70%. However, in the follow-up study (**IV**) five autoantibody-positive patients did not proceed to villous atrophy during the follow-up, though it is possible that a longer follow-up might have revealed progression of the disease in these individuals. HLA DQ is applied in the identification of patients with genetic gluten intolerance, and patients with HLA DQ2 or DQ8 having positive coeliac autoantibodies in the serum are highly likely to be suffering from coeliac disease. However, HLA determination alone is of little value except when excluding coeliac disease, since the specificity of this method is low (Table 13).

This study showed that autoantibodies (equivalent to EmA) targeted against TG2 were deposited in the small bowel mucosa of all coeliac disease patients with overt villous atrophy, regardless of serology, and further, these deposits were gluten-dependent (Figure 3) (**II**). None of the non-coeliac controls with intestinal diseases was shown to have similar IgA deposits in their mucosa. Thus the detection of intestinal IgA deposits proved to be highly valuable in differentiating between coeliac disease and other causes of villous atrophy such as autoimmune enteropathy. Furthermore, the follow-up study (**IV**) showed that intestinal IgA deposits targeted against TG2 are currently the best method in revealing early developing coeliac disease compared to determination of the densities of CD3+, αβ+, γδ+ or villous tip IELs. By investigating these deposits in small bowel biopsy specimens, when coeliac disease was first suspected, it was possible to detect early developing coeliac disease in 93% of the subjects before the development of forthcoming villous atrophy (**IV**). Investigation of intestinal IgA deposits is a special method requiring frozen small bowel biopsy specimens, which limits its utility. Nonetheless, this method should be available at least in special centres, since it is clearly beneficial in cases where the conventional histology is not diagnostic, and a follow-up investigations with a second small bowel biopsy are needed to confirm or to exclude coeliac disease.

Moreover, it was also demonstrated here that the in vivo deposited IgA is functional towards TG2, as it was able also to bind externally added recombinant human TG2 (**II**). It would appear that in seronegative coeliac disease patients autoantibodies are sequestered in the bowel and their presence in the serum is caused by "spill-over" from the gut. The results also indicate that coeliac antibodies are bound to intestinal TG2 with considerably high avidity. IgA antibodies of EmA-negative patients could not be removed from the gut tissue by moderate amounts of KSCN, which is frequently used to test the avidity of antigen-antibody binding (Jones et al. 1987). During a long-standing immune reaction antibodies with increasing avidity are produced, which might result in seronegativity in long-standing coeliac disease.

5.4. Re-evaluation of the diagnostic criteria for coeliac disease

This study has shown that the ESPGAN diagnostic criteria for coeliac disease are no longer valid. First, villous atrophy and crypt hyperplasia can also be present in other disorders, and there is especially uncertainty in the diagnosis of coeliac disease when serum EmA is negative (Korponay-Szabo et al. 1997, Kwiecien et al. 2005). Clinical response to gluten-free diet does not always solve this clinical problem, since patients without coeliac disease have been known to benefit from gluten-free dietary treatment. Also, the gluten challenge is laborious and time-consuming and hence at present not very often applied. Secondly, it is currently recognized that many patients suffer from gluten-dependent symptoms (Table 10) and even coeliac disease complications such as osteoporosis before the development of villous atrophy (Kaukinen et al. 2001, Tursi and Brandimarte 2003, Paparo et al. 2005). These patients with early developing coeliac disease do not fulfil the traditional ESPGAN diagnostic criteria, which only detected 77% of untreated coeliac disease patients in this study. The current diagnostic criteria should thus be revised. On the other hand, it is important not to advise patients to adhere to a life-long gluten-free diet without firm evidence. Up to this point reliable means of ascertaining coeliac disease diagnosis in seronegative patients and especially in early developing coeliac disease have been lacking.

The results of this study should be taken into consideration when the diagnostic criteria for coeliac disease are revised. Table 14 shows the suggested actions to take based on this study when coeliac disease is suspected but the histology is not diagnostic or when early developing coeliac disease is suspected. When the histology is ambiguous but suggestive of coeliac disease an increased density of γδ+ and villous tip IELs indicates coeliac disease; in contrast, increased densities of CD3+ or αβ+ IELs are an unspecific finding and further evidence is needed before gluten-free dietary treatment can be advised. Serum and especially intestinal coeliac autoantibodies strengthen the diagnosis of coeliac disease and dietary treatment is justified. However, when there is any ambiguity in the diagnosis of coeliac disease in the presence of villous atrophy and further evidence is needed, intestinal TG2-specific IgA deposits should be investigated. These deposits should also invariably be investigated when early developing coeliac disease is suspected, and in the presence of the deposits gluten-free dietary treatment might be beneficial, at least in symptomatic patients. However, more prospective studies of the benefits of gluten-free dietary treatment in early developing coeliac disease patients must be undertaken before dietary treatment can be advised to all such patients. In cases where early developing coeliac disease is suspected on clinical grounds, and the density of γδ+ or villous tip IELs is increased or serum coeliac autoantibodies are positive, patients should be followed up. Alternatively, intestinal autoantibody deposits

can be investigated. However, Marsh I lesion without any other evidence of coeliac disease does not require routine surveillance (Table 14).

Table 14. *Measures suggested on the basis of the results of this study when histology is ambiguous but villous atrophy and coeliac disease (CD) are suspected or when villous architecture is normal in the small bowel mucosa but early developing CD is suspected*

	Histology suggestive but not diagnostic for CD	Normal villous architecture; early developing CD suspected on clinical grounds
The density of γδ+ IELs increased	CD diagnosis probable, consider GFD	Follow-up to detect possible subsequent CD
The density of αβ+ IELs increased	No CD diagnosis, further evidence needed	Unspecific finding, no routine follow-up
The density of CD3+ IELs increased	No CD diagnosis, further evidence needed	Unspecific finding, no routine follow-up
The density of villous tip IELs increased	CD diagnosis probable, consider GFD*	Follow-up to detect possible subsequent CD
Serum IgA-class ARA/EmA elevated	CD diagnosis can be made and GFD advised	Follow-up; the majority of patients will develop subsequent CD
Intestinal TG2-specific IgA deposits present	CD diagnosis can be made and GFD advised	CD diagnosis probable, consider GFD

GFD=Gluten-free diet
* Investigation of villous tip IELs not possible if total or subtotal villous atrophy is present

6. CONCLUSIONS AND FUTURE ASPECTS

This study showed that the ESPGAN diagnostic criteria relying on conventional histology are not very reliable in current coeliac disease diagnosis. It is important to emphasize that the majority of coeliac disease patients missed by conventional histology in this study suffered from clinical symptoms.

This study also shed light on the value of determination of IELs in coeliac disease diagnosis. It was shown that even though the density of CD3+, αβ+ and γδ+ IELs is usually elevated in untreated coeliac disease, it may also be normal. Further, these cells are not pathognomic for coeliac disease, as their density can be elevated in other disorders and their value in coeliac disease diagnostics is thus limited. However, in borderline cases in the presence of villous atrophy the determination of γδ+ IELs or villous tip IELs (severe partial villous atrophy) can support the diagnosis of coeliac disease. This study also assessed the value of IELs in early developing coeliac disease; previously no statistical analysis of the reliability of different markers in early developing coeliac disease has been undertaken, since the literature has mainly consisted of case reports. It was shown that in early developing coeliac disease increased density of villous tip IELs or γδ+ IELs supports the diagnosis but does not confirm it, since these cells are not specific for coeliac disease. On the other hand, the value of determinating CD3+ IELs in early developing coeliac disease seems to be minimal.

This study has shown that the determination of coeliac autoantibodies from the serum, and especially from the intestine where the antibodies are produced, is highly valuable in the diagnosis of coeliac disease with villous atrophy and also in early developing coeliac disease. The detection of intestinal autoantibody deposits proved a most reliable marker of coeliac disease in this study compared to methods utilized hitherto.

In the future conventional histology may no longer remain the gold standard in the coeliac disease diagnosis, and partly based on the results of this study a new diagnostic algorithm for coeliac disease diagnosis is suggested in Figure 6. Such an algorithm might be applicable in the future, but not before more prospective studies have been made of the value of serum coeliac autoantibodies and the determination of intestinal IgA deposits in larger patient material. In the algorithm it is suggested that when coeliac disease is suspected the first step would be to investigate serum EmA or TG2 antibodies. If one of these autoantibodies is positive, genetic gluten intolerance should be confirmed by showing the presence of HLA DQ2 and DQ8, and a clinical and serological response to a gluten-free diet will eventually confirm the diagnosis of coeliac disease. In cases were response to dietary treatment cannot be demonstrated, small bowel biopsy should be performed, which is also the second-step procedure if serum coeliac autoantibodies or HLA DQ2 and DQ8 remain negative despite clinical suspicion of the disease. Small bowel specimens should

be studied for the presence of villous atrophy, and TG2-specific IgA deposits should also be investigated in frozen sections. If a small bowel specimen shows villous atrophy with TG2-targeted IgA deposits, a coeliac disease diagnosis can be established and a gluten-free diet advised. Also, the presence of intestinal IgA deposits even in the absence of villous atrophy is highly indicative of early developing coeliac disease and a gluten-free diet should be considered, at least in symptomatic patients. When intestinal TG2-specific IgA deposits are not detectable in the presence of villous atrophy causes of villous atrophy other than coeliac disease should also be considered and investigated.

The diagnostic algorithm shown here has one important benefit; coeliac disease diagnosis in the majority of patients could be established without invasive small bowel biopsy. However, more studies supporting the value of serum and intestinal coeliac autoantibodies in the diagnosis of coeliac disease must be undertaken before the diagnostic criteria for coeliac disease can be revised in such a dramatic manner.

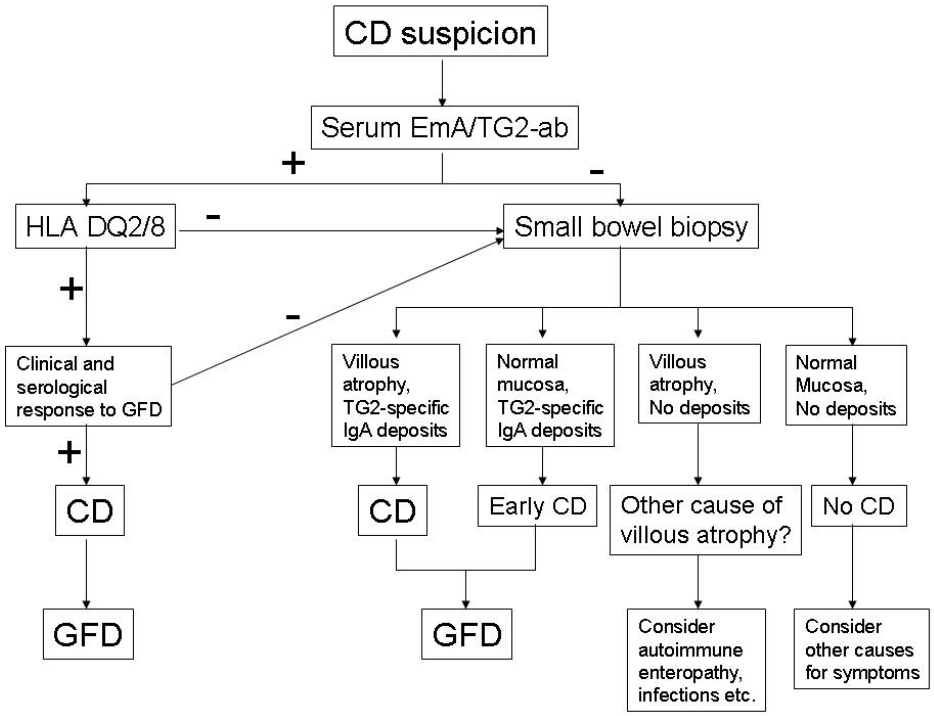

Figure 6. *Proposal for a diagnostic algorithm for coeliac disease (CD = coeliac disease, ab = antibodies, GFD = gluten-free diet)*

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8. REFERENCES

Abrams JA, Diamond B, Rotterdam H and Green PHR (2004): Seronegative celiac disease: increased prevalence with lesser degrees of villous atrophy. Dig Dis Sci 49:546-550.

Aine L, Mäki M, Collin P and Keyriläinen O (1990): Dental enamel defects in celiac disease. J Oral Pathol Med 19:241-245.

Ambrus A, Banyai I, Weiss MS, Hilgenfeld R, Keresztessy Z, Muszbek L and Fesus L (2001): Calcium binding of transglutaminases: a 43Ca NMR study combined with surface polarity analysis. J Biomol Struct Dyn 19:59-74.

Arato A, Hacsek G and Savilahti E (1998): Immunohistochemical findings in the jejunal mucosa of patients with coeliac disease. Scand J Gastroenterol Suppl 228:3-10.

Arentz-Hansen H, Körner R, Molberg O, Quarsten H, Vader W, Kooy YMC, Lundin KEA, Koning F, Roepstorff P, Sollid LM and McAdam SN (2000): The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J Exp Med 191:603-612.

Arnaud-Battandier F, Cerf-Bensussan N, Amsellem R and Schmitz J (1986): Increased HLA-DR expression by enterocytes in children with celiac disease. Gastroenterology 91:1206-1212.

Arranz E and Ferguson A (1993): Intestinal antibody pattern of celiac disease: occurrence in patients with normal jejunal biopsy histology. Gastroenterology 104:1263-1272.

Arranz E, Bode J, Kingstone K and Ferguson A (1994): Intestinal antibody pattern of coeliac disease: association with gamma/delta T cell receptor expression by intraepithelial lymphocytes, and other indices of potential coeliac disease. Gut 35:476-482.

Ascher H, Hahn-Zoric M, Hanson LÅ, Kilander AF, Nilsson LÅ and Tlaskalova H (1996): Value of serologic markers for clinical diagnosis and population studies of coeliac disease. Scand J Gastroenterol 31:61-67.

Askling J, Linet M, Gridley G, Halstensen TS, Ekström K and Ekbom A (2002): Cancer incidence in a population-based cohort of individuals hospitalized with celiac disease or dermatitis herpetiformis. Gastroenterology 123:1428-1435.

Augustin MT, Kokkonen J and Karttunen TJ (2005): Duodenal cytotoxic lymphocytes in cow's milk protein sensitive enteropathy and coeliac disease. Scand J Gastroenterol 40:1398-1406.

Auricchio R, Paparo F, Maglio M, Franzese A, Lombardi F, Valerio G, Nardone G, Percopo S, Greco L and Troncone R (2004): In vitro-deranged intestinal immune response to gliadin in type I diabetes. Diabetes 53:1680-1683.

Ballinger A, Hughes C, Kumar P, Hutchinson I and Clark M (1994): Dental enamel defects in coeliac disease. Lancet 343:230-231.

Bardella MT, Vecchi M, Conte D, Del Ninno E, Fraquelli M, Pacchetti S, Minola E, Landoni M, Cesana BM and De Franchis R (1999): Chronic unexplained hypertransaminasemia may be caused by occult celiac disease. Hepatology 29:654-657.

Bardella MT, Trovato C, Cesana BM, Pagliari C, Gebbia C and Peracchi M (2001): Serological markers for coeliac disease: is it time to change? Dig Liver Dis 33:426-431.

Bernstein CN and Leslie WD (2003): The pathophysiology of bone disease in gastrointestinal disease. Eur J Gastoenterol Hepatol 15:857-864.

Biagi F, Ellis HJ, Yiannakou JY, Brusco G, Swift G, Smith PM, Corazza GR and Ciclitira PJ (1999): Tissue transglutaminase antibodies in celiac disease. Am J Gastroenterol 94:2187-2192.

Biagi F, Pezzimenti D, Campanella J, Vadacca GB and Corazza GR (2001): Endomysial and tissue transglutaminase antibodies in coeliac sera: a comparison not influenced by previous serological testing. Scand J Gastroenterol 36:955-958.

Bister V, Kolho K-L, Karikoski R, Westerholm-Ormio M, Savilahti E and Saarialho-Kere U (2005): Metalloelastase (MMP-12) is upregulated in the gut of pediatric patients with potential celiac disease and in type 1 diabetes. Scand J Gastroenterol 40:1413-1422.

Bode S and Gudmand-Hoyer E (1996): Symptoms and haematological features in consecutive adult coeliac patients. Scand J Gastroenterol 31:54-60.

Boismenu R and Havran WL (1994): Modulation of epithelial growth by intraepithelial gamma/delta T cells. Science 266:1253-1255.

Bonamico M, Mariani P, Danesi HM, Crisogianni M, Failla P, Gemme G, Quartino AR, Giannotti A, Castro M, Balli F, Lecora M, Andria G, Guariso G, Gabrielli O, Catassi C, Lazzari R, Balocco NA, De Virgiliis S, Culasso F and Romano C (2001a): Prevalence and clinical picture of celiac disease in Italian Down syndrome patients: a multicentre study. J Pediatr Gastroenterol Nutr 33:139-143.

Bonamico M, Tiberti C, Picarelli A, Mariani P, Rossi D, Cipolletta E, Greco M, Di Tola M, Sabbatella L, Carabba B, Magliocca FM, Strisciuglio P and Di Mario U (2001b): Radioimmunoassay to detect antitransglutaminase autoantibodies is the most sensitive and specific screening method for celiac disease. Am J Gastroenterol 96:1536-1540.

Bonamico M, Pasquino AM, Mariani P, Danesi HM, Culasso F, Mazzanti L, Petri A and Bona G (2002): Prevalence and clinical picture of celiac disease in Turner syndrome. J Clin Endocrinol Metab 87:5495-5498.

Bottaro G, Volta U, Spina M, Rotolo N, Sciacca A and Musumeci S (1997): Antibody pattern in childhood celiac disease. J Pediatr Gastroenterol Nutr 24:559-562.

Bourne JT, Kumar P, Huskisson EC, Mageed R, Unsworth DJ and Wojtulewski JA (1985): Arthritis and coeliac disease. Ann Rheum Dis 44:592-598.

Burgin-Wolff A, Bertele RM, Berger R, Gaze H, Harms HK, Just M, Khanna S, Schurmann K, Signer E and Tomovic D (1983): A reliable screening test for childhood celiac disease: fluorescent immunosorbent test for gliadin antibodies. A prospective multicenter study. J Pediatr 102:655-660.

Burgin-Wolff A, Gaze H, Hadziselimovic F, Lentze M, Nussle MD and Reymond-Berthet C (1988): The diagnostic significance of gliadin and endomysium antibodies in coeliac disease of children and adults. In: Coeliac disease: one hundred years, pp 106-109. Eds. PJ Kumar and JA Walker-Smith. Leeds University Press, Leeds.

Camarero C, Eiras P, Asensio A, Leon F, Olivares F, Escobar H and Roy G (2000): Intraepithelial lymphocytes and coeliac disease: permanent changes in CD3-/CD7+ and T cell receptor gammadelta subsets studied by flow cytometry. Acta Paediatr 89:285-290.

Carlsson A, Axelsson I, Borulf S, Bredberg A, Forslund M, Lindberg B, Sjöberg K and Ivarsson S-A (1998): Prevalence of IgA -antigliadin antibodies and IgA-antiendomysium antibodies related to celiac disease in children with Down's syndrome. Pediatrics 101:272-275.

Carroccio A, Iacono G, Lerro P, Cavataio F, Malorgio E, Soresi M, Baldassarre M, Notarbartolo A, Ansaldi N and Montalto G (1997): Role of pancreatic impairment in growth recovery during gluten-free diet in childhood coeliac disease. Gastroenterology 112:1839-1844.

Carroccio A, Vitale G, Di Prima L, Chifari N, Napoli S, La Russa C, Gulotta G, Averna MR, Montalto G, Mansueto S and Notarbartolo A (2002): Comparison of anti-transglutaminase ELISAs and an anti-endomysial antibody assay in the diagnosis of celiac disease: a prospective study. Clin Chem 48:1546-1550.

Cataldo F, Marino V, Bottaro G, Greco P and Ventura A (1997): Celiac disease and selective immunoglobulin A deficiency. J Pediatr 131:306-308.

Cataldo F, Marino V, Ventura A, Bottaro G and Corazza GR (1998): Prevalence and clinical features of selective immunoglobulin A deficiency in coeliac disease: an Italian multicentre study. Gut 42:362-365.

Catassi C, Rätsch I-M, Gandolfi L, Pratesi R, Fabiani E, El Asmar R, Frijia M, Bearzi I and Vizzoni L (1999): Why is coeliac disease endemic in the people of the Sahara? Lancet 354:647-648.

Cellier C, Patey N, Mauvieux L, Jabri B, Delabesse E, Cervoni J-P, Burtin M-L, Guy-Grand D, Bouhnik Y, Modigliani R, Barbier J-P, Macintyre E, Brousse N and Cerf-Bensussan N (1998): Abnormal intestinal intraepithelial lymphocytes in refractory sprue. Gastroenterology 114:471-481.

Challacombe DN and Bayliss JM (1980): Childhood coeliac disease is disappearing. Lancet 2:1360.

Chan KN, Phillips AD, Walker-Smith JA, Koskimies S and Spencer J (1993): Density of gamma/delta T cells in small bowel mucosa related to HLA-DQ status without coeliac disease. Lancet 342:492-493.

Chartrand LJ, Russo PA, Duhaime AG and Seidman EG (1997): Wheat starch intolerance in patients with celiac disease. J Am Diet Assoc 97:612-618.

Chorzelski TP, Beutner EH, Sulej J, Tchorzewska H, Jablonska S, Kumar V and Kapuscinska A (1984): IgA anti-endomysium antibody. A new immunological marker of dermatitis herpetiformis and coeliac disease. Br J Dermatol 111:395-402.

Ciacci C, Iavarone A, Mazzacca G and De Rosa A (1998): Depressive symptoms in adult coeliac disease. Scand J Gastroenterol 33:247-250.

Ciccocioppo R, Di Sabatino A and Corazza GR (2005): The immune recognition of gluten in coeliac disease. Clin Exp Immunol 140:408-416.

Ciclitira PJ, Ellis HJ and Evans DJ (1983): A solid-phase radioimmunoassay for measurement of circulating antibody titres to wheat gliadin and its subfractions in patients with adult coeliac disease. J Immunol Methods 62:231-239.

Ciclitira PJ, Nelufer JM, Ellis HJ and Evans DJ (1986): The effect of gluten on HLA-DR in the small intestinal epithelium of patients with coeliac disease. Clin Exp Immunol 63:101-104.

Collin P, Pirttilä T, Nurmikko T, Somer H, Erilä T and Keyriläinen O (1991): Celiac disease, brain atrophy and dementia. Neurology 41:372-375.

Collin P, Korpela M, Hällström O, Viander M, Keyriläinen O and Mäki M (1992): Rheumatic complaints as a presenting symptom in patients with coeliac disease. Scand J Rheumatol 21:20-23.

Collin P, Helin H, Mäki M, Hällström O and Karvonen A-L (1993): Follow-up of patients positive in reticulin and gliadin antibody tests with normal small bowel biopsy findings. Scand J Gastroenterol 28:595-598.

Collin P, Reunala T, Pukkala E, Laippala P, Keyriläinen O and Pasternack A (1994a): Coeliac disease - associated disorders and survival. Gut 35:1215-1218.

Collin P, Salmi J, Hällström O, Reunala T and Pasternack A (1994b): Autoimmune thyroid disorders and coeliac disease. Eur J Endocrinol 130:137-140.

Collin P, Pukkala E and Reunala T (1996a): Malignancy and survival in dermatitis herpetiformis: a comparison with coeliac disease. Gut 38:528-530.

Collin P, Vilska S, Heinonen PK, Hällström O and Pikkarainen P (1996b): Infertility and coeliac disease. Gut 39:382-384.

Collin P, Kaukinen K, Välimäki M and Salmi J (2002): Endocrinological disorders and celiac disease. Endocrine Reviews 23:464-483.

Collin P, Kaukinen K, Vogelsang H, Korponay-Szabo I, Sommer R, Schreier E, Volta U, Granito A, Veronesi L, Mascart F, Ocmant A, Ivarsson A, Lagerqvist C, Burgin-Wolff A, Hadziselimovic F, Furlano RI, Sidler MA, Mulder CJJ, Goerres MS, Mearin ML, Ninaber MK, Gudmand-Hoyer E, Fabiani E, Catassi C, Tidlund H, Alainentalo L and Mäki M (2005): Anti-endomysial and anti-human recombinant tissue transglutaminase antibodies in the diagnosis of coeliac disease: a biopsy-proven European multicentre study. Eur J Gastoenterol Hepatol 17:85-91.

Cook HB, Burt MJ, Collet JA, Whitebread MR, Frampton CM and Chapman BA (2000): Adult coeliac disease: prevalence and clinical significance. J Gastroenterol Hepatol 15:1032-1036.

Cooke WT and Holmes GKT (1984): Coeliac disease. Churchill Livingstone, Edinburgh.

Cooper BT, Holmes GKT, Ferguson R, Thompson RA, Allan RN and Cooke WT (1980): Gluten-sensitive diarrhea without evidence of celiac disease. Gastroenterology 79:801-806.

Cooper BT, Holmes GKT and Cooke WT (1982): Lymphoma risk in coeliac disease of later life. Digestion 23:89-92.

Corazza GR, Frisoni M, Treggiari EA, Valentini RA, Filipponi C, Volta U and Gasbarrini G (1993): Subclinical celiac sprue. Increasing occurrence and clues to its diagnosis. J Clin Gastroenterol 16:16-21.

Corazza GR, Andreani ML, Venturo N, Bernardi M, Tosti A and Gasbarrini G (1995a): Celiac disease and alopecia areata: report of a new association. Gastroenterology 109:1333-1337.

Corazza GR, Di Sario A, Cecchetti L, Tarozzi C, Corrao G, Bernardi M and Gasbarrini G (1995b): Bone mass and metabolism in patients with celiac disease. Gastroenterology 109:122-128.

Corazza GR, Andreani ML, Biagi F, Bonvicini F, Bernardi M and Gasbarrini G (1996): Clinical, pathological, and antibody pattern of latent celiac disease: report of three adult cases. Am J Gastroenterol 91:2203-2207.

Corazza GR, Biagi F, Volta U, Andreani ML, De Franceschi L and Gasbarrini G (1997): Autoimmune enteropathy and villous atrophy in adults. Lancet 350:106-109.

Corazza GR, Zoli G, Di Sabatino A, Ciccocioppo R and Gasbarrini G (1999): A reassessment of splenic hypofunction in celiac disease. Am J Gastroenterol 94:391-397.

Cronin CC, Jackson LM, Feighery C, Shanahan F, Abuzakouk M, Ryder DQ, Whelton M and Callaghan N (1998): Coeliac disease and epilepsy. QJM 91:303-308.

Csizmadia CGDS, Mearin ML, von Blomberg BME, Brand R and Verloove-Vanhorick SP (1999): An iceberg of childhood coeliac disease in the Netherlands. Lancet 353:813-814.

Dahele A, Kingstone K, Bode J, Anderson D and Ghosh S (2001): Anti-endomysial antibody negative celiac disease. Does additional serological testing help? Dig Dis Sci 46:214-221.

Dalton TA and Bennet JC (1992): Autoimmune disease and major histocompatibility complex: therapeutic implications. Am J Med 92:183-188.

Daum S, Cellier C and Mulder CJJ (2005): Refractory coeliac disease. Best Pract Res Clin Gastroenterol 19:413-424.

Dicke WK (1950): Coeliakie. M.D. Thesis, Utrecht.

Dickey W (2002): Low serum vitamin B12 is common in coeliac diseaese and is not due to autoimmune gastritis. Eur J Gastoenterol Hepatol 14:425-427.

Dickey W and Bodkin S (1998): Prospective study of body mass index in patients with coeliac disease. BMJ 317:1290.

Dickey W, Hughes DF and McMillan SA (2000): Reliance on serum endomysial antibody testing underestimates the true prevalence of coeliac disease by one fifth. Scand J Gastroenterol 35:181-183.

Dickey W, Hughes DF and McMillan SA (2005): Patients with serum IgA endomysial antibodies and intact duodenal villi: clinical characteristics and management options. Scand J Gastroenterol 40:1240-1243.

Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO and Schuppan D (1997): Identification of tissue transglutaminase as the autoantigen of celiac disease. Nat Med 3:797-801.

Djilali-Saiah I, Schmitz J, Harfouch-Hammound E, Mougenot J-F, Bach J-F and Caillat-Zucman S (1998): CTLA-4 gene polymorphism is associated with predisposition to coeliac disease. Gut 43:187-189.

Duhring L (1884): Dermatitis herpetiformis. JAMA 3:225-228.

Eade OE, Lloyd RS, Lang C and Wright R (1977): IgA and IgG reticulin antibodies in coeliac disease and non-coeliac patients. Gut 18:991-993.

Ebert EC (1998): Interleukin 15 is a potent stimulant of intraepithelial lymphocytes. Gastroenterology 115:1439-1445.

Egan-Mitchell B, Fottrell PF and McNicholl P (1981): Early or pre-coeliac mucosa: development of gluten enteropathy. Gut 22:65-69.

Fabiani E, Taccari LM, Rätsch I-M, DiGiuseppe S, Coppa GV and Catassi C (2000): Compliance with gluten-free diet in adolescents with screening-detected celiac disease: a 5-year follow-up study. J Pediatr 136:841-843.

Farstad IN, Johansen F-E, Vlatkovic L, Jahnsen J, Scott H, Fausa O, Bjorneklett A, Brandtzaeg P and Halstensen TS (2002): Heterogeneity of intraepithelial lymphocytes in refractory sprue: potential implications of CD30 expression. Gut 51:372-378.

Farthing MJ, Edwards CR, Rees LH and Dawson AM (1982): Male gonadal function in coeliac disease: 1. Sexual dysfunction, infertility and semen quality. Gut 23:608-614.

Fasano A, Not T, Wang W, Uzzau S, Berti I, Tommasini A and Glodblum SE (2000): Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. Lancet 355:1518-1519.

Feighery C, Weir DG, Whelan A, Willoughby R, Youngprapakorn S, Lynch S, O'Morain C, McEneany P and O'Farrelly C (1998): Diagnosis of gluten-sensitive enteropathy: is exclusive reliance on histology appropriate? Eur J Gastroenterol Hepatol 10:919-925.

Ferguson A and Murray D (1971): Quantitation of intraepithelial lymphocytes in human jejunum. Gut 12:988-994.

Ferguson A, Arranz E and O'Mahony S (1993): Clinical and pathological spectrum of coeliac disease - active, silent, latent, potential. Gut 34:150-151.

Ferguson MM, Wray D, Carmichael HA, Russell RI and Lee FD (1980): Coeliac disease associated with recurrent aphthae. Gut 21:223-226.

Ferguson R, Basu MK, Asquith P and Cooke WT (1976): Jejunal mucosal abnormalities in patients with recurrent aphthous ulceration. BMJ 1:11-13.

Ferreira M, Davies SL, Butler M, Scott D, Clark M and Kumar P (1992): Endomysial antibody: is it the best screening test for coeliac disease? Gut 33:1633-1637.

Floreani A, Betterle C, Baragiotta A, Martini S, Venturi C, Basso D, Pittoni M, Chiarelli S and Sategna Guidetti C (2001): Prevalence of coeliac disease in primary biliary cirrhosis and of antimitochondrial antibodies in adult coeliac disease patients in Italy. Dig Liver Dis 33:258-261.

Freeman HJ (2004): Adult celiac disease and the severe "flat" small bowel biopsy lesion. Dig Dis Sci 49:535-545.

Fry L, Seah PP, McMinn RMH and Hoffbrand AV (1972): Lymphocytic infiltration of epithelium in diagnosis of gluten-sensitive enteropathy. BMJ 3:371-374.

Garioch JJ, Lewis HM, Sargent SA, Leonard JN and Fry L (1994): 25 years' experience of a gluten-free diet in the treatment of dermatitis herpetiformis. Br J Dermatol 131:541-545.

Gee S (1888): On the coeliac disease. St Bart Hosp Rep 24:17-20.

Goldstein NS (2004): Non-gluten sensitivity-related small bowel villous flattening with increased intraepithelial lymphocytes: not all that flattens is celiac sprue. Am J Clin Pathol 121:546-550.

Goldstein NS and Underhill J (2001): Morphologic features suggestive of gluten sensitivity in architecturally normal duodenal biopsy specimens. Am J Clin Pathol 116:63-71.

Greco L, Corazza G, Babron M-C, Clot F, Fulchignoni-Lataud M-C, Percopo S, Zavattari P, Bouguerra F, Dib C, Tosi R, Troncone R, Ventura A, Mantavoni W, Magazzu G, Gatti R, Lazzari R, Giunta A, Perri F, Iacono G, Cardi E, de Virgiliis S, Cataldo F, De Angelis G, Musumeci S, Ferrari R, Balli F, Bardella M-T, Volta U, Catassi C, Torre G, Eliaou J-F, Serre J-L and Clerget-Darpoux F (1998): Genome search in celiac disease. Am J Hum Genet 62:669-675.

Greco L, Romino R, Coto I, Di Cosmo N, Percopo S, Maglio M, Paparo F, Gasperi V, Limongelli MG, Cotichini R, D'Agate C, Tinto N, Sacchetti L, Tosi R and Stazi MA (2002): The first large population based twin study of coeliac disease. Gut 50:624-628.

Green PHR, Fleischauer AT, Bhagat G, Goyal R, Jabri B and Neugut AI (2003): Risk of malignancy in patients with celiac disease. Am J Med 115:191-195.

Grefte JMM, Bouman JG, Grond J, Jansen W and Kleibeuker JH (1988): Slow and incomplete histological and functional recovery in adult gluten sensitive enteropathy. J Clin Pathol 41:886-891.

Guandalini S, Ventura A, Ansaldi N, Giunta AM, Greco L, Lazzari R, Mastella G and Rubino A (1989): Diagnosis of coeliac disease: time for a change? Arch Dis Child 64:1320-1325.

Hadjivassiliou M, Gibson A, Davies-Jones GAB, Lobo AJ, Stephenson TJ and Milford-Wars A (1996): Does cryptic gluten sensitivity play a part in neurological illness? Lancet 347:369-371.

Hallert C and Derefeldt T (1982): Psychic disturbances in adult coeliac disease. I. Clinical observations. Scand J Gastroenterol 17:17-19.

Hällström O (1989): Comparison of IgA-class reticulin and endomysium antibodies in coeliac disease and dermatitis herpetiformis. Gut 30:1225-1232.

Halstensen TS, Scott H and Brandtzaeg P (1989): Intraepithelial T cells of the TcR gamma/delta+ CD8- and V delta 1/J delta 1+ phenotypes are increased in coeliac disease. Scand J Immunol 30:665-672.

Halttunen T and Mäki M (1999): Serum immunoglobulin A from patients with celiac disease inhibits human T84 intestinal crypt epithelial cell differentiation. Gastroenterology 116:566-572.

Hardman CM, Garioch JJ, Leonard JN, Thomas HJW, Walker MM, Lortan JE, Lister A and Fry L (1997): Absence of toxicity of oats in patients with dermatitis herpetiformis. N Engl J Med 337:1884-1887.

Hervonen K, Hakanen M, Kaukinen K, Collin P and Reunala T (2002): First-degree relatives are frequently affected in coeliac disease and dermatitis herpetiformis. Scand J Gastroenterol 37:51-55.

Hill ID (2005): What are the sensitivity and specificity of serological tests for celiac disease? Do sensitivity and specificity vary in different populations? Gastroenterology 128:S25-32.

Hin H, Bird G, Fisher P, Mahy N and Jewell D (1999): Coeliac disease in primary care: case finding study. BMJ 318:164-167.

Högberg L, Laurin P, Fälth-Magnusson K, Grant C, Grodzinsky E, Jansson G, Ascher H, Browaldh L, Hammersjö J-Å, Lindberg E, Myrdal U and Stenhammar L (2004): Oats to children with newly diagnosed coeliac disease: a randomised double blind study. Gut 53:649-654.

Holmes GKT, Prior P, Lane MR, Pope D and Allan RN (1989): Malignancy in coeliac disease - effect of a gluten free diet. Gut 30:333-338.

Holopainen P, Arvas M, Sistonen P, Mustalahti K, Collin P, Mäki M and Partanen J (1999): CD28/CTLA4 gene region on chromosome 2q3 confers genetic susceptibility to celiac disease. A linkage and family-based association study. Tissue Antigens 53:470-475.

Hue S, Mention J-J, Monteiro RC, Zhang S, Cellier C, Schmitz J, Verkarre V, Fodil N, Bahram S, Cerf-Bensussan N and Caillat-Zucman S (2004): A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity 21:367-377.

Iltanen S, Collin P, Korpela M, Holm K, Partanen J, Polvi A and Mäki M (1999a): Celiac disease and markers of celiac disease latency in patients with primary Sjögren's syndrome. Am J Gastroenterol 94:1042-1046.

Iltanen S, Holm K, Ashorn M, Ruuska T, Laippala P and Mäki M (1999b): Changing jejunal gamma/delta T cell receptor (TCR)-bearing intraepithelial lymphocyte density in coeliac disease. Clin Exp Immunol 117:51-55.

Iltanen S, Holm K, Partanen J, Laippala P and Mäki M (1999c): Increased density of jejunal gamma/delta+ T cells in patients having normal mucosa - marker of operative autoimmune mechanisms? Autoimmunity 29:179-187.

Ivarsson A, Persson LÅ, Juto P, Peltonen M, Suhr O and Hernell O (1999a): High prevalence of undiagnosed coeliac disease in adults: a Swedish population-based study. J Intern Med 245:63-68.

Ivarsson S-A, Carlsson A, Bredberg A, Alm J, Aronsson S, Gustafsson J, Hagenäs L, Häger A, Kriström B, Marcus C, Moell C, Nilsson KO, Tuvemo T, Westphal O, Albertsson-Wikland K and Åman J (1999b): Prevalence of coeliac diseaes in Turner syndrome. Acta Paediatr 88:933-936.

Jabri B, de Serre NP, Cellier C, Evans K, Gache C, Carvalho C, Mourgenot J-F, Allez M, Jian R, Desreumaux P, Colombel J-F, Matuchansky C, Cugnenc H, Lopez-Botet M, Vivier E, Moretta A, Roberts AI, Ebert EC, Guy-Grand D, Brousse N, Schmitz J and Cerf-Bensussan N (2000): Selective expansion of intraepithelial lymphocytes expressing the HLA-E-specific natural killer receptor CD94 in celiac disease. Gastroenterology 118:867-879.

Janatuinen EK, Pikkarainen PH, Kemppainen TA, Kosma V-M, Järvinen RMK, Uusitupa MIJ and Julkunen RJK (1995): A comparison of diets with and without oats in adults with celiac disease. N Engl J Med 333:1033-1037.

Janatuinen EK, Kemppainen TA, Julkunen RJK, Kosma V-M, Mäki M, Heikkinen M and Uusitupa MIJ (2002): No harm from five year ingestion of oats in coeliac disease. Gut 50:332-335.

Jones CL, MacDonald RA, Hosking CS and Roberton DM (1987): Estimating the relative avidity of mucosal IgA for antigen. J Immunol Methods 105:111-117.

Jos J, Labbe F, Geny B and Griscelli C (1979): Immunoelectron-microscopic localization of immunoglobulin A and secretory component in jejunal mucosa from children with coeliac disease. Scand J Immunol 9:441-450.

Kakar S, Nehra V, Murray JA, Dayharsh GA and Burgart LJ (2003): Significance of intraepithelial lymphocytosis in small bowel biopsy samples with normal mucosal architecture. Am J Gastroenterol 98:2027-2033.

Kamar N, Faure P, Dupuis E, Cointault O, Joseph-Hein K, Durand D, Moreau J and Rostaing L (2004): Villous atrophy induced by mycophenolate mofetil in renal-transplant patients. Transpl Int 17:463-467.

Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, Ciclitira PJ, Sollid LM and Partanen J (2003): HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European genetics cluster on celiac disease. Hum Immunol 64:469-477.

Karpati S, Kosnai I, Török E and Kovacs JB (1988): Immunoglobulin A deposition in jejunal mucosa of children with dermatitis herpetiformis. J Invest Dermatol 91:336-339.

Karpati S, Burgin-Wolff A, Krieg T, Meurer M, Stolz W and Braun-Falco O (1990): Binding to human jejunum of serum IgA antibody from children with coeliac disease. Lancet 336:1335-1338.

Kaukinen K, Collin P, Holm K, Karvonen A-L, Pikkarainen P and Mäki M (1998): Small bowel mucosal inflammation in reticulin or gliadin antibody-positive patients without villous atrophy. Scand J Gastroenterol 33:944-949.

Kaukinen K, Collin P, Holm K, Rantala I, Vuolteenaho N, Reunala T and Mäki M (1999): Wheat starch-containing gluten-free flour products in the treatment of coeliac disease and dermatitis herpetiformis. A long-term follow-up study. Scand J Gastroenterol 34:163-169.

Kaukinen K, Turjanmaa K, Mäki M, Partanen J, Venäläinen R, Reunala T and Collin P (2000): Intolerance to cereals is not specific for coeliac disease. Scand J Gastroenterol 35:942-946.

Kaukinen K, Mäki M, Partanen J, Sievänen H and Collin P (2001): Celiac disease without villous atrophy: revision of criteria called for. Dig Dis Sci 46:879-887.

Kaukinen K, Halme L, Collin P, Färkkilä M, Mäki M, Vehmanen P, Partanen J and Höckerstedt K (2002): Celiac disease in patients with severe liver diseases: gluten-free diet may reverse hepatic failure. Gastroenterology 122:881-888.

Kaukinen K, Peräaho M, Collin P, Partanen J, Woolley N, Kaartinen T, Nuutinen T, Halttunen T, Mäki M and Korponay-Szabo IR (2005): Small-bowel mucosal transglutaminase 2-specific IgA-deposits in coeliac disease without villous atrophy: a prospective and randomized clinical study. Scand J Gastroenterol 40:564-572.

Kilander AF, Dotevall G, Fällstrom SP, Gillberg RE, Nilsson LA and Tarkowski A (1983): Evaluation of gliadin antibodies for detection of coeliac disease. Scand J Gastroenterol 18:377-383.

Kokkonen J, Holm K, Karttunen TJ and Mäki M (2000): Children with untreated food allergy express a relative increment in the density of duodenal γδ+T cells. Scand J Gastroenterol 35:1137-1142.

Kolho K-L and Savilahti E (1997): IgA endomysium antibodies on human umbilical cord: an excellent diagnostic tool for celiac disease in childhood. J Pediatr Gastroenterol Nutr 24:563-567.

Kolho K-L, Färkkilä MA and Savilahti E (1998): Undiagnosed coeliac disease is common in Finnish adults. Scand J Gastroenterol 33:1280-1283.

Korponay-Szabo IR, Kovacs JB, Lorincz M, Goracz G, Szabados K and Balogh M (1997): Prospective significance of antiendomysium antibody positivity in subsequently verified celiac disease. J Pediatr Gastroenterol Nutr 25:56-63.

Korponay-Szabo IR, Sulkanen S, Halttunen T, Maurano F, Rossi M, Mazzarella G, Laurila K, Troncone R and Mäki M (2000): Tissue transglutaminase is the target in both rodent and primate tissues for celiac disease-specific autoantibodies. J Pediatr Gastroenterol Nutr 31:520-527.

Korponay-Szabo IR, Laurila K, Szondy Z, Halttunen T, Szalai Z, Dahlbom I, Rantala I, Kovacs JB, Fesus L and Mäki M (2003): Missing endomysial and reticulin binding of coeliac antibodies in transglutaminase 2 knockout tissues. Gut 52:199-204.

Korponay-Szabo IR, Halttunen T, Szalai Z, Laurila K, Kiraly R, Kovacs JB, Fesus L and Mäki M (2004): In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies. Gut 53:641-648.

Korponay-Szabo IR, Raivio T, Laurila K, Opre J, Kiraly R, Kovacs JB, Kaukinen K, Fesus L and Mäki M (2005): Coeliac disease case finding and diet monitoring by point-of-care testing. Aliment Pharmacol Ther 22:729-737.

Kuitunen P, Kosnai I and Savilahti E (1982): Morphometric study of the jejunal mucosa in various childhood enteropathies with special reference to intraepithelial lymphocytes. J Pediatr Gastroenterol Nutr 1:525-531.

Kumar PJ, Walker-Smith J, Milla P, Harris G, Colyer J and Halliday R (1988): The teenage coeliac: follow up study of 102 patients. Arch Dis Child 63:916-920.

Kutlu T, Brousse N, Rambaud C, Le Deist F, Schmitz J and Cerf-Bensussan N (1993): Numbers of T cell receptor (TCR) alpha beta+ but not of TcR gamma delta+ intraepithelial lymphocytes correlate with the grade of villous atrophy in coeliac patients on a long term normal diet. Gut 34:208-214.

Kwiecien J, Karczewska K, Lukasik M, Kasner J, Dyduch A, Zabka A, Sulej J and Ronckowski S (2005): Negative results of antiendomysial antibodies: long term follow-up. Arch Dis Child 90:41-42.

Ladinser B, Rossipal E and Pittschieler K (1994): Endomysium antibodies in coeliac disease: an improved method. Gut 35:776-778.

Lähdeaho M-L, Kaukinen K, Collin P, Ruuska T, Partanen J, Haapala A-M and Mäki M (2005): Celiac disease: from inflammation to atrophy: a long-term follow-up study. J Pediatr Gastroenterol Nutr 41:44-48.

Lancaster-Smith M, Packer S, Kumar PJ and Harries JT (1976): Immunological phenomena in the jejunum and serum after reintroduction of dietary gluten in children with treated coeliac disease. J Clin Pathol 29:592-597.

Larizza D, Calcaterra V, De Giacomo C, De Silvestri A, Asti M, Badulli C, Autelli M, Coslovich E and Martinetti M (2001): Celiac disease in children with autoimmune thyroid disease. J Pediatr 139:738-740.

Lerner A, Kumar V and Iancu TC (1994): Immunological diagnosis of childhood coeliac disease: comparison between antigliadin, antireticulin and antiendomysial antibodies. Clin Exp Immunol 95:78-82.

Liu J, Juo S-H, Holopainen P, Terwilliger J, Tong X, Grunn A, Brito M, Green P, Mustalahti K, Mäki M, Gilliam TC and Partanen J (2002): Genomewide linkage analysis of celiac disease in Finnish families. Am J Hum Genet 70:51-59.

Lo W, Sano K, Lebwohl B, Diamond B and Green PH (2003): Changing presentation of adult celiac disease. Dig Dis Sci 48:395-398.

Lock RJ, Pitcher MC and Unsworth DJ (1999): IgA anti-tissue transglutaminase as a diagnostic marker of gluten sensitive enteropathy. J Clin Pathol 52:274-277.

Logan RFA, Tucker G, Rifkind EA, Heading RC and Ferguson A (1983): Changes in clinical features of coeliac disease in adults in Edinburgh and the Lothians 1960-79. BMJ 286:95-97.

Louka AS, Nilsson S, Olsson M, Talseth B, Lie BA, Ek J, Gudjonsdottir AH, Ascher H and Sollid LM (2002): HLA in coeliac disease families: a novel test of risk modification by the ´other´ haplotype when at least one DQA1*05-DQB1*02 haplotype is carried. Tissue Antigens 60:147-154.

Lundin KEA, Nilsen EM, Scott HG, Loberg EM, Gjoen A, Bratlie J, Skar V, Mendez E, Lovik A and Kett K (2003): Oats induced villous atrophy in coeliac disease. Gut 52:1649-1652.

Lundqvist C, Melgar S, Yeung MM, Hammarström S and Hammarström M-L (1996): Intraepithelial lymphocytes in human gut have lytic potential and a cytokine profile that suggest T Helper 1 and cytotoxic functions. J Immunol 157:1926-1934.

Luostarinen L, Pirttila T and Collin P (1999): Coeliac disease presenting with neurological disorders. Eur Neurol 42:132-135.

Luostarinen L, Collin P, Peräaho M, Mäki M and Pirttilä TA (2001a): Coeliac disease in patients with cerebellar ataxia of unkown origin. Ann Med 33:445-449.

Luostarinen L, Dastidar P, Collin P, Peräaho M, Mäki M, Erilä T and Pirttilä T (2001b): Association between coeliac disease, epilepsy and brain atrophy. Eur Neurol 46:187-191.

MacDonald WC, Brandborg LL, Flick AL, Trier JS and Rubin CE (1964): Studies of celiac disease sprue. IV. The response of whole length of the small bowel to a gluten-free diet. Gastroenterology 47:573-589.

MacGowan DJL, Hourihane DOB, Tanner WA and O'Morain C (1996): Duodeno-jejunal adenocarcinoma as a first presentation of coeliac disease. J Clin Pathol 49:602-604.

Mahadeva S, Wyatt JI and Howdle PD (2002): Is a raised intraepithelial lymphocyte count with normal duodenal villous architecture clinically relevant? J Clin Pathol 55:424-428.

Maiuri L, Ciacci C, Raia V, Vacca L, Ricciardelli I, Raimondi F, Auricchio S, Quaratino S and Londei M (2001a): FAS engagement drives apoptosis of enterocytes of coeliac patients. Gut 48:418-424.

Maiuri L, Ciacci C, Vacca L, Ricciardelli I, Auricchio S, Quarantino S and Londei M (2001b): IL-15 drives the specific migration of CD94+ and TCR-gammadelta+ intraepithelial lymphocytes in organ cultures of treated celiac patients. Am J Gastroenterol 96:150-156.

Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, Picard J, Osman M, Quaratino S and Londei M (2003): Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. Lancet 362:30-37.

Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Rispo A, Griffin M, Issekutz T, Quaratino S and Londei M (2005): Unexpected role of surface transglutaminase type II in celiac disease. Gastroenterology 129:1400-1413.

Mäki M (1995): The humoral immune system in coeliac disease. Baillieres Clin Gastroenterol 9:231-249.

Mäki M and Holm K (1990): Incidence and prevalence of coeliac disease in Tampere. Coeliac disease is not disappearing. Acta Paediatr Scand 79:980-982.

Mäki M, Hällström O, Vesikari T and Visakorpi JK (1984): Evaluation of a serum IgA-class reticulin antibody test for the detection of childhood celiac disease. J Pediatr 105:901-905.

Mäki M, Kallonen K, Lähdeaho M-L and Visakorpi JK (1988): Changing pattern of childhood coeliac disease in Finland. Acta Paediatr Scand 77:408-412.

Mäki M, Holm K, Koskimies S, Hällström O and Visakorpi JK (1990): Normal small bowel biopsy followed by coeliac disease. Arch Dis Child 65:1137-1141.

Mäki M, Holm K, Collin P and Savilahti E (1991a): Increase in gamma/delta T cell receptor bearing lymphocytes in normal small bowel mucosa in latent coeliac disease. Gut 32:1412-1414.

Mäki M, Holm K, Lipsanen V, Hällström O, Viander M, Collin P, Savilahti E and Koskimies S (1991b): Serological markers and HLA genes among healthy first-degree relatives of patients with coeliac disease. Lancet 338:1350-1353.

Mäki M, Huupponen T, Holm K and Hällstrom O (1995): Seroconversion of reticulin autoantibodies predicts coeliac disease in insulin dependent diabetes mellitus. Gut 36:239-242.

Mäki M, Mustalahti K, Kokkonen J, Kulmala P, Haapalahti M, Karttunen T, Ilonen J, Laurila K, Dahlbom I, Hansson T, Höpfl P and Knip M (2003): Prevalence of celiac disease among children in Finland. N Engl J Med 348:2517-2524.

Mankai A, Sakly W, Landolsi H, Gueddah L, Sriha B, Ayadi A, Sfar MT, Skandrani K, Harbi A, Essoussi AS, Korbi S, Fabien N, Jeddi M and Ghedira I (2005): Tissue transglutaminase antibodies in celiac disease, comparison of an enzyme linked immunosorbent assay and a dot blot assay. Pathol Biol 53:204-209.

Marks J, Shuster S and Watson AJ (1966): Small-bowel changes in dermatitis herpetiformis. Lancet 2:1280-1282.

Marsh MN (1989): Studies of intestinal lymphoid tissue. XIII. Immunopathology of the evolving celiac sprue lesion. Pathol Res Pract 185:774-777.

Marsh MN (1992): Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology 102:330-354.

Marsh MN and Crowe PT (1995): Morphology of the mucosal lesion in gluten sensitivity. Baillieres Clin Gastroenterol 9:273-293.

Marzari R, Sblattero D, Florian F, Tongiorgi E, Not T, Tommasini A, Ventura A and Bradbury A (2001): Molecular dissection of tissue transglutaminase autoantibody response in celiac disease. J Immunol 166:4170-4176.

Mawhinney H and Love AHG (1975): Anti-reticulin antibody in jejunal juice in coeliac disease. Clin Exp Immunol 21:394-398.

Mayer M, Greco L, Troncone R, Auricchio S and Marsh MN (1991): Compliance of adolescents with coeliac disease with a gluten free diet. Gut 32:881-885.

Mazure R, Vazquez H, Gonzalez D, Mautalen C, Pedreira S, Boerr L and Bai JC (1994): Bone mineral affection in asymptomatic adult patients with celiac disease. Am J Gastroenterol 89:2130-2134.

Mazzarella G, Maglio M, Paparo F, Nardone G, Stefanile R, Greco L, van de Wal Y, Kooy Y, Koning F, Auricchio S and Troncone R (2003): An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative coeliac patients. Gut 52:57-62.

McMillan SA, Haughton DJ, Biggart JD, Edgar JD, Porter KG and McNeill TA (1991): Predictive value for coeliac disease of antibodies to gliadin, endomysium, and jejunum in patients attending for jejunal biopsy. BMJ 303:1163-1165.

McNeish AS, Harms HK, Rey J, Scherling DH, Visakorpi JK and Walker-Smith JA (1979): The diagnosis of coeliac disease. A commentary on the current practices of members of the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN). Arch Dis Child 54:783-786.

Meeuwisse GW (1970): Diagnostic criteria in coeliac disease. Acta Paediatr Scand 59:461-463.

Meini A, Pillan NM, Villanacci V, Monafo V, Ugazio AG and Plebani A (1996): Prevalence and diagnosis of celiac disease in IgA-deficient children. Ann Allergy Asthma Immunol 77:333-336.

Meloni G, Dore A, Fanciulli G, Tanda F and Bottazzo GF (1999a): Subclinical coeliac disease in schoolchildren from Northern Sardinia. Lancet 353:37.

Meloni GF, Dessole S, Vargiu N, Tomasi PA and Musumeci S (1999b): The prevalence of coeliac disease in infertility. Hum Reprod 14:2759-2761.

Meresse B, Chen Z, Ciszewski C, Tretiakova M, Bhagat G, Krausz TN, Raulet DH, Lanier LL, Groh V, Spies T, Ebert EC, Green PH and Jabri B (2004): Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. Immunity 21:357-366.

Molberg O, McAdam SN, Körner R, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Noren O, Roepstorff P, Lundin KEA, Sjöström H and Sollid LM (1998): Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nature Med 4:713-717.

Monsuur AJ, de Bakker PIW, Alizadeh BZ, Zhernakova A, Bevova MR, Strengman E, Franke L, van't Slot R, Van Belzen MJ, Lavrijsen ICM, Diosdado B, Daly MJ, Mulder CJJ, Mearin ML, Meijer JW, Meijer GA, van Oort E, Wapenaar MC, Koeleman BPC and Wijmenga C (2005): Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. Nat Genet 37:1341-1344.

Mustalahti K, Collin P, Sievänen H, Salmi J and Mäki M (1999): Osteopenia in patients with clinically silent coeliac disease warrants screening. Lancet 354:744-745.

Mustalahti K, Lohiniemi S, Collin P, Vuolteenaho N, Laippala P and Mäki M (2002): Gluten-free diet and quality of life in patients with screen-detected celiac disease. Eff Clin Pract 5:105-113.

Myhre AG, Aarsetoy H, Undlien DE, Hovdenak N, Aksnes L and Husebye ES (2003): High frequency of coeliac disease among patients with autoimmune adrenocortical failure. Scand J Gastroenterol 38:511-515.

Niveloni S, Pedreira S, Sugai E, Vazquez H, Smecuol E, Fiorini A, Cabanne A, Dezi R, Valero J, Kogan Z, Maurino E and Bai JC (2000): The natural history of gluten sensitivity: report of two new celiac disease patients resulting from long-term follow-up of nonatrophic, first-degree relatives. Am J Gastroenterol 95:463-468.

Not T, Ventura A, Peticarari S, Basile S, Torre G and Dragovic D (1993): A new, rapid, noninvasive screening test for celiac disease. J Pediatr 123:425-427.

Nuti R, Martini G, Valenti R, Giovani S, Salvadori S and Avanzati A (2001): Prevalence of undiagnosed coeliac syndrome in osteoporotic women. J Intern Med 250:361-366.

Oberhuber G, Vogelsang H, Stolte M, Muthenthaler S, Kummer AJ and Radaszkiewicz T (1996): Evidence that intestinal intraepithelial lymphocytes are activated cytotoxic T cells in celiac disease but not in giardiasis. Am J Pathol 148:1351-1357.

O'Farrelly C, Feighery C, O'Briain DS, Stevens F, Connolly CE, McCarthy C and Weir DG (1986): Humoral response to wheat protein in patients with coeliac disease and enteropathy associated T cell lymphoma. BMJ 293:908-910.

O'Grady JG, Stevens FM, Harding B, O'Gorman TA, McNicholl B and McCarthy CF (1984): Hyposplenism and gluten-sensitive enteropathy. Natural history, incidence, and relationship to diet and small bowel morphology. Gastroenterology 87:1326-1331.

O'Leary C, Walsh CH, Wieneke P, O'Regan P, Buckley B, O'Halloran DJ, Ferriss JB, Quigley EMM, Annis P, Shanahan FL and Cronin CC (2002): Celiac disease and autoimmune Addison´s disease: a clinical pitfall. QJM 95:79-82.

Paparo F, Petrone E, Tosco A, Maglio M, Borrelli M, Salvati VM, Miele E, Greco L, Auricchio S and Troncone R (2005): Clinical, HLA, and small bowel immunohistochemical features of children with positive serum antiendomysium antibodies and architecturally normal small intestinal mucosa. Am J Gastroenterol 100:2294-2298.

Pare P, Douville P, Caron D and Lagace R (1988): Adult celiac sprue: changes in the pattern of clinical recognition. J Clin Gastroenterol 10:395-400.

Paulley JW (1954): Observations on the aetiology of idiopathic steatorrhoea; jejunal and lymph node biopsies. BMJ 2:1318-1321.

Peräaho M, Kaukinen K, Paasikivi K, Sievänen H, Lohiniemi S, Mäki M and Collin P (2003): Wheat-starch-based gluten-free products in the treatment of newly detected coeliac disease. Prospective and randomized study. Aliment Pharmacol Therapy 17:587-594.

Picarelli A, Maiuri L, Frate A, Greco M, Auricchio S and Londei M (1996a): Production of antiendomysial antibodies after in-vitro gliadin challenge of small intestine biopsy samples from patients with coeliac disease. Lancet 348:1065-1067.

Picarelli A, Maiuri L, Mazzilli MC, Coletta S, Ferrante P, Di Giovambattista F, Greco M, Torsoli A and Auricchio S (1996b): Gluten-sensitive disease with mild enteropathy. Gastroenterology 111:608-616.

Ploski R, Ek J, Thorsby E and Sollid LM (1993): On the HLA-DQ(alpha 1*0501, beta 1*0201)-associated susceptibility in celiac disease: a possible gene dosage effect of DQB1*0201. Tissue Antigens 41:173-177.

Polvi A, Eland C, Koskimies S, Mäki M and Partanen J (1996): HLA DQ and DP in Finnish families with coeliac disease. Eur J Immunogen 23:221-234.

Polvi A, Arranz E, Fernandez-Arquero M, Collin P, Mäki M, Sanz A, Calvo C, Maluenda C, Westman P, de la Concha EG and Partanen J (1998): HLA-DQ2-negative celiac disease in Finland and Spain. Hum Immunol 59:169-175.

Pynnönen PA, Isometsä ET, Aronen ET, Verkasalo MA, Savilahti E and Aalberg VA (2004): Mental disorders in adolescents with celiac disease. Psychosomatics 45:325-335.

Pynnönen PA, Isometsä ET, Verkasalo MA, Kähkönen SA, Sipilä I, Savilahti E and Aalberg VA (2005): Gluten-free diet may alleviate depressive and behavioral symptoms in adolescents with coeliac disease: a prospective follow-up case-series study. BMC Psychiatry 5:14.

Radek JT, Jeong J-M, Murthy SNP, Ingham KC and Lorand L (1993): Affinity of human erythrocyte transglutaminase for a 42-kDa gelatin-binding fragment of human plasma fibronectin. Proc Natl Acad Sci U SA 90:3152-3156.

Rantala I, Mäki M, Laasonen A and Visakorpi JK (1985): Periodate-lysine-paraformaldehyde as fixative for the study of duodenal mucosa. Morphologic and immunohistochemical results at light and electron microscopic levels. Acta Pathol Microbiol Immunol Scand [A] 93:165-173.

Regan PT and DiMagno EP (1980): Exocrine pancreatic insufficiency in celiac sprue: a cause of treatment failure. Gastroenterology 78:484-487.

Reif S and Lerner A (2004): Tissue transglutaminase- the key player in celiac disease: a review. Autoimmun Rev 3:40-45.

Reunala T, Kosnai I, Karpati S, Kuitunen P, Török E and Savilahti E (1984): Dermatitis herpetiformis: jejunal findings and skin response to gluten-free diet. Arch Dis Child 59:517-522.

Reunala T, Collin P, Holm K, Pikkarainen P, Miettinen A, Vuolteenaho N and Mäki M (1998): Tolerance to oats in dermatitis herpetiformis. Gut 43:490-493.

Risdon RA and Keeling JW (1974): Quantitation of the histological changes found in small intestinal biopsy specimens from children with suspected coeliac disease. Gut 15:9-18.

Rizzetto M and Doniah D (1973): Types of 'reticulin' antibodies detected in human sera by immunofluorescence. J Clin Pathol 26:841-851.

Roberts AI, Lee L, Schwarz E, Groh V, Spies T, Ebert EC and Jabri B (2001): NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. J Immunol 167:5527-5530.

Rostami K, Kerckhaert J, Tiemessen R, von Blomberg ME, Meijer JWR and Mulder CJJ (1999): Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. Am J Gastroenterol 94:888-894.

Sacchetti L, Ferrajolo A, Salerno G, Esposito P, Lofrano MM, Oriani G, Micillo M, Paparo F, Troncone R, Auricchio S and Salvatore F (1996): Diagnostic value of various serum antibodies detected by diverse methods in childhood celiac disease. Clin Chem 42:1838-1842.

Salmaso C, Ocmant A, Pesce G, Altrinetti V, Montagna P, Descalzi D, Martino S, Bagnasco M and Mascart F (2001): Comparison of ELISA for tissue transglutaminase autoantibodies with antiendomysium antibodies in pediatric and adult patients with celiac disease. Allergy 56:544-547.

Sategna-Guidetti C, Grosso S, Bruno M and Grosso SB (1995): Comparison of serum anti-gliadin, anti-endomysium, and anti-jejunum antibodies in adult celiac sprue. J Clin Gastroenterol 20:17-21.

Sategna-Guidetti C, Bruno M, Mazza E, Carlino A, Predebon S, Tagliabue M and Brossa C (1998): Autoimmune thyroid diseases and coeliac disease. Eur J Gastroenterol Hepatol 10:927-931.

Savilahti E (1972): Intestinal immunoglobulins in children with coeliac disease. Gut 13:958-964.

Savilahti E, Arato A and Verkasalo M (1990): Intestinal gamma/delta receptor-bearing T lymphocytes in celiac disease and inflammatory bowel diseases in children. Constant increase in celiac disease. Pediatr Res 28:579-581.

Savilahti E, Reunala T and Mäki M (1992): Increase of lymphocytes bearing the gamma/delta T cell receptor in the jejunum of patients with dermatitis herpetiformis. Gut 33:206-211.

Sbarbati A, Valletta E, Bertini M, Cipolli M, Morroni M, Pinelli L and Tato L (2003): Gluten sensitivity and ´normal´ histology: is the intestinal mucosa really normal? Dig Liver Dis 35:768-773.

Sblattero D, Berti I, Trevisiol C, Marzari R, Tommasini A, Bradbury A, Fasano A, Ventura A and Not T (2000): Human recombinant tissue transglutaminase ELISA: an innovative diagnostic assay for celiac disease. Am J Gastroenterol 95:1253-1257.

Sblattero D, Florian F, Azzoni E, Ziberna F, Tommasini A, Not T, Ventura A, Bradbury A and Marzari R (2004): One-step cloning of anti tissue transglutaminase scFv from subjects with celiac disease. J Autoimmunity 22:65-72.

Sblattero D, Ventura A, Tommasini A, Cattin L, Martelossi S, Florian F, Marzari R, Bradbury A and Not T (2006): Cryptic gluten intolerance in type I diabetes: identifying suitable candidates for a gluten free diet. Gut 55:133-134.

Schesinger MJ (1994): How the cell copes with stress and the function of heat shock proteins. Pediatr Res 36:1-6.

Schuppan D (2000): Current concepts of celiac disease pathogenesis. Gastroenterology 119:234-242.

Scoglio R, Di Pasquale G, Pagano G, Lucanto MC, Magazzu G and Sferlazzas C (2003): Is intestinal biopsy always needed for diagnosis of celiac disease? Am J Gastroenterol 98:1325-1331.

Scott BB and Losowsky MS (1976): Patchiness and duodenal-jejunal variation of the mucosal abnormality in coeliac disease and dermatitis herpetiformis. Gut 17:984-992.

Seah PP, Fry LL, Rossiter MA, Hoffbrand AV and Holborow EJ (1971): Anti-reticulin antibodies in childhood coeliac disease. Lancet 2:681-682.

Selby WS, Janossy G, Bofill M and Jewell DP (1983): Lymphocyte subpopulations in the human small intestine. The findings in normal mucosa and in the mucosa of patients with adult coeliac disease. Clin Exp Immunol 52:219-228.

Sher KS and Mayberry JF (1996): Female fertility, obstetric and gynaegological history in coeliac disese: a case control study. Acta Paediatr Suppl 412:76-77.

Shidrawi RG, Przemioslo R, Davies DR, Tighe MR and Ciclitira PJ (1994): Pitfalls in diagnosing coeliac disease. J Clin Pathol 47:693-694.

Shiner M (1957): Small intestinal biopsies by the oral route. J Mt Sinai Hosp 24:273-277.

Shiner M and Ballard J (1972): Antigen-antibody reactions in jejunal mucosa in childhood coeliac disease after gluten challenge. Lancet 1:1202-1205.

Sjöström H, Lundin KEA, Molberg O, Körner R, McAdam SN, Anthonsen D, Quarsten H, Noren O, Roepstorff P, Thorsby E and Sollid LM (1998): Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. Scand J Immunol 48:111-115.

Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F and Thorsby E (1989): Evidence for a primary association of celiac disease to a particular HLA-DQ α/β heterodimer. J Exp Med 169:345-350.

Spencer J, Isaacson PG, Diss TC and MacDonald TT (1989): Expression of disulfide-linked and non-disulfide-linked forms of the T cell receptor gamma/delta heterodimer in human intestinal intraepithelial lymphocytes. Eur J Immunol 19:1335-1338.

Spencer J, Isaacson PG, MacDonald TT, Thomas AJ and Walker-Smith JA (1991): Gamma/delta T cells and the diagnosis of coeliac disease. Clin Exp Immunol 85:109-113.

Spurkland A, Sollid LM, Polanco I, Vartdal F and Thorsby E (1992): HLA -DR and -DQ genotypes of celiac disease patients serologically typed to be non-DR3 or non-DR 5/7. Human Immunol 35:188-192.

Stenson WF, Newberry R, Lorenz R, Baldus C and Civitelli R (2005): Increased prevalence of celiac disease and need for routine screening among patients with osteoporosis. Arch Intern Med 165:393-399.

Stern M, Fischer K and Gruttner R (1979): Immunofluorescent serum gliadin antibodies in children with coeliac disease and various malabsorptive disorders. Eur J Pediatr 130:155-164.

Stevens FM, Egan-Mitchell B, Cryan E, McCarthy CF and McNicholl B (1987): Decreasing incidence of coeliac disease. Arch Dis Child 62:465-468.

Storsrud S, Olsson M, Arvidsson Lenner R, Nilsson LÅ, Nilsson O and Kilander A (2003): Adult coeliac patients do tolerate large amounts of oats. Eur J Clin Nutr 57:163-169.

Sulkanen S, Collin P, Laurila K and Mäki M (1998a): IgA- and IgG-class antihuman umbilical cord antibody tests in adult coeliac disease. Scand J Gastroenterol 33:251-254.

Sulkanen S, Halttunen T, Laurila K, Kolho K-L, Korponay-Szabo IR, Sarnesto A, Savilahti E, Collin P and Mäki M (1998b): Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology 115:1322-1328.

Szodoray P, Barta Z, Lakos G, Szakall S and Zeher M (2004): Coeliac disease in Sjögren's syndrome-a study of 111 Hungarian patients. Rheumatol Int 24:278-282.

Tesei N, Sugai E, Vazquez H, Smecuol E, Niveloni S, Mazure R, Moreno ML, Gomez JC, Maurino E and Bai JC (2003): Antibodies to human recombinant tissue transglutaminase may detect coeliac disease patients undiagnosed by endomysial antibodies. Aliment Pharmacol Therapy 17:1415-1423.

Tommasini A, Not T, Kiren V, Baldas V, Santon D, Trevisiol C, Berti I, Neri E, Gerarduzzi T, Bruno I, Lenhardt A, Zamuner E, Spano A, Crovella S, Martelossi S, Torre G, Sblattero D, Marzari R, Bradbury A, Tamburlini G and Ventura A (2004): Mass screening for coeliac disease using antihuman transglutaminase antibody assay. Arch Dis Child 89:512-515.

Trejo-Skalli AV, Velasco PT, Murthy SNP, Lorand L and Goldman RD (1995): Association of a transglutaminase-related antigen with intermediate filaments. Proc Natl Acad Sci U SA 92:8940-8944.

Troncone R (1995): Latent coeliac disease in Italy. Acta Paediatr 84:1252-1257.

Tursi A and Brandimarte G (2003): The symptomatic and histologic response to a gluten-free diet in patients with borderline enteropathy. J Clin Gastroenterol 36:13-17.

Tursi A, Brandimarte G, Giorgetti G, Gigliobianco A, Lombardi D and Gasbarrini G (2001): Low prevalence of antigliadin and anti-endomysium antibodies in subclinical/silent celiac disease. Am J Gastroenterol 96:1507-1510.

Uibo O, Uibo R, Kleimola V, Jögi T and Mäki M (1993): Serum IgA anti-gliadin antibodies in an adult population sample. High prevalence without celiac disease. Dig Dis Sci 38:2034-2037.

Vainio E, Kalimo K, Reunala T, Viander M and Palosuo T (1983): Circulating IgA- and IgG-class antigliadin antibodies in dermatitis herpetiformis detected by enzyme-linked immunosorbent assay. Arch Dermatol Res 275:15-18.

Valdimarsson T, Löfman O, Toss G and Ström M (1996): Reversal of osteopenia with diet in adult coeliac disease. Gut 38:322-327.

Valentino R, Savastano S, Maglio M, Paparo F, Ferrara F, Dorato M, Lombardi G and Troncone R (2002): Markers of potential coeliac disease in patients with Hashimoto's thyroiditis. Eur J Endocrinol 146:479-483.

Valletta E, Bertini M, Piccoli R, Capelli P and Zanoni G (2002): Latent celiac disease or low-gluten-containing diet? J Pediatr Gastroenterol Nutr 34:91-92.

Van Belzen MJ, Meijer JWR, Sandkuijl LA, Bardoel AFJ, Mulder CJJ, Pearson PL, Houwen RHJ and Wijmenga C (2003): A major non-HLA locus in celiac disease maps to chromosome 19. Gastroenterology 125:1032-1041.

van de Kamer JH, Weijers HA and Dicke WK (1953): Coeliac disease. IV. An investigation into the injurious constituents of wheat in connection with their action on patients with coeliac disease. Acta Paediatr 42:223-231.

van der Meer JB (1969): Granular deposits of immunoglobulins in the skin of patients with dermatitis herpetiformis. An immunofluorescent study. Br J Dermatol 81:493-503.

Vazquez H, Mazure R, Gonzalez D, Flores D, Pedreira S, Niveloni S, Smecuol E, Maurino E and Bai JC (2000): Risk of fractures in celiac disease patients: a cross-sectional, case-control study. Am J Gastroenterol 95:183-189.

Ventura A, Magazzu G and Greco L (1999): Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. Gastroenterology 117:297-303.

Verkasalo MA, Arato A, Savilahti E and Tainio VM (1990): Effect of diet and age on jejunal and circulating lymphocyte subsets in children with coeliac disease: persistence of CD4-8- intraepithelial T cells through treatment. Gut 31:422-425.

Viljamaa M, Collin P, Huhtala H, Sievänen H, Mäki M and Kaukinen K (2005): Is coeliac disease screening in risk groups justified? A fourteen-year follow-up with a special focus on compliance and quality of life. Aliment Pharmacol Ther 22:317-324.

Visakorpi JK, Immonen P and Kuitunen P (1967): Malabsorption syndrome in childhood. Acta Paediatr Scand 56:1-9.

Vogelsang H, Genser D, Wyatt J, Lochs H, Ferenci P, Granditsch G and Penner E (1995): Screening for celiac disease: a prospective study on the value of noninvasive tests. Am J Gastroenterol 90:394-398.

Vogelsang H, Schwarzenhofer M, Granditsch G and Oberhuber G (1999): In vitro production of endomysial antibodies in cultured duodenal mucosa from patients with celiac disease. Am J Gastroenterol 94:1057-1061.

Volta U, Molinaro N, Fusconi M, Cassani F and Bianchi FB (1991): IgA antiendomysial antibody test. A step forward in celiac disease screening. Dig Dis Sci 36:752-756.

Volta U, Bardazzi F, Zauli D, DeFranceschi L, Tosti A, Molinaro N, Ghetti S, Tetta C, Grassi A and Bianchi FB (1997): Serological screening for coeliac disease in vitiligo and alopecia areata. Br J Dermatol 136:801-802.

Volta U, De Franceschi L, Lari F, Molinaro N, Zoli M and Bianchi FB (1998a): Coeliac disease hidden by cryptogenic hypertransaminasemia. Lancet 352:26-29.

Volta U, De Franceschi L, Molinaro N, Cassani F, Muratori L, Lenzi M, Bianchi FB and Czaja AJ (1998b): Frequency and significance of anti-gliadin and anti-endomycial antibodies in autoimmune hepatitis. Dig Dis Sci 43:2190-2195.

Volta U, Bellentani S, Bianchi FB, Brandi G, De Franceschi L, Miglioli L, Granito A, Balli F and Tiribelli C (2001): High prevalence of celiac disease in Italian general population. Dig Dis Sci 46:1500-1505.

Volta U, Rodrigo L, Granito A, Petrolini N, Muratori P, Muratori L, Linares A, Veronesi L, Fuentes D, Zauli D and Bianchi FB (2002): Celiac disease in autoimmune cholestatic liver disorders. Am J Gastroenterol 97:2609-2613.

Wahab PJ, Crusius JBA, Meijer JWR and Mulder CJJ (2001): Gluten challenge in borderline gluten-sensitive enteropathy. Am J Gastroenterol 96:1464-1469.

Wahnschaffe U, Ullrich R, Riecken EO and Schulzke JD (2001): Celiac disease-like abnormalities in a subgroup of patients with irritabile bowel syndrome. Gastroenterology 121:1329-1338.

Walker-Smith JA (1997): Discussion of diagnostic criteria for coeliac disease at Tampere meeting. In: Coeliac disease: proceedings of the Seventh symposium on coeliac disease, pp 191-193. Eds. M Mäki, P Collin and J Visakorpi. Coeliac disease study group, Tampere.

Walker-Smith JA, Guandalini S, Schmitz J, Shmerling DH and Visakorpi JK (1990): Revised criteria for diagnosis of coeliac disease. Arch Dis Child 65:909-911.

Weinstein WM (1974): Latent celiac sprue. Gastroenterology 66:489-493.

West J, Logan RFA, Card TR, Smith C and Hubbard R (2003): Fracture risk in people with celiac disease: a population-based cohort study. Gastroenterology 125:429-436.

Wolters V, Vooijs-Moulaert A-F, Burger H, Brooimans R, De Schryver J, Rijkers G and Houwen R (2002): Human tissue transglutaminase enzyme linked immunosorbent assay outperforms both the guinea pig based tissue transglutaminase assay and anti-endomysium antibodies when screening for coeliac disease. Eur J Pediatr 161:284-287.

Young WF and Pringle EM (1971): 110 children with coeliac disease. Arch Dis Child 46:421-436.

Ziegler TR, Fernandez-Estivariz C, Gu LH, Fried MW and Leader LM (2003): Severe villus atrophy and chronic malabsorption induced by azathioprine. Gastroenterology 124:1950-1957.

Zimmer K-P, Naim H, Weber P, Ellis HJ and Ciclitira PJ (1998): Targeting of gliadin peptides, CD8, alpha/beta-TCR and gamma/delta-TCR to Golgi complexes and vacuoles within celiac disease enterocytes. FASEB J 12:1349-1357.

Zipser RD, Patel S, Yahya KZ, Baisch DW and Monarch E (2003): Presentations of adult celiac disease in a nationwide patient support group. Dig Dis Sci 48:761-764.

9. ORIGINAL PUBLICATIONS

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Intraepithelial Lymphocytes in Celiac Disease

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OBJECTIVE: The aim of this study was to investigate the value of immunohistochemical characterization of different intraepithelial lymphocytes (IELs) in the diagnostic workup of celiac disease (CD).

METHODS: The study involved 928 consecutive adult patients undergoing endoscopy undertaken on suspicion of CD or to ascertain the dietary compliance; the control group consisted of 59 adults who underwent endoscopy because of indigestion. Small bowel mucosal morphology, CD3+, $\alpha\beta$+, and $\gamma\delta$+ IELs were determined.

RESULTS: CD was detected in 138 and excluded in 545 adults. CD3+ and $\gamma\delta$+ IELs both showed a sensitivity of 93% for CD; specificity was 73% and 88%, respectively. For $\alpha\beta$+ cells, the sensitivity was 83% and specificity, 66%. The mucosal morphology recovered on a gluten-free diet and the densities of different IELs, even $\gamma\delta$+ cells, decreased. Only the density of $\gamma\delta$+ cells remained elevated compared with controls.

CONCLUSIONS: Counting of IELs is recommended in borderline cases where the histology is difficult to interpret. An increase especially in $\gamma\delta$+ cells strengthens the probability of CD. However, IELs are not invariably increased in CD. (Am J Gastroenterol 2003;98:1332–1337. © 2003 by Am. Coll. of Gastroenterology)

INTRODUCTION

The diagnosis of celiac disease (CD) is currently based on small bowel mucosal biopsy (1). Usually, no difficulties are encountered when the biopsy shows severe villous atrophy and crypt hyperplasia. In clinical practice, however, the findings are often less straightforward. Diagnostic difficulties arise especially when the biopsy findings are borderline. Diagnosis should be made from well-orientated high-quality samples (1), but often sampling is compromised, and conclusions may be difficult to draw. Patients may have reduced their gluten intake on their own account, and the mucosal damage may have recovered at least to some extent, making a proper diagnosis difficult (2). Occasionally, villous shortening can be seen only in certain parts of the small bowel mucosa (3, 4); this patchy form of villous atrophy may be easily overlooked.

The diagnosis is rendered even more complicated by the fact that the mucosal deterioration in CD occurs gradually. First, infiltration of lymphocytes into the epithelium and lamina propria can be seen (Marsh I), this being followed by hypertrophy of the crypts (Marsh II), and ultimately by villous atrophy (Marsh III) (5). There are numerous examples in the literature showing that the mucosal lesion can be normal (latent CD), and the atrophy develops by time (6, 7).

These diagnostic difficulties clearly call for closer study of the inflammatory intestinal mucosal changes taking place in CD. Intraepithelial lymphocytes (IELs) are increased in the mucosa of untreated celiac patients (8). In general, these IELs are CD3+ $\alpha\beta$+ T-cell receptor-bearing cells. However, in CD, 20–30% of CD3+ IELs bear $\gamma\delta$+ T-cell receptor-bearing cells, which comprise less than 10% of the IELs in nonceliac subjects (9, 10). Because $\gamma\delta$+ IELs are considered markers of CD latency and specific for potential or overt CD (11, 12), this may provide an improved means of detecting the disease in cases where the histological diagnosis remains equivocal. The number of patients in previous studies has been relatively small, and the value of IELs has never been tested in a large clinical series. The aim here was, therefore, to evaluate systematically in a prospective study the usefulness of immunohistochemical characterization of IELs in the diagnostic workup of CD.

MATERIALS AND METHODS

Patients

The study comprised 928 adult patients who underwent small intestinal biopsy at the Department of Medicine in Tampere University Hospital from 1995 to 1999 because of a suspicion of CD or for control of histological recovery with a gluten-free diet (Table 1). The control group comprised 59 patients who underwent endoscopy because of indigestion; they had no suspicion of CD nor any relatives with CD, and all were CD antibody negative.

Methods

All patients and controls underwent upper GI endoscopy at the Department of Medicine in Tampere University Hospi-

Table 1. Demographic Data and Indications for Endoscopy

	CD Untreated	DH Untreated	CD Treated	DH Treated	CD Suspicion, Normal Villi	Controls
Number of patients	138	24	198	23	545	59
Age; mean (range), yr	42 (16–81)	41 (17–70)	45 (16–83)	48 (24–69)	43 (15–88)	50 (22–74)
Female, %	69	63	65	44	71	50
Indication, %						
GI complaints	42		14	26	54	100
Anemia	11		4		13	
Screening in associated diseases*	17				20	
To control mucosal recovery on diet			73	22		
Other†	30	100	11	52	13	
Duration of gluten-free diet; mean (range), yr	0	0	4 (0.1–22)	9 (0.1–32)	0	0

* Sjögren's syndrome, thyroid diseases, insulin-dependent diabetes mellitus, neurological symptoms, infertility.
† Skin, arthritis, osteoporosis, gluten challenge required to establish the diagnosis, suspicion of celiac disease latency, poor dietary complicance, family history.

tal. Seven forceps biopsy specimens were taken from the distal part of the duodenum; five of these were processed, stained with hematoxylin and eosin and studied under light microscopy, and the villous height crypt depth ratio was counted. Two small bowel biopsy specimens were freshly embedded in optimal cutting temperature compound (OTC, Tissue-Tec, Miles, Elkhart, IN) and stored at 70°C. Immunohistochemical stainings were carried out on 5 μm-thick frozen sections. CD3+ IELs were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA), $\alpha\beta$+ IELs with monoclonal antibody αF1 (T Cell Diagnostics, Woburn, MA), and $\gamma\delta$+ IELs with T-cell receptor-bearing cell γ antibody (T Cell Diagnostics). Positive IELs were counted with a $\times$100 flat field light microscope objective in the surface epithelium; at least 30 fields of 1.6 mm epithelial length were counted, and IEL density expressed as cells/mm of epithelium, as previously described (13, 14). This method has widely been used, and it correlates well with the counting of IELs per 100 epithelial cells (13). All specimens were evaluated by the same investigator without prior knowledge of disease history or laboratory findings. In our laboratory, the correlation coefficients for intraobserver variation for CD3+, for $\alpha\beta$+, and for $\gamma\delta$+ IELs were 0.95, 0.85, and 0.98, and those for interobserver variation were 0.92, 0.82, and 0.98, respectively.

The diagnosis of CD was based on small intestinal biopsy, but also a histological or clinical response was later observed in virtually all individuals. IgA class antiendomysial (EmA) and antigliadin (AGA) antibodies (15) were also determined in many cases. However, antibody testing had not been carried out systematically, and a negative antibody result was not considered exclusive when there was a clinical suspicion of CD.

The calculation of IELs and antibody tests was part of routine clinical practice. The local ethical committee approved the taking of specimens from controls, and written informed consent was obtained from each control.

Statistical Analysis

The cell densities were expressed as mean and 95% CIs. The χ^2 test was used in cross tabulations.

RESULTS

Altogether, 138 were found to be suffering from this disease, and all fulfilled the current diagnostic criteria (16) (Table 1). In 545, the villous structure was interpreted as normal. Twenty-four had newly detected untreated and 23 treated dermatitis herpetiformis (DH). Control biopsy taken during a gluten-free diet was available in altogether 198 patients with CD. The densities of CD3+, $\alpha\beta$+, and $\gamma\delta$+ cells were significantly higher in patients with untreated CD than in controls (Table 2). In antibody-negative untreated CD patients (n = 10), the mean densities of IELs (CD3+, 61; $\alpha\beta$+, 36; $\gamma\delta$+, 18 cells/mm) were not different from those in all untreated patients (Table 2). Villous atrophy was less severe in DH than in CD, but the densities of different IELs were similarly increased in both conditions.

In celiac patients on a gluten-free diet, the densities were lower, nonetheless higher than in control subjects. Mucosal recovery occurred on a gluten-free diet, but the villous height crypt depth ratio remained lower than in controls.

Table 2. Mean Values and 95% CIs of IELs and Villous Height Crypt Depth Ratios (V/C)

	CD Untreated	DH Untreated	CD Treated	DH Treated	CD Suspicion, Normal Villi	Controls
CD3+ IELs	68, 64–73*	57, 47–68*	40, 37–43*	40, 33–46	26, 24–27	30, 26–34
$\alpha\beta$+ IELs	42, 39–45*	37, 29–45*	23, 21–24	25, 20–29	17, 17–18	22, 18–25
$\gamma\delta$+ IELs	19.8, 17.8–21.9*	16.2, 11.9–20.5*	12.1, 10.9–13.3*	13.1, 9.5–16.7*	3.2, 2.8–3.6	2.3, 1.6–3.1
V/C	0.6, 0.5–0.7*	1.3, 0.8–1.8*	1.9, 1.7–2.0*	1.7, 1.3–2.1*	2.8, 2.7–2.9	3.0, 2.8–3.1

* Statistically significant increase compared with controls.

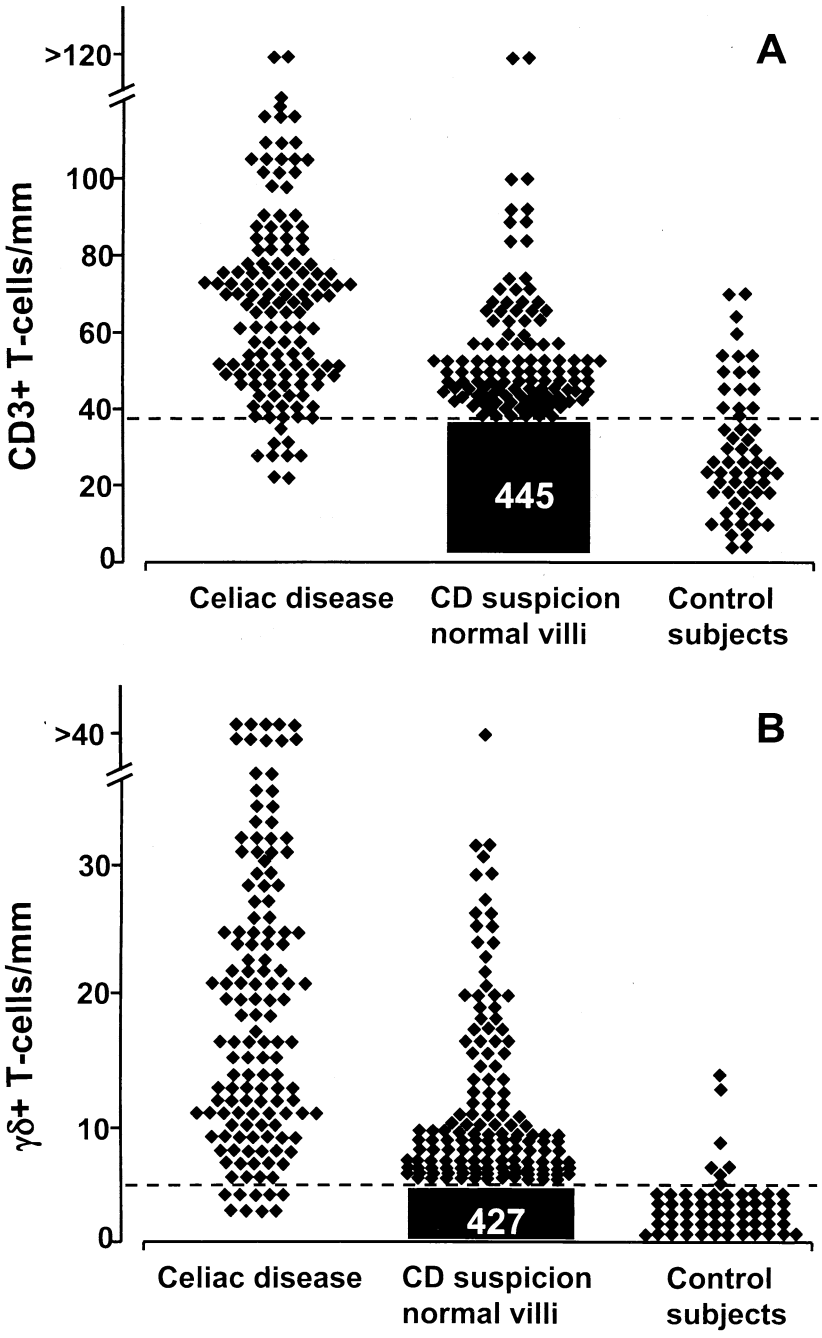

Figure 1. The density of CD3+ (*A*) and γδ+ (*B*) IELs in patients with confirmed CD, in patients with celiac suspicion but normal villous atrophy, and in nonceliac controls.

CD3+ and αβ+ IELs decreased to normal levels, whereas γδ+ cells remained elevated on a gluten-free diet. However, even these cells seemed to respond to the dietary treatment (Table 2).

There was some overlap in IELs between CD patients and controls, even in γδ+ cells, as seen in Figure 1. The cutoff values were set to get the best sensitivity without losing the specificity, at 37 cells/mm for CD3+ cells, 25 for αβ+ cells, and 4.3 for γδ+ cells. By this approach, CD3+ and γδ+ IELs showed a sensitivity of 93%; the specificity was 73% for CD3+ and 88 for γδ+ IELs (Table 3). γδ+ IELs had the best diagnostic value, as depicted by the receiver operation curve (Fig. 2).

The sensitivity of EmA was higher than that of AGA, and patients with CD were more often antibody positive than those with DH (Table 4). The specificity of antibodies was in general good, but AGA positivity was common in subjects with celiac suspicion, this probably because of selection bias. CD may often have been suspected because of positive gliadin antibodies, although a biopsy was always

Table 3. Sensitivities, Specificities, and Positive and Negative Predictive Values of IELs in the Detection of CD

	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
CD3+ IELs > 37 cells/mm	0.93	0.73	0.89	0.83
$\alpha\beta$+ IELs > 25 cells/mm	0.83	0.66	0.85	0.62
$\gamma\delta$+ IELs > 4.3 cells/mm	0.93	0.88	0.95	0.85

Comparisons were made to subjects with no suspicion of CD (control group). Patients with DH were not included.

taken when there was a suspicion of CD, even in antibody-negative cases.

Among patients with a suspicion of CD but evincing a normal villous architecture, many even had a relatively high density of IELs, especially of $\gamma\delta$+ cells (Fig. 1). When focusing on these subjects without villous atrophy but evincing intense intraepithelial $\gamma\delta$+ cell infiltration, EmA but not AGA were more often positive than in the rest of the group (Table 5).

DISCUSSION

Ferguson and Murray, in 1971, showed that the number of IELs was clearly increased in the intestinal mucosa of patients with untreated CD (8). The appearance of these cells seems to be dependent on the amount of ingested gluten (17–19). The aim of the present study was to reevaluate the diagnostic value of IELs in the current form of CD, where symptoms are often subtle and the mucosal lesion only partial. We have frequently encountered problems in interpreting whether a borderline histology is compatible with CD or not. In such cases, there is a danger of both under- and overdiagnosis.

Small bowel IELs are for the most part CD3+ lymphocytes, and thus closely correspond to IELs counted in ordinary hematoxylin and eosin biopsy samples. $\gamma\delta$+ IELs are considered to be highly sensitive and specific for CD and, further, remain elevated despite a gluten-free diet (20–22). The present study partially confirmed this conception. In borderline cases, an increase in CD3+ IELs is indicative of CD, and especially an increase in $\gamma\delta$+ cells further strengthens the probability of gluten intolerance. Immunohistochemical characterization of IELs is thus of value in the diagnosis of CD, but the limitations of this method must be borne in mind. IELs may be within normal limits in patients with untreated CD (Fig. 1). Even $\gamma\delta$+ IELs are not as specific for the disease as recently suggested (23). Theoretically, patients with untreated CD may for some reason have consumed less cereal for even a short period of time before the biopsy, and the densities of IELs may thereby have been reduced. This possibility must be confirmed in further studies.

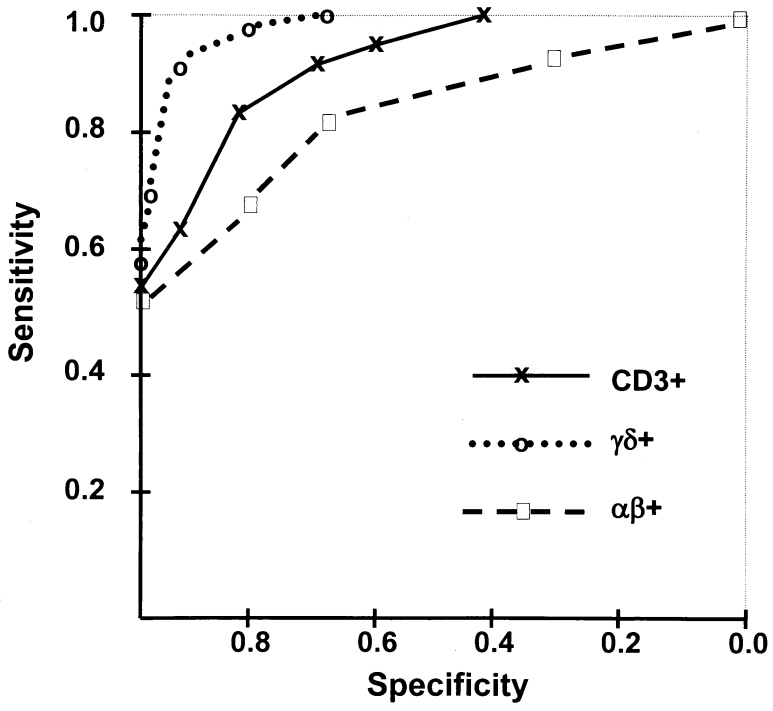

Figure 2. Receiver operating curve depicting the value of counting the densities of different IELs in the diagnosis of CD.

Table 4. Antibody-Positive Patients in Study Groups

	CD Untreated (n = 138)	DH Untreated (n = 24)	CD Suspicion, Normal Villi (n = 545)
IgA–AGA, n (%)	76/103 (74)*	12/22 (55)	197/321 (61)
IgA–EmA, n (%)	116/131 (89)†	15/22 (68)†	41/314 (13)

* $p = 0.03$ compared with patients with CD suspicion but normal villi.
† $p < 0.0001$ compared with patients with CD suspicion but normal villi.

The role of the γδ+ T-cells in the mucosa of celiac patients remains unclear. Some studies suggest that these cells function in surveillance and in repair of damaged epithelial tissues (24), whereas others consider it possible that γδ+ T-cells are cytotoxic for epithelial cells in the presence of gluten (25). γδ+ T-cells are also raised in the intestinal epithelium of patients with latent CD, when the small intestinal mucosa shows no histological abnormalities (11, 26). The increase may be a precursor or a prerequisite for the development of an intestinal lesion, but it is likely that the lesion is only manifested in the presence of gluten and some so far unrecognized environmental agents (12). In the present study, patients with a suspicion of CD who evinced an increase in γδ cells had more often positive EmA (Table 5), this indicating a tendency to develop CD. It is, therefore, likely that some patients with "false-positive" γδ+ cells subsequently develop CD, as has been shown elsewhere (26–28). Here, however, we would emphasize that the main object of this study was to investigate the density of IELs in a confirmed celiac population.

The density of all IELs, even that of γδ+ cells, was lower in treated than in untreated CD, which indicates that the lymphocytic infiltration was gluten dependent: the cell density normalized on a gluten-free diet. In cases where a gluten-free dietary treatment has been advocated when the mucosal lesion is only borderline, it is essential to show this gluten dependence during the diet (29). The present findings (Table 2) further indicate that the determination of γδ+ cells can be used in those unfortunately common cases where patients have started a gluten-reduced diet without intestinal biopsy and refuse to revert to a normal diet. An increase in γδ+ cells was often present when the mucosal structure had recovered completely or partially, and the presence of these cells is therefore suggestive of, whereas absence speaks against, CD. The higher specificity makes γδ+ IELs superior to CD3+ cells in these cases.

Our data further show that the intestinal damage and inflammation in DH is no different from that in CD, only less severe on the average. The mucosal lesion in DH is often consistent with Marsh I–II lesions and can then be considered to represent latent CD.

Our results indicated that the counting of IELs might be helpful in making the diagnosis of CD in some clinical conditions: 1) in cases where the mucosal lesion is equivocal, and in particular when the patient has on this account to undergo a new biopsy to confirm the diagnosis; 2) when patients have reduced their gluten intake, and are not willing to proceed to adequate gluten challenge; and 3) when there is a suspicion of early mucosal lesion in symptomatic patients (the increase of IELs strengthens the likelihood of CD, even in antibody-negative cases). Especially the counting of γδ+ cells offers both a sensitive and specific diagnostic adjunct in current clinical practice.

Table 5. Patients With a Suspicion of CD But Normal Villous Structure

	γδ+ Cells > 4.3/mm (n = 132)	γδ+ Cells ≤ 4.3/mm (n = 428)
IgA–AGA+, n (%)	45/74 (61)	152/247 (62)
IgA–EmA+, n (%)	22/79 (28)*	19/235 (8)

Celiac serology in patients with a high density of γδ+ IELs compared with those with a normal γδ+ IELs density.
* $p < 0.05$.

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REFERENCES

1. Walker-Smith JA, Guandalini S, Schmitz J, et al. Revised criteria for diagnosis of coeliac disease. Arch Dis Child 1990; 65:909–11.
2. Collin P. New diagnostic findings in coeliac disease. Ann Med 1999;31:399–405.
3. Scott BB, Losowsky MS. Patchiness and duodenal-jejunal variation of the mucosal abnormality in coeliac disease and dermatitis herpetiformis. Gut 1976;17:948–92.
4. Maiuri L, Ciacci C, Raia V, et al. FAS engagement drives apoptosis of enterocytes of coeliac patients. Gut 2001;48:418–24.
5. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology 1992;102:330–54.
6. Weinstein WM. Latent celiac sprue. Gastroenterology 1974; 66:489–93.
7. Ferguson A, Arranz E, O'Mahony S. Clinical and pathological spectrum of coeliac disease—Active, silent, latent, potential. Gut 1993;34:150–1.
8. Ferguson A, Murray D. Quantitation of intraepithelial lymphocytes in human jejunum. Gut 1971;12:988–94.

9. Brandtzaeg P, Halstensen TS, Kett K, et al. Immunobiology and immunopathology of human gut mucosa: Humoral immunity and intraepithelial lymphocytes. Gastroenterology 1989; 97:1562–84.
10. Spencer J, Isaacson PG, Diss TC, et al. Expression of disulfide-linked and non-disulfide-linked forms of the T cell receptor gamma/delta heterodimer in human intestinal intraepithelial lymphocytes. Eur J Immunol 1989;19:1335–8.
11. Mäki M, Holm K, Collin P, et al. Increase in gamma/delta T cell receptor bearing lymphocytes in normal small bowel mucosa in latent coeliac disease. Gut 1991;32:1412–4.
12. Savilahti E, Reunala T, Mäki M. Increase of lymphocytes bearing the gamma/delta T cell receptor in the jejunum of patients with dermatitis herpetiformis. Gut 1992;33:206–11.
13. Arranz E, Bode J, Kingstone K, et al. Intestinal antibody pattern of coeliac disease: Association with gamma/delta T cell receptor expression by intraepithelial lymphocytes, and other indices of potential coeliac disease. Gut 1994;35:476–82.
14. Savilahti E, Örmälä T, Arato A, et al. Density of gamma/delta T cells in jejunal epithelium of patients with coeliac disease and dermatitis herpetiformis is increased with age. Clin Exp Immunol 1997;109:464–7.
15. Sulkanen S, Collin P, Laurila K, et al. IgA- and IgG-class antihuman umbilical cord antibody tests in adult coeliac disease. Scand J Gastroenterol 1998;33:251–4.
16. When is a coeliac a coeliac. Report of a working group of the United European Gastroenterology Week in Amsterdam 2001. Eur J Gastroenterol Hepatol 2001;13:1123–8.
17. Verkasalo MA, Arato A, Savilahti E, et al. Effect of diet and age on jejunal and circulating lymphocyte subsets in children with coeliac disease: Persistence of CD4−8-intraepithelial T cells through treatment. Gut 1990;31:422–5.
18. Selby WS, Janossy G, Bofill M, et al. Lymphocyte subpopulations in the human small intestine. The findings in normal mucosa and in the mucosa of patients with adult coeliac disease. Clin Exp Immunol 1983;52:219–28.
19. Marsh MN. The immunopathology of the small intestinal reaction in gluten-sensitivity. Immunol Invest 1989;18:509–31.
20. Halstensen TS, Farstad IN, Scott H, et al. Intraepithelial TcR alpha/beta+ lymphocytes express CD45RO more often than the TcR gamma/delta+ counterparts in coeliac disease. Immunology 1990;71:460–6.
21. Kutlu T, Brousse N, Rambaud C, et al. Numbers of T cell receptor (TCR) alpha beta+ but not of TcR gamma delta+ intraepithelial lymphocytes correlate with the grade of villous atrophy in coeliac patients on a long term normal diet. Gut 1993;34:208–14.
22. Savilahti E, Arato A, Verkasalo M. Intestinal gamma/delta receptor-bearing T lymphocytes in celiac disease and inflammatory bowel diseases in children. Constant increase in celiac disease. Pediatr Res 1990;28:579–81.
23. Leon F, Eiras P, Roy G, et al. Intestinal intraepithelial lymphocytes and antitransglutaminase in a screening algorithm for coeliac disease. Gut 2002;50:740–1.
24. Boismenu R, Havran WL. Modulation of epithelial growth by intraepithelial gamma/delta T cells. Science 1994;266:1253–5.
25. Viney J, MacDonald TT, Spencer J. Gamma-delta T-cells in gut epithelium. Gut 1990;31:841–4.
26. Kaukinen K, Collin P, Holm K, et al. Small bowel mucosal inflammation in reticulin or gliadin antibody-positive patients without villous atrophy. Scand J Gastroenterol 1998;33:944–9.
27. Corazza GR, Andreani ML, Biagi F, et al. Clinical, pathological, and antibody pattern of latent celiac disease: Report of three adult cases. Am J Gastroenterol 1996;91:2203–7.
28. Troncone R. Latent coeliac disease in Italy. Acta Paediatr 1995;84:1252–7.
29. Kaukinen K, Mäki M, Partanen J, et al. Celiac disease without villous atrophy. Revision of criteria called for. Dig Dis Sci 2001;46:879–87.

ORIGINAL ARTICLE

Endomysial antibody-negative coeliac disease: clinical characteristics and intestinal autoantibody deposits

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Background: Some patients with untreated coeliac disease are negative for serum endomysial autoantibodies (EmA) targeted against transglutaminase 2 (TG2).
Aims: To evaluate the clinical and histological features of EmA-negative coeliac disease, and to examine whether EmA-equivalent autoantibodies against TG2 can be seen in the small-bowel mucosa when absent in serum.
Patients: Serum EmA was studied in 177 biopsy-proved specimens from adult patients with coeliac disease. 20 patients with intestinal diseases served as non-coeliac controls; three had autoimmune enteropathy with villous atrophy.
Methods: Clinical manifestations, small-bowel mucosal morphology, intraepithelial inflammation and TG2-specific extracellular immunoglobulin A (IgA) deposits were investigated in both serum EmA-negative and EmA-positive patients.
Results: 22 patients with IgA-competent coeliac disease were negative for serum EmA. Three of these had small-bowel lymphoma. Patients with EmA-negative coeliac disease were older, had abdominal symptoms more often, and the density of $\gamma\delta+$ intraepithelial lymphocytes in their intestinal mucosa was lower than in EmA-positive patients; otherwise the histology was similar. All serum EmA-negative patients with coeliac disease, but none of the disease controls, had gluten-dependent mucosal IgA deposits alongside TG2 in the small-bowel mucosal specimens. In vivo deposited IgA was shown to be TG2-specific by its ability to bind recombinant TG2.
Conclusions: Negative serum EmA might be associated with advanced coeliac disease. TG2-targeted autoantibodies were deposited in the small-bowel mucosa even when absent in serum. This finding can be used in the diagnosis of seronegative coeliac disease when the histology is equivocal. It may also be helpful in the differential diagnosis between autoimmune enteropathy and coeliac disease.

Small-bowel mucosal villous atrophy and crypt hyperplasia remain the golden standard in the diagnosis of coeliac disease.[1] However, coeliac disease has no pathognomic histological features,[2 3] and diagnosis can be difficult especially in the presence of borderline histology. Serology clearly has a supportive role,[1] as a specific feature in coeliac disease is the presence of serum immunoglobulin A (IgA)-class endomysial antibodies (EmA) targeted against transglutaminase 2 (TG2). Negative EmA in the serum on coeliac disease suspicion always brings about some ambiguity.[4 5] In obscure cases, a histological or clinical response to a gluten-free diet (GFD) or a laborious and time-consuming gluten challenge is required to ascertain the diagnosis.[5] 1

Although a positive serum EmA has a close to 100% specific association with coeliac disease,[6] approximately 10–20% of patients with untreated coeliac disease remain negative for serum EmA.[7 8] On the other hand, when patients with negative serum EmA and borderline histological lesions are treated with a GFD, there is always a possibility for a false diagnosis of coeliac disease.[3 5] Data suggesting whether EmA negativity is related to a specific clinical or histological course of coeliac disease are conflicting. Most studies suggest that EmA negativity is commonly associated with mild histological lesions,[9–11] which would contradict the notion that EmA is a marker for early-stage coeliac disease without obvious villous atrophy.[12]

EmA-binding patterns in serum samples from patients with coeliac disease have proved to be exclusively TG2-targeted,[13 14] and the correlation between EmA and TG2 antibodies is therefore good.[15 16] Evidence shows that coeliac autoantibodies are produced in the small-bowel mucosa. In phage antibody libraries from the peripheral and intestinal lymphocytes of patients with coeliac disease, the humoral response against TG2 was shown to occur at the local level in the intestinal mucosa but not peripherally.[17] This has also been shown by detecting EmA in duodenal biopsy organ culture supernatants from patients with untreated coeliac disease, and also from patients with treated coeliac disease after in vitro gliadin challenge.[18] The concept of local production of coeliac autoantibodies was reinforced in our previous study showing the presence of TG2-targeted extracellular IgA deposits detected by direct immunofluorescence from the small-bowel mucosa of patients with untreated coeliac disease.[19 20] It is intriguing to hypothesise that TG2-targeted autoantibodies would be present in the small-bowel mucosa of patients with untreated coeliac disease even when serum autoantibodies (EmA) are not detectable.

Abbreviations: EATL, enteropathy-associated T cell lymphoma; EmA, endomysial antibodies; GFD, gluten-free diet; GST, glutathione S-transferase-tagged; HLA, human leucocyte antigen; IEL, intraepithelial lymphocyte; IgA, immunoglobulin A; KSCN, potassium thiocyanate; PBS, phosphate-buffered saline; TG2, transglutaminase 2

Our study aimed to compare the clinical and histological features of IgA-competent serum EmA-negative patients with coeliac disease with those in EmA-positive patients. Further, we investigated whether TG2-specific IgA deposits can be found in the small-bowel mucosa even in seronegative patients with coeliac disease. This would have a diagnostic value in EmA-negative people suspected of coeliac disease yielding ambiguous histology, and would in most cases make the laborious gluten challenge unnecessary.

MATERIALS AND METHODS

Patients and controls

The participants were enrolled from among 833 consecutive adult patients who underwent upper gastrointestinal endoscopy at Tampere University Hospital, Tampere, Finland, between 1995 and 2000 because of suspicion of coeliac disease. Endoscopy and small-bowel biopsy were performed when coeliac disease was suspected regardless of the antibody result. Villous atrophy and crypt hyperplasia compatible with coeliac disease[1] were found in 177 of 833 (21%) patients. Patients with selective IgA deficiency were excluded from further evaluations. Signs and symptoms leading to suspicion of coeliac disease, family history of coeliac disease and the number of patients deceased after the diagnosis of coeliac disease were recorded. For the examination of small-bowel mucosal TG2-targeted IgA deposits and for the comparison of histological response to GFD, an age-matched and sex-matched EmA-positive patient with coeliac disease was selected for each IgA-competent EmA-negative patient with coeliac disease.

In all, 20 patients with intestinal disorders, but not with coeliac disease, served as controls, three of whom had autoimmune enteropathy-evinced villous atrophy (negative for human leucocyte antigen (HLA) DQ2 and DQ8), and the remaining 11 with dyspepsia, 3 with collagen colitis, 2 with ulcerative colitis and 1 with Crohn's disease had normal villous architecture. All 20 controls were negative for serum IgA-class EmA.

Serology

Serum IgA-class EmA samples were measured in the same laboratory. An indirect immunofluorescence method was used with human umbilical cord as substrate; a dilution of 1:$\geqslant$5 was considered to be positive. Positive and negative controls were included in every test batch.[6] Assessment of serum IgA-class TG2 antibodies was carried out by ELISA using guinea pig liver TG2[15] (Inova Diagnostics, San Diego, California, USA; a unit value (U) $\geqslant$20 U being positive) and human recombinant TG2[16] (Celikey, Pharmacia Diagnostics, GmbH, Freiburg, Germany; $\geqslant$5 U positive) as antigens.

Small-bowel mucosal morphology and inflammation

On endoscopy, seven forceps biopsy specimens were taken from the distal part of the duodenum. Five were processed, stained with haematoxylin and eosin, and studied under light microscopy. The specimens were interpreted according to the criteria of Marsh.[11] Marsh III lesion was further classified into three subgroups: Marsh IIIa indicated severe partial, Marsh IIIb subtotal and Marsh IIIc total villous atrophy. In addition, to study the mucosal histology more objectively, the villous height:crypt depth ratio was determined from well-oriented biopsy samples from multiple sites.[21] A ratio $<$2 was considered to be compatible with coeliac disease.

Two small-bowel biopsy specimens were freshly embedded in optimal cutting temperature compound (Tissue-Tec, Miles Elkhart, Indiana, USA), snap-frozen in liquid nitrogen and stored at -70°C. Immunohistochemical stainings for CD3+ and γδ+ intraeplithelial lymphocyte (IEL) densities were determined as described previously.[20 22] The reference values were set at 37 cells/mm for CD3+ and at 4.3 cells/mm for γδ+ IELs.[23] In our laboratory, the correlation coefficients for intraobserver variation for CD3+ and γδ+ IELs were 0.95 and 0.98, and those for interobserver variation 0.92 and 0.98, respectively.

Small-bowel mucosal TG2-targeted IgA deposits

In earlier studies, we have shown that EmA-positive patients with coeliac disease have in vivo in situ IgA deposits on TG2 in their small-bowel mucosa, and when this IgA was eluted from the tissues, it targeted purified TG2 both in ELISA and in western blot.[19] The method used here was based on our previous experiments to detect TG2-specific antibodies in situ in tissue sections by their colocalisation with TG2 when double labelled by immunofluorescence.

Frozen duodenum specimens were available in 18 of 22 EmA-negative and 17 of 22 EmA-positive patients with coeliac disease, and in all 20 controls. From each of these patients, altogether six unfixed, 5-μm-thick sections from frozen small-bowel specimens were processed, three for investigating IgA deposits and three for double-colour labelling for both IgA and TG2. IgA was detected by direct immunofluorescence using fluorescein isothiocyanate-labelled rabbit antibody against human IgA (Dako AS, Glostrup, Denmark) at a dilution of 1:40 in phosphate-buffered saline (PBS), pH 7.4. In coeliac disease, a clear subepithelial IgA deposition can be found below the basement membrane along the villous and crypt epithelium and around mucosal vessels; this is in contrast with normal small-bowel samples, where IgA is detected only inside the plasma and epithelial cells.[19 20] These coeliac disease-type IgA deposits were graded from 0 to 3 on the basis of the intensity along basement membranes in the villous–crypt area. The evaluation was carried out blinded to the disease history or laboratory findings. For the double labelling, sections were stained for human IgA (green, as above) and for TG2 (red) using monoclonal mouse antibodies against TG2 (CUB7402, NeoMarkers, Fremont, California, USA) followed by rhodamine-conjugated anti-mouse Ig antibodies (Dako), both diluted 1:200 in PBS. More than 500 small-bowel specimens have been investigated for IgA deposits in our laboratory so far, and intraobserver and interobserver variations have both been 98% in the detection of the presence or absence of TG2-targeted IgA deposits between five investigators.

Investigation of target specificity of small-bowel mucosal IgA deposits

Unfixed frozen duodenum sections from seven serum EmA-negative and six EmA-positive patients with coeliac disease were washed in PBS, pH 7.4, and incubated for 30 min with 0.1 M sodium citrate buffer (pH 5) or with 0.5–1 M potassium thiocyanate (KSCN), which dissolves non-specific protein complexes as a chaotropic agent.[24] After further washing in PBS, the sections were stained for human IgA and TG2 as described in the previous section.

In further experiments, extracellular TG2 was removed from the sections using 0.25% chloroacetic acid (Fluka Chemie AG, Buchs, Switzerland) in 0.2 M NaCl, pH 2.7, after treatment with KSCN. Chloroacetic acid is needed to disrupt the tight binding of TG2 to fibronectin[25] and to remove TG2 from the tissues.[19] The sections were thereafter similarly stained for remaining IgA and TG2.

To prove that extracellular IgA deposits in the small bowel of EmA-negative patients with coeliac disease was targeted against TG2, we investigated whether it would bind labelled TG2 added to the tissue. Glutathione S-transferase-tagged full-length human recombinant TG2 (GST-TG2) was expressed in *Escherichia coli* as described previously.[26]

Table 1 Demographic data and signs and symptoms leading to suspicion of coeliac disease in 173 immunoglobulin-competent patients with coeliac disease

	EmA-negative patients, n = 22	EmA-positive patients, n = 151	p Value
Female	9 (41)	106 (70)	0.014*
Median age (range), years	55 (20–79)	40 (16–81)	0.001*
Coeliac disease in first-degree relatives	5/14 (36)	40/101 (40)	1.000
Signs and symptoms leading to suspicion of coeliac disease			
Abdominal symptoms†	16 (73)	72 (48)	0.039*
Anaemia or malabsorption	5 (23)	43 (28)	0.799
Atypical symptoms or associated conditions‡	6 (27)	92 (61)	0.005*

EmA, endomysial antibody.
Values are n (%), unless otherwise specified.
*Significant difference between study groups ($p<0.05$).
†Diarrhoea, flatulence, indigestion, abdominal distension and abdominal pain.
‡Arthritis, skin symptoms, mouth ulcerations, neurological symptoms, increased liver enzymes, osteoporosis, alopecia areata, autoimmune thyroid disorders, Sjögren's disease, insulin-dependent diabetes mellitus, family history of coeliac disease.

Unfixed frozen small-bowel sections from patients with coeliac disease and controls were washed in PBS and incubated for 15 min at room temperature with GST-TG2 at a concentration of 0.01 mg/ml. After extensive washing, GST-TG2 bound to the tissue was labelled red by goat antibodies against GST (Pharmacia Biotech, Uppsala, Sweden) followed by Alexa Fluor 594-conjugated chicken antibodies against goat immunoglobulins (Molecular Probes, Leiden, The Netherlands). Human IgA in the tissue was labelled green as described previously. The anti-GST antibody used did not cross react with natural TG2 in the tissues. To block the binding of GST-TG2 to tissue fibronectin, GST-TG2 was also added to the sections with the 45-kDa gelatine-binding fragment of human fibronectin (Sigma F-0162, Sigma-Aldrich, St Louis, Missouri, USA; 0.2 mg/ml) and monoclonal antibodies G92 (0.4 mg/ml).[27] These antibodies recognise the blocked N-terminal segment of TG2 with high specificity.

HLA typing

HLA DQB1* allele groups were investigated using the Olerup SSP DQ low-resolution kit (Olerup SSP AB, Saltsjöbaden, Sweden). This method determines HLA DQ2, DQ4, DQ5, DQ6, DQ7, DQ8 and DQ9 allele groups.

Statistical analysis

Quantitative data were expressed as medians and ranges. Statistical differences between study groups were evaluated using Pearson's χ^2 test, Fisher's exact test or Mann–Whitney U test, as appropriate. Values of $p<0.05$ were considered to be significant.

Table 2 Severity of small-bowel mucosal villous atrophy according to Marsh classification in 173 immunoglobulin A-competent patients with coeliac disease

	EmA-negative patients, n = 22	EmA-positive patients, n = 151
Marsh IIIa	5 (23)	31 (21)
Marsh IIIb	6 (27)	53 (35)
Marsh IIIc	11 (50)	67 (44)

EmA, endomysial antibody.
Values are n (%).
No significant differences between study groups (p = 0.769).

Ethical considerations

The study protocol was approved by the ethics committee of Tampere University Hospital and informed consent was obtained from all study participants.

RESULTS

Of the 177 patients with coeliac disease, 26 (15%) had negative serum EmA, and 4 of these were IgA-deficient. Thus, 22 EmA-negative patients with coeliac disease constituted the study group. HLA DQ2 or DQ8 was detected in each of 12 patients with available sample (HLA DQ2 in 11 and DQ8 in 1). Among EmA-negative patients with coeliac disease, 13 (59%) were men and the median age was higher than in EmA-positive patients (table 1).

Abdominal symptoms were significantly more common in the EmA-negative group. Three EmA-negative patients with coeliac disease were found to have enteropathy-associated T cell lymphoma (EATL), which was detected at the same time as the diagnosis of coeliac disease was established. Two of

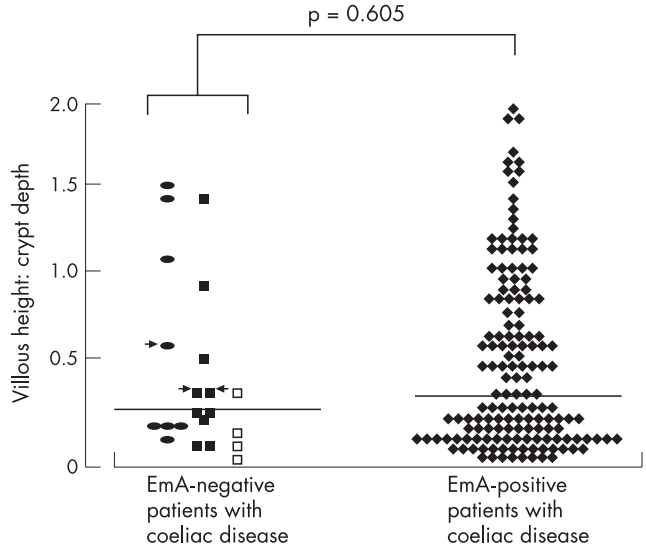

Figure 1 Villous height:crypt depth ratios in immunoglobulin A (IgA)-competent endomysial antibody (EmA)-negative and EmA-positive patients with coeliac disease. Median values are shown by solid lines. In the EmA-negative group, filled squares denote transglutaminase 2 (TG2) antibody-negative patients, open squares TG2 antibody-positive patients and filled ellipses patients without available TG2 antibody result. Furthermore, EmA-negative patients with small-bowel lymphoma are indicated with an arrow.

these patients had HLA DQ2 and in one there were no data available. All three patients had proximal small-bowel villous atrophy and crypt hyperplasia compatible with coeliac disease while on a gluten-containing diet. Furthermore, two of these patients had small-bowel biopsy taken earlier, 2 and 6 years before the diagnosis of coeliac disease and EATL. Even at that time, both showed partial villous atrophy and crypt hyperplasia, but the diagnosis of coeliac disease was overlooked. In all, 6 (27%) of the 22 EmA-negative patients and 6 (4%) of the 151 EmA-positive patients with coeliac disease died after the diagnosis of coeliac disease.

No differences were observed between EmA-negative and EmA-positive patients with coeliac disease in Marsh classification (table 2) or villous height:crypt depth ratios (fig 1).

The median density of CD3+ IELs (fig 2A) was similar, whereas the density of γδ+ IELs was statistically significantly higher in EmA-positive than in EmA-negative patients (fig 2B). Of the three EmA-negative patients with EATL, two had normal densities of γδ+ IELs.

Small-bowel mucosal IgA deposits in colocalisation with extracellular TG2 were detected in all EmA-negative (n = 18) and EmA-positive (n = 17) examined patients with coeliac disease (figs 3 and 4). The intensity of intestinal IgA deposits did not correlate with the severity of the mucosal lesion—that is, villous height:crypt depth ratios. For example, three EmA-negative patients with coeliac disease with villous height:crypt depth ratios close to 1.5 had IgA deposits with 2.5+ to 3+ intensity. Figure 4 shows that the intensity of mucosal TG2-targeted IgA deposits decreased after adopting a GFD. In contrast, TG2-targeted IgA deposits were not detected in any of the controls with intestinal diseases, not even in patients having autoimmune enteropathy with severe villous atrophy (fig 4).

To obtain direct evidence of the TG2 specificity of IgA deposits, further experiments were carried out. The small-bowel mucosal subepithelial and pericryptal IgA deposits along TG2 in both EmA-negative and EmA-positive patients with coeliac disease remained unchanged after treatment with citrate buffer and 0.5–1 M KSCN (fig 5A). In contrast, the amount of IgA deposits substantially decreased in eight samples and almost completely disappeared in five samples (fig 5B) when the sections were treated additionally with chloroacetic acid, which removes TG2 from its fibronectin binding sites. The amount of detectable TG2 also decreased in parallel (fig 5C), whereas IgA in the brush border of epithelial cells remained essentially unchanged (fig 5A, B; asterisks). Chloroacetic acid had similar effects in EmA-negative and EmA-positive samples.

When the small-bowel sections were incubated in vitro with human recombinant GST-TG2, binding of GST-TG2 was observed both in coeliac and in non-coeliac tissue sections along fibronectin (data not shown). This non-specific binding to fibronectin could be blocked by pre-incubating GST-TG2 with a soluble 45-kDa fragment of fibronectin as well as the G92 monoclonal anti-TG2 mouse antibodies. Under these conditions, GST-TG2 bound only to the coeliac tissue, colocalising with the IgA deposits (fig 5D,E), but did not bind to the duodenum sections from controls without extracellular IgA deposition (fig 5F). Small-bowel sections from the seven serum EmA-negative patients with coeliac disease gave similar results as the six EmA-positive coeliac samples. These experiments collectively show that coeliac IgA antibodies were specifically bound in situ to TG2 target antigen in the duodenum samples of both serum EmA-negative and EmA-positive patients with coeliac disease.

Serum TG2 antibody test results were available in 14 of 22 EmA-negative patients with coeliac disease; five were tested using guinea pig liver and nine using human recombinant as antigen. Four were positive and 10 negative for TG2

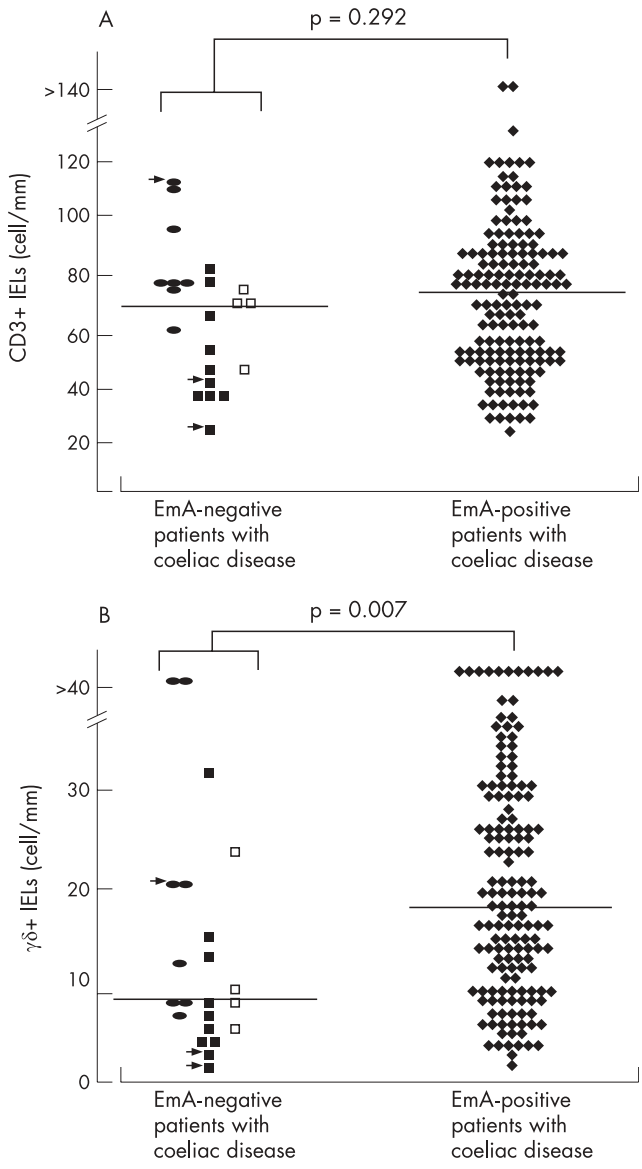

Figure 2 The density of CD3+ (A) and γδ+ (B) intraepithelial lymphocytes (IELs) in immunoglobulin A (IgA)-competent endomysial antibody (EmA)-negative and EmA-positive patients with coeliac disease. The reference values were 37 cells/mm of epithelium for CD3+ and 4.3 cells/mm for γδ+ IELs. Median values of IELs are shown by solid lines. In the EmA-negative group, filled squares denote transglutaminase 2 (TG2) antibody-negative patients, open squares TG2 antibody-positive patients and filled ellipses patients without available TG2 antibody result. EmA-negative patients with small-bowel lymphoma are indicated by arrows.

antibodies. Three of four EmA-negative TG2 antibody-positive patients had only low TG2 antibody levels using human recombinant as antigen (5.4, 6.8 and 12.8 U; normal values <5 U, median titre in untreated patients with coeliac disease 70.3, range 8.8–680).[16] Only one had a high TG2 antibody level using guinea pig liver as antigen (159 U, normal value <20 U). In the EmA-negative group, patients having positive TG2 antibodies in the serum did not show more intense intestinal IgA deposits than in TG2 antibody-negative patients.

After a median of 13 months on a GFD, there were no differences in small-bowel histological recovery between EmA-negative and EmA-positive patients with coeliac disease. Histological improvement was observed in all patients who underwent small-bowel biopsy while on a GFD, except in the three affected by EATL. Three EmA-negative and four

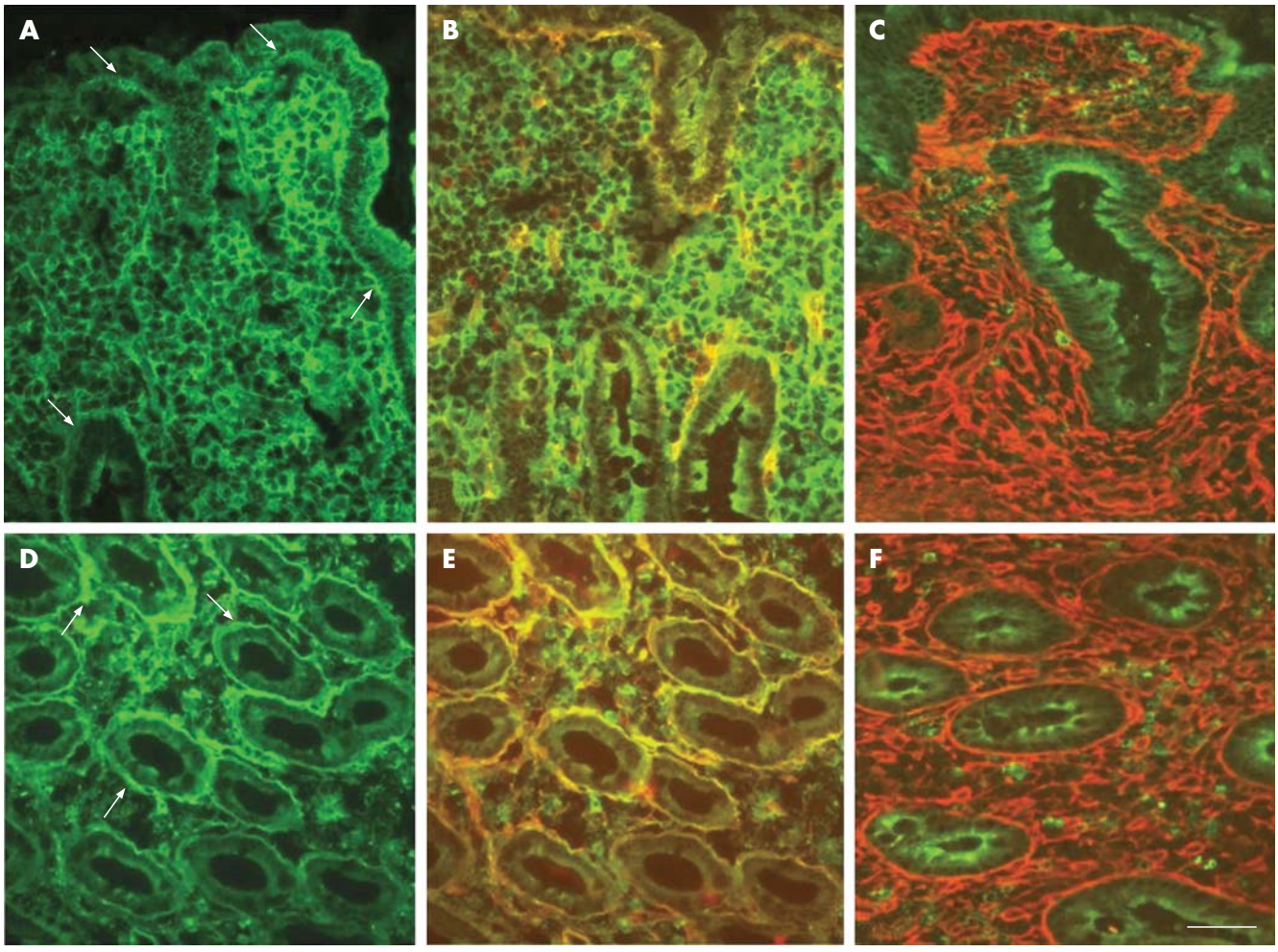

Figure 3 Subepithelial coeliac-type small-bowel mucosal immunoglobulin A (IgA) deposits (A, D, green, arrow) in IgA-competent patients with coeliac disease, with human leucocyte antigen DQ2 and negative serum endomysial (EmA) and transglutaminase 2 (TG2) antibodies. Yellow colour in composite pictures (B, E) indicates colocalisation of coeliac-type IgA deposits (green) and TG2 (red). Composite pictures of small-bowel biopsy specimens of a patient with autoimmune enteropathy having villous atrophy (C, F); IgA deposition or colocalisation of IgA and TG2 (yellow) was not detected. Bar = 50 μm.

EmA-positive patients did not undergo small-bowel biopsy, but clinical recovery on a GFD was evident in all; one EmA-

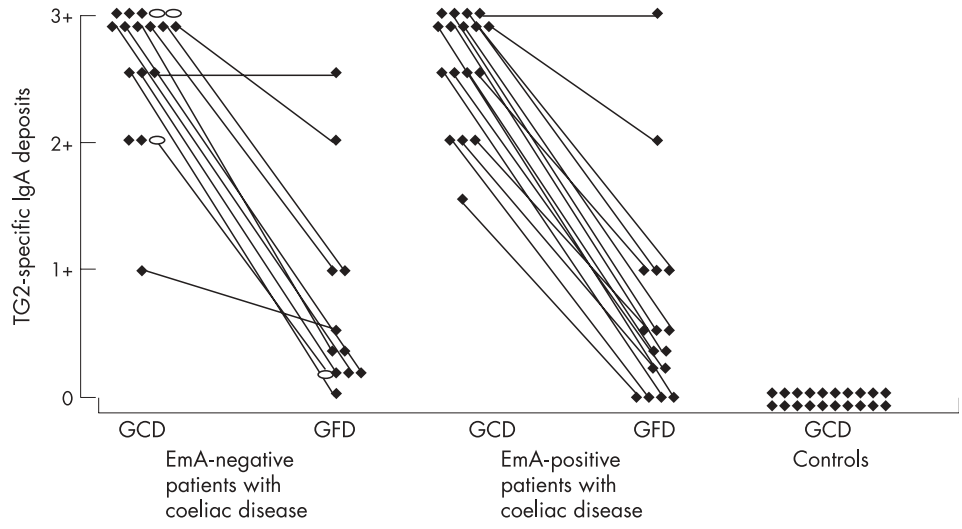

Figure 4 Transglutaminase 2 (TG2)-specific immunoglobulin A (IgA) deposits in the small-bowel mucosa of IgA-competent, endomysial antibody (EmA)-negative patients with coeliac disease and age-matched and sex-matched EmA-positive patients with coeliac disease on a normal gluten-containing diet (GCD) and after a median of 1 year on a gluten-free diet (GFD). All available intestinal IgA deposit results are shown; some patients had results available only while on GCD or while on GFD. Patients with intestinal diseases other than coeliac disease and maintaining a GCD served as controls. Open ellipses denote EmA-negative patients with coeliac disease and small-bowel lymphoma.

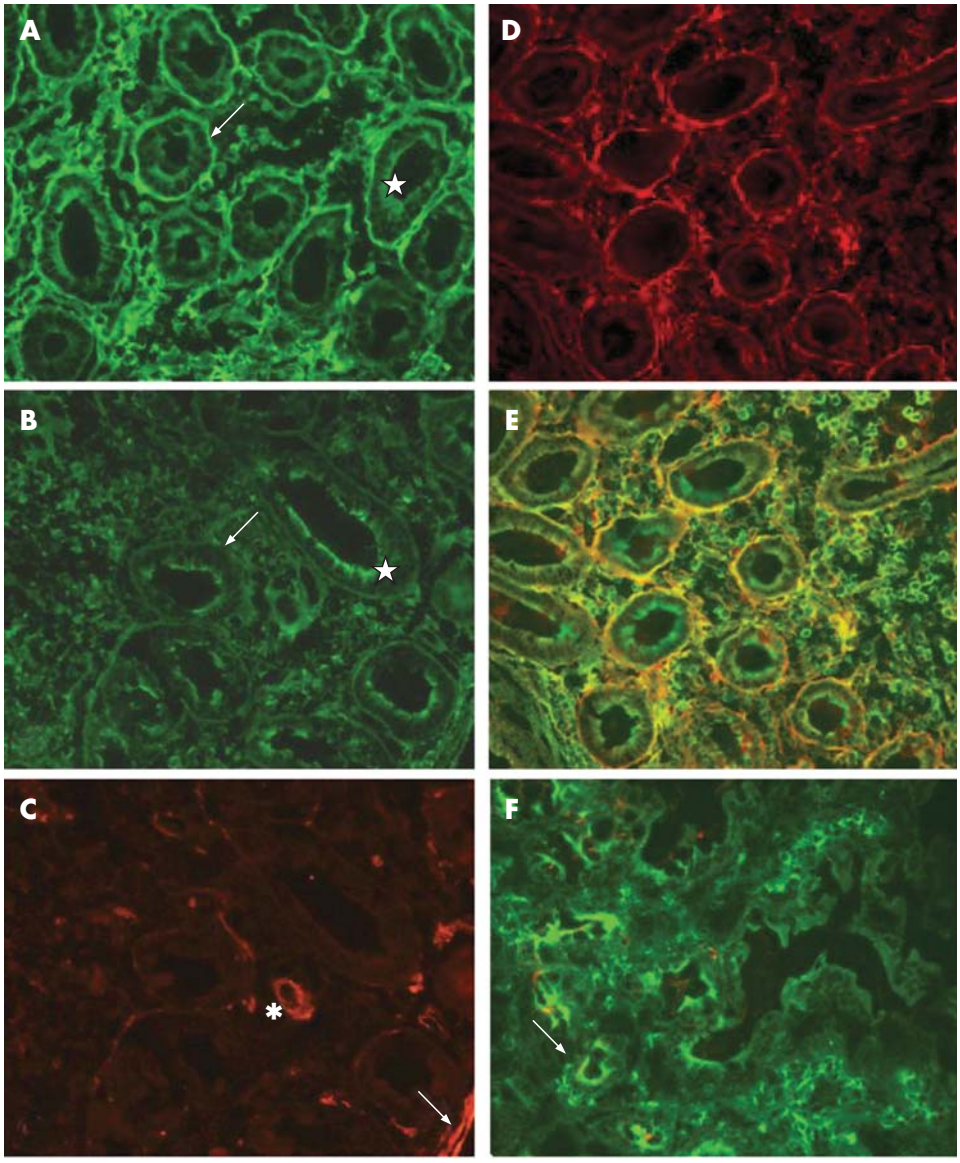

Figure 5 Investigation of specificity of deposited immunoglobulin A (IgA) for transglutaminase 2 (TG2). (A) Pretreatment of coeliac duodenum section with 0.5 M potassium thiocyanate (KSCN) does not affect extracellularly deposited IgA (green). After incubation with chloroacetic acid, which removes intrinsic TG2 from its fibronectin-binding sites, most deposited IgA (B) and extracellular TG2 (red, C) disappear from the sections (arrows), but IgA in the epithelial cells remains unchanged (A, B; asterisks). Some TG2 is visible only in vessels (C; asterisk) and smooth-muscle cells (arrow, for comparison see panel F in fig 3). Exposure time for B and C was four times longer than for A. (D, E) Glutathione S-transferase-tagged full-length human recombinant TG2 (GST-TG2) shown in red by anti-GST antibodies binds to coeliac duodenum section (D) along extracellularly deposited IgA, shown in green (E). Merging of green and red labels to yellow indicates colocalisation. Direct binding of GST-TG2 to fibronectin was blocked with 45 kDa fibronectin fragment and with monoclonal antibodies specifically targeted against the N-terminal epitope of TG2. (A–E) Specimens are from the same endomysial antibody-negative patient with coeliac disease as in fig 3. (F) No binding of GST-TG2 to control duodenum without IgA deposition, double stained for IgA (green) and GST-TG (red). Arrow shows the crypt region. Natural TG2 in the tissue is not recognised by the anti-GST antibody. Bar = 50 μm.

negative patient and one EmA-positive patient were lost to follow-up.

DISCUSSION

This study yielded two major findings: firstly, EmA-negative patients with coeliac disease were older and had more abdominal symptoms and complications than EmA-positive patients, which suggest that they had more advanced coeliac disease. Secondly, even when autoantibodies (EmA) against TG2 were not measurable in the serum, TG2-specific gluten-dependent autoantibodies were deposited and detectable in the small-bowel mucosa in all patients with coeliac disease.

The frequency of EmA-negative coeliac disease has been markedly different in previous studies.[6 11 28 29] These divergences are obviously dependent on the populations tested and on the likelihood of the disease. Further, there exists a possibility of selection bias, as EmA-negative patients are less likely than EmA-positive patients to be examined rigorously for coeliac disease. This might be an explanation for the more evident clinical manifestations in EmA-negative patients with coeliac disease in this study. On the other hand, in our department small-bowel biopsy was taken every time the patient underwent endoscopy, regardless of the indication, and endoscopy was performed in all patients suspected of coeliac disease, and also in seronegative cases. The prevalence figure (15%) for EmA-negative coeliac disease in our cohort was comparable to that reported in many other studies,[7 28 29] indicating that our series would be representative. Most EmA-negative patients were men (table 1). However, 117 (66%) of all 177 patients with coeliac disease in the present series were female, which is the typical sex distribution found in coeliac disease. Thus, the male predominance seems to be a true finding rather than a result of selection bias.

It has been proposed that coeliac autoantibodies might have a biological role in the immunopathology of the coeliac mucosal lesion,[30] but the fact that these autoantibodies are not present in the serum of every patient with coeliac disease contradicts this concept.[31 32] The current study does not exclude the possible importance of autoantibodies in the pathogenesis of coeliac disease, as we showed that auto-antibodies (equivalent to EmA) targeted against TG2 were deposited in the small-bowel mucosa of even seronegative patients with coeliac disease, and also that these deposits were gluten dependent. Moreover, we could also show that the in vivo deposited IgA is functional towards TG2, as it was also able to bind externally added recombinant human TG2. Thus, it appears that autoantibodies seem to be sequestered in the bowel of seronegative patients and autoantibodies present in the serum seem to be caused by spill-over from the gut. IgA antibodies of EmA-negative patients could not be

removed from the gut tissue by moderate amounts of KSCN. As KSCN is often used to test the avidity of antigen–antibody binding,[24] our results also indicate that coeliac antibodies are bound to intestinal TG2 with considerably high avidity. During a longstanding immune reaction, antibodies with increasing avidity are produced, which makes it understandable why older patients with coeliac disease may have lower serum EmA levels than the younger ones. Thus longstanding coeliac disease might even result in seronegativity.

Some uncertainty often exists in the diagnosis of coeliac disease when serum EmA is negative, as villous atrophy can also be present in other disorders.[3–5] Also, the poor quality of biopsy specimens makes erroneous diagnosis possible.[28] In this study, none of the EmA-negative patients with coeliac disease were HLA DQ2 and DQ8 negative, and histological or clinical recovery on a GFD was shown. The presence of small-bowel mucosal TG2 autoantibodies eventually confirmed the diagnosis of coeliac disease in EmA-negative patients. The absence of intestinal TG2-targeted autoantibodies in controls, especially in patients with autoimmune enteropathy and villous atrophy, is certainly of value in the differential diagnosis between autoimmune enteropathy and coeliac disease.

The older age and more severe clinical symptoms of EmA-negative patients with coeliac disease compared with EmA-positive patients suggest that coeliac disease has remained unrecognised for a long time in EmA-negative people. The disappearance of gliadin antibodies from the serum of patients with coeliac disease who had discontinued their GFD for a long time has been shown previously .[33] Further, the lack of humoral immune response typical of coeliac disease in patients with EATL has also been seen.[34] In the present study, negative EmA in three patients with untreated coeliac disease with EATL also supports the conclusion that EmA negativity is connected with longlasting, severe disease. We emphasise that the normal density of γδ+ IELs in two patients with EATL does not exclude coeliac disease. The sensitivity of γδ+ IELs in the diagnosis of coeliac disease has been shown to be 93–94%.[23 35] In patients with coeliac disease and EATL, the sensitivity may be even lower; rearrangement in the T cell receptor γ gene, with low densities of γδ+ IELs, has been documented in patients with refractory sprue or EATL.[36 37] On the other hand, an increased density of γδ+ IELs is not restricted to HLA DQ2 or DQ8[38] and hence is not a finding specific for coeliac disease.[23]

Collection of the current data began in 1995, and TG2 was not identified as the main and probably the sole autoantigen for EmA until 1997.[39] EmA and TG2 antibody tests correlate closely,[15 16] and the sensitivity and specificity values of these tests have been equal. Regardless of that, some patients with coeliac disease positive for EmA remain negative for TG2 antibodies and vice versa. One explanation for this fact could be that EmA and TG2-ELISA test systems expose TG2 antigenic epitopes in different ways. In this study, retrospective measurement of TG2 antibodies was not possible in every patient, but in those tested TG2 antibodies were increased in only one third of the EmA-negative patients with coeliac disease. Thus the detection of serum TG2 antibodies did not solve the problem of EmA-negative coeliac disease, as also shown previously.[40]

CONCLUSIONS

Our study suggests that serum EmA negativity might be related to a longlasting, complicated coeliac disease. Further, the results indicate that EmA-negative patients with coeliac disease had gluten-dependent TG2-specific IgA deposits in the small-bowel mucosa, which were not detected in any of the controls. The presence of these intestinal autoantibodies strengthens the diagnosis of coeliac disease. We suggest that this method could be used in the diagnostic investigation of seronegative coeliac disease instead of the time-consuming and laborious follow-up or gluten challenge, and also in the differential diagnosis of autoimmune enteropathy.

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REFERENCES

1 **Walker-Smith JA**, Guandalini S, Schmitz J, *et al*. Revised criteria for diagnosis of coeliac disease. *Arch Dis Child* 1990;**65**:909–11.
2 **Freeman HJ**. Adult celiac disease and the severe ''flat'' small bowel biopsy lesion. *Dig Dis Sci* 2004;**49**:535–45.
3 **Goldstein NS**. Non-gluten sensitivity-related small bowel villous flattening with increased intraepithelial lymphocytes: not all that flattens is celiac sprue. *Am J Clin Pathol* 2004;**121**:546–50.
4 **Kwiecien J**, Karczewska K, Lukasik M, *et al*. Negative results of antiendomysial antibodies: long term follow-up. *Arch Dis Child* 2005;**90**:41–2.
5 **Korponay-Szabo IR**, Kovacs JB, Lorincz M, *et al*. Prospective significance of antiendomysium antibody positivity in subsequently verified celiac disease. *J Pediatr Gastroenterol Nutr* 1997;**25**:56–63.
6 **Ladinser B**, Rossipal E, Pittschieler K. Endomysium antibodies in coeliac disease: an improved method. *Gut* 1994;**35**:776–8.
7 **McMillan SA**, Haughton DJ, Biggart JD, *et al*. Predictive value for coeliac disease of antibodies to gliadin, endomysium, and jejunum in patients attending for jejunal biopsy. *BMJ* 1991;**303**:1163–5.
8 **Dickey W**, Hughes DF, McMillan SA. Reliance on serum endomysial antibody testing underestimates the true prevalence of coeliac disease by one fifth. *Scand J Gastroenterol* 2000;**35**:181–3.
9 **Abrams JA**, Diamond B, Rotterdam H, *et al*. Seronegative celiac disease: increased prevalence with lesser degrees of villous atrophy. *Dig Dis Sci* 2004;**49**:546–50.
10 **Tursi A**, Brandimarte G, Giorgetti G, *et al*. Low prevalence of antigliadin and anti-endomysium antibodies in subclinical/silent celiac disease. *Am J Gastroenterol* 2001;**96**:1507–10.
11 **Rostami K**, Kerckhaert J, Tiemessen R, *et al*. Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. *Am J Gastroenterol* 1999;**94**:888–94.
12 **Kaukinen K**, Mäki M, Partanen J, *et al*. Celiac disease without villous atrophy. Revision of criteria called for. *Dig Dis Sci* 2001;**46**:879–87.

13 **Korponay-Szabo IR**, Sulkanen s, Halttunen T, et al. Tissue transglutaminase is the target in both rodent and primate tissues for celiac disease-specific autoantibodies. *J Pediatr Gastroenterol Nutr* 2000;**31**:520–7.
14 **Korponay-Szabo IR**, Laurila K, Szondy Z, *et al.* Missing endomysial and reticulin binding of coeliac antibodies in transglutaminase 2 knockout tissues. *Gut* 2003;**52**:199–204.
15 **Sulkanen S**, Halttunen T, Laurila K, *et al.* Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* 1998;**115**:1322–8.
16 **Maki M**, Mustalahti K, Kokkonen J, *et al.* Prevalence of celiac disease among children in Finland. *N Engl J Med* 2003;**348**:2517–24.
17 **Marzari R**, Sblattero D, Florian F, *et al.* Molecular dissection of tissue transglutaminase autoantibody response in celiac disease. *J Immunol* 2001;**166**:4170–6.
18 **Picarelli A**, Maiuri L, Frate A, *et al.* Production of antiendomysial antibodies after in-vitro gliadin challenge of small intestine biopsy samples from patients with coeliac disease. *Lancet* 1996;**348**:1065–7.
19 **Korponay-Szabo IR**, Halttunen T, Szalai Z, *et al.* In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies. *Gut* 2004;**53**:641–8.
20 **Kaukinen K**, Peräaho M, Collin P, *et al.* Small-bowel mucosal transglutaminase 2-specific IgA-deposits in coeliac disease without villous atrophy: prospective and randomized clinical study. *Scand J Gastroenterol* 2005;**40**:564–72.
21 **Kuitunen P**, Kosnai I, Savilahti E. Morphometric study of the jejunal mucosa in various childhood enteropathies with special reference to intraepithelial lymphocytes. *J Pediatr Gastroenterol Nutr* 1982;**1**:525–31.
22 **Arranz E**, Bode J, Kingstone K, *et al.* Intestinal antibody pattern of coeliac disease: association with gamma/delta T cell receptor expression by intraepithelial lymphocytes, and other indices of potential coeliac disease. *Gut* 1994;**35**:476–82.
23 **Järvinen TT**, Kaukinen K, Laurila K, *et al.* Intraepithelial lymphocytes in celiac disease. *Am J Gastroenterol* 2003;**98**:1332–7.
24 **Jones CL**, Macdonald RA, Hosking CS, *et al.* Estimating the relative avidity of mucosal IgA for antigen. *J Immunol Methods* 1987;**105**:111–17.
25 **Radek JT**, Jeong JM, Murthy SN, *et al.* Affinity of human erythrocyte transglutaminase for a 42-kDa gelatin-binding fragment of human plasma fibronectin. *Proc Natl Acad Sci USA* 1993;**90**:3152–6.
26 **Ambrus A**, Banyai I, Weiss MS, *et al.* Calcium binding of transglutaminases: a 43Ca NMR study combined with surface polarity analysis. *J Biomol Struct Dyn* 2001;**19**:59–74.
27 **Trejo-Skalli AV**, Velasco PT, Murthy SN, *et al.* Association of a transglutaminase-related antigen with intermediate filaments. *Proc Natl Acad Sci USA* 1995;**92**:8940–4.
28 **Collin P**, Kaukinen K, Vogelsang H, *et al.* Anti-endomysial and anti-human recombinant tissue transglutaminase antibodies in the diagnosis of coeliac disease: a biopsy-proven European multicentre study. *Eur J Gastoenterol Hepatol* 2005;**17**:85–91.
29 **Feighery C**, Weir DG, Whelan A, *et al.* Diagnosis of gluten-sensitive enteropathy: is exclusive reliance on histology appropriate? *Eur J Gastroenterol Hepatol* 1998;**10**:919–25.
30 **Halttunen T**, Mäki M. Serum immunoglobulin A from patients with celiac disease inhibits human T84 intestinal crypt epithelial cell differentiation. *Gastroenterology* 1999;**116**:566–72.
31 **Feighery C**, Abuzakouk M, Jackson J, *et al.* Coeliac disease serology - Ema negative disease. In: Cerf-Bensussan N, Brousse N, Caillat-Zucman S, Cellier C, Schmitz J, eds. *Coeliac disease: Proceedings of the Xth International Symposium on Coeliac Disease*. Montrouge: John Libbey Eurotext, 2003:183–90.
32 **Prasad S**, Thomas P, Nicholas DS, *et al.* Adult endomysial antibody-negative coeliac disease and cigarette smoking. *Eur J Gastoenterol Hepatol* 2001;**13**:667–71.
33 **Burgin-Wolff A**, Gaze H, Hadziselimovic F, *et al.* The diagnostic significance of gliadin and endomysium antibodies in coeliac disease of children and adults. In: Kumar PJ, Walker-Smith JA, eds. *Coeliac disease: one hundred years*. Leeds: Leeds University Press, 1988:106–9.
34 **O'Farrelly C**, Feighery C, O'Briain DS, *et al.* Humoral response to wheat protein in patients with coeliac disease and enteropathy associated T cell lymphoma. *BMJ* 1986;**293**:908–10.
35 **Camarero C**, Eiras P, Asensio A, *et al.* Intraepithelial lymphocytes and coeliac disease: permanent changes in CD3−/CD7+ and T cell receptor gamma delta subsets studied by flow cytometry. *Acta Paediatr* 2000;**89**:285–90.
36 **Cellier C**, Patey N, Mauvieux L, *et al.* Abnormal intestinal intraepithelial lymphocytes in refractory sprue. *Gastroenterology* 1998;**114**:471–81.
37 **Farstad IN**, Johansen F-E, Vlatkovic L, *et al.* Heterogeneity of intraepithelial lymphocytes in refractory sprue: potential implications of CD30 expression. *Gut* 2002;**51**:372–8.
38 **Chan KN**, Philips AD, Walker-Smith JA, *et al.* Density of gamma/delta T cells in small bowel mucosa related to HLA-DQ status without coeliac disease. *Lancet* 1993;**342**:492–3.
39 **Dieterich W**, Ehnis T, Bauer M, *et al.* Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 1997;**3**:797–801.
40 **Weiss B**, Bujanover Y, Avidan B, *et al.* Positive tissue transglutaminase antibodies with negative endomysial antibodies: low rate of celiac disease. *Isr Med Assoc J* 2004;**6**:9–12.

ORIGINAL ARTICLE

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Villous Tip Intraepithelial Lymphocytes as Markers of Early-Stage Coeliac Disease

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Järvinen TT, Collin P, Rasmussen M, Kyrönpalo S, Mäki M, Partanen J, Reunala T, Kaukinen K. Villous tip intraepithelial lymphocytes as markers of early-stage coeliac disease. Scand J Gastroenterol 2004;39:428–433.

Background: An investigation was conducted to determine whether the density of small-intestinal villous tip intraepithelial lymphocytes would be of value in clinical practice in uncovering early-stage coeliac disease. **Methods:** Villous tip, CD3+ and $\gamma\delta$+ intraepithelial lymphocytes were counted in patients with definite early-stage coeliac disease without villous atrophy, in classic coeliac disease with manifest mucosal lesion and in non-coeliac controls with normal mucosal structure. Villous tip analysis was made of haematoxylin-eosin specimens and CD3+ and $\gamma\delta$+ of immunohistochemical stainings from frozen samples. **Results:** The villous tip intraepithelial lymphocyte count was statistically significantly higher in patients with early-stage coeliac disease than in non-coeliac controls. The sensitivity of this method to detect untreated coeliac disease with normal villous architecture was 0.84; the specificity was 0.88. This method proved superior to CD3+ analysis and was at least as good as $\gamma\delta$+ analysis in detecting early-stage coeliac disease. In detecting classic coeliac disease, villous tip analysis also reached a higher sensitivity than CD3+ and $\gamma\delta$+ cells. **Conclusions:** Villous tip analysis seems to distinguish early coeliac from non-specific changes, thus providing a valuable tool in routine practice, especially when borderline findings are involved. Its value appears to be similar to counting of $\gamma\delta$+ cells, which, however, requires frozen biopsy samples.

Key words: Coeliac disease; dermatitis herpetiformis; gamma delta cells; intraepithelial lymphocytes; latency; villous atrophy

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Current criteria for coeliac disease comprise small-bowel mucosal villous atrophy with crypt hyperplasia and further, clinical or histological recovery on a gluten-free diet (1). However, villous atrophy (Marsh III) is only the end stage in the clinical course of the disease; the early changes, frequently referred to as latent coeliac disease, are characterized by infiltration of the lymphocytes (Marsh I) and subsequently hypertrophy of the crypts (Marsh II) (2). Coeliac disease is strongly associated with HLA DQ2 or DQ8, one or other of which is to be found in 96%–100% of patients. The use of HLA in the diagnosis is limited, since 30%–40% of the general population also have HLA DQ2 or DQ8 (3–5).

There is some evidence that even patients with Marsh I-II small-intestinal lesions should be recognized and treated with a gluten-free diet. They may suffer from abdominal symptoms and osteopenia or osteoporosis, which respond to a gluten-free diet (6–8). These changes are non-specific, since similar lesions can be found in several other diseases (9, 10). This again may lead to over-diagnosis of coeliac disease even when only subjects with the appropriate HLA DQ are considered.

Efforts have been made to characterize early small-bowel lesions in patients with suspected gene-linked coeliac disease. Intraepithelial lymphocytes (IELs) are for the most part CD3+ lymphocytes, and the correlation between haematoxylin-eosin stained and immunohistochemical CD3+ stainings of IELs is good (11). As expected, an elevated density of CD3+ IELs has a fairly low specificity for coeliac disease, whereas $\gamma\delta$+ T-cell receptor-bearing (TCR) IELs are considered relatively sensitive and specific, and also good candidates for markers of coeliac disease latency (12, 13). However, morphologic TCR analysis requires frozen tissue staining, which renders the method too expensive and laborious in everyday clinics. Clearly, a reliable means of discovering early coeliac disease changes in formalin-fixed tissue samples would be of value.

A study by Goldstein & Underhill showed that counting IELs from the tips of the villi might improve the diagnostic

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accuracy in coeliac disease (14). These investigators studied 78 formalin-fixed small-bowel biopsy samples from patients with suspected coeliac disease but architecturally normal villi. Twelve patients fulfilled their criteria for gluten sensitivity, and in these patients the number of villous tip IELs was significantly greater than the number in the remainder. Furthermore, the distribution of IELs was found to be distinct: patients with gluten sensitivity had an even distribution of IELs along villous sides and over tips compared to the descrendo pattern of non-gluten-sensitive patients.

The aim of this study was to evaluate the method of counting the tip IELs in a large series of patients with early-stage coeliac disease without villous atrophy. The cohort comprised patients with dermatitis herpetiformis, with biopsy-proven latent coeliac disease, and patients with probable genetic coeliac disease without atrophy. The gluten sensitivity of all patients had been ascertained by clinical or histological improvement during a gluten-free diet and in some by gluten challenge. The tip IEL method was compared with the immunohistochemical analysis of CD3+ and $\gamma\delta$+ IELs.

Materials and Methods

Patients

The study cohort comprised 183 adult patients who had undergone endoscopy at Tampere University Hospital or Tampere Health Centre during the years 1995–2002 (Table I). Forty-nine patients had proven gluten sensitivity without villous atrophy. Twelve of these patients had dermatitis herpetiformis, 5 of whom had partial villous atrophy and 7 an architecturally normal villous structure. Seventeen patients had normal villous architecture while on a gluten-containing diet, but subsequently developed small-bowel villous atrophy compatible with coeliac disease, and were hence at the time of the study diagnosed as having definite latent coeliac disease. Twenty symptomatic patients had normal villous structure, despite several other indicators of coeliac disease (Table II) and were considered to have coeliac disease without atrophy. It is noteworthy that positive EmA was only one criterion among others. Patients known to be negative for both HLA DQ2 and DQ8 were not enrolled. All 20 patients had been advised to adopt a gluten-free diet and clinical recovery was evident in all. Some patients also underwent later gluten challenge to confirm the diagnosis of coeliac disease.

Control groups comprised classic coeliac disease and non-coeliac patients (Table I). The coeliac disease group comprised 22 untreated patients with severe partial villous atrophy and crypt hyperplasia and 20 patients treated with gluten-free diet for a median of 1 year (range 0.75–10 years). Patients with subtotal or total villous atrophy were excluded, as counting tip IELs from their specimens would be impossible. Non-coeliac controls included 34 patients who had been investigated because of dyspepsia and had no suspicion of coeliac disease nor relatives with coeliac disease, 33 patients with suspected coeliac disease but with negative serum-endomysial antibody and no HLA DQ2 or DQ8 haplotypes, and 25 patients with ulcerative colitis, Crohn disease, collagen colitis or giardiasis lamblia; all non-coeliac controls had normal small intestinal villous architecture.

Methods

All patients and controls underwent upper gastrointestinal endoscopy; seven forceps biopsy specimens were taken from the distal part of the duodenum. To ensure that well-orientated

Table I. Demographic data and indications for endoscopy

	Study group			Coeliac controls		Non-coeliac controls		
	Probable coeliac disease without atrophy	Latent coeliac disease	Dermatitis herpetiformis	Untreated coeliac disease	Treated coeliac disease	Coeliac disease excluded*	Dyspepsia controls	Intestinal disease controls†
No. of patients	20	17	12	22	20	33	34	25
Female; *n* (%)	15 (75)	13 (76)	5 (42)	15 (68)	15 (75)	24 (73)	21 (62)	18 (72)
Age; mean (range), years	46 (21–74)	48 (26–69)	48 (29–70)	44 (16–72)	43 (21–77)	38 (19–63)	51 (22–72)	41 (19–67)
Indications for endoscopy; *n* (%)								
Abdominal complaints‡	13 (65)	12 (71)		9 (41)		30 (91)	34 (100)	22 (88)
Anaemia	1 (5)	1 (6)		3 (14)		2 (6)		3 (12)
Skin symptoms	1 (5)		12 (100)	1 (4)		1 (3)		
Screening at-risk groups§	5 (25)	4 (23)		9 (41)				
To control mucosal recovery on diet					20 (100)			

*Suspected clinical coeliac disease with normal villous morphology, negative HLA DQ2 and DQ8 haplotypes and negative EmA.
†Patients with colitis ulcerosa ($n = 9$), collagen colitis ($n = 6$), Crohn disease ($n = 9$) or giardiasis lamblia ($n = 1$).
‡Indigestion, flatulence, abdominal distension, loose stools; subjective experience.
§Autoimmune thyroid disorders, Sjögren's disease, neurological symptoms, mouth ulcerations, dental enamel defects, 1st-degree relatives of coeliac patients.

Table II. Indicators of coeliac disease in patients with probable coeliac disease. All had normal small-bowel villous architecture

Patients	HLADQ2 or DQ8	EmA on gluten-containing diet	EmA on gluten-free diet	Density of CD3+ IELs	Density of $\gamma\delta$+ IELs	Clinical gluten dependence	Autoimmune diseases
1	+	−	−	+	+	+*	−
2	+	+	−	−	−	+*	−
3	+	+	−	+	+	+*	−
4	+	+	−	−	+	+*	−
5	+	+	−	+	+	+	−
6	+	+	−	−	+	+	Thyroid disease
7	+	+	−	+	+	+	−
8	+	+	−	+	+	+	−
9	+	+	−	−	+	+	IDDM
10	+	+	−	+	+	+	Sjögren's disease
11	+	+	−	+	+	+	−
12	ND	+	−	+	+	+	−
13	ND	+	ND	+	+	+	−
14	ND	+	+†	+	+	+	−
15	+	+	−	−	+	+*	−
16	+	+	−	+	+	+	−
17	+	−	−	+	+	+*	−
18	+	+	−	−	+	+	−
19	+	+	−	+	+	+	−
20	ND	+	−	+	+	+	−

IELs = intraepithelial lymphocytes; ND = no data; IDDM = insulin-dependent diabetes mellitus; + = positive/increased density; − = negative/normal density.

* Patient also underwent gluten challenge afterwards with a positive result.

†Serum-EmA titre decreased.

samples would be available, five of these samples were processed, stained with haematoxylin-eosin and studied under light microscopy. The villous height crypt depth ratio (Vh/CrD) was counted. The villous tip IEL score per 20 enterocytes was calculated from the mean value of five random villi, if available, as described by Goldstein & Underhill (14). The distribution of villous IELs along the sides of the villi was determined and considered even when the numbers of IELs were similar over the tips and at the base of the villi. A descrendo-like pattern was present when the numbers of IELs were greater in the basal portions of the villi and decreased towards the tip of the villi.

Two small-bowel biopsy specimens were freshly embedded in optimal cutting temperature compound (OTC, Tissue-Tec, Miles Inc., Elkhart, Ind., USA) and stored at −70 °C. Immunohistochemical stainings were carried out on 5-µm-thick frozen sections. CD3+ IELs were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, Calif., USA) and $\gamma\delta$+ IELs with TCR $\gamma\delta$ (Endogen, Woburn, Mass., USA). Positive IELs were counted with a ×100 flat field light microscope objective throughout the surface epithelium; at least 30 fields measuring 1.6 mm in epithelial length were counted and IEL density expressed as cells per millimeter of epithelium, as described elsewhere (11, 15).

The specimens were evaluated by the same investigator, who had no knowledge of the disease history or laboratory findings. The correlation coefficients for intraobserver variation for CD3+ and for $\gamma\delta$+ IELs were 0.95 and 0.98, and those for interobserver variation 0.92 and 0.98, respectively. The cut-off values for CD3+ and $\gamma\delta$+ IELs used in the calculations had been previously determined; at 37 cells/mm for CD3+ cells and 4.3 for $\gamma\delta$+ cells (13).

Serum immunoglobulin A (IgA) endomysial antibodies were determined using an indirect immunofluorescence method with human umbilical cord as substrate; a dilution of 1:≥5 was considered positive (16). HLA DQ alleles encoding HLA DQ2 and DQ8 were determined using the Dynal SSP low-resolution DQ typing kit (Dynal AS, Oslo, Norway).

Ethical considerations

All study subjects gave informed consent and the study protocol was approved by the Ethics Committee of Tampere University Hospital.

Statistics

Quantitative data were expressed as means, ranges and 95% confidence intervals (CI). The Pearson two-tailed *t* test was used to determine the correlations between tip IELs and CD3+ and $\gamma\delta$+ IELs and intra- and interobserver variations. *P* values lower than 0.05 were considered statistically significant.

Results

The Vh/CrD values in the study group were higher than those in patients with untreated classic coeliac disease and comparable with those of non-coeliac controls and patients

Table III. Mean values and 95% confidence intervals (CI) of villous height crypt depth ratios (Vh/CrD), serum endomysium positivity, HLA DQ haplotypes and response to gluten-free diet. Untreated coeliac disease group comprised patients with severe partial villous atrophy

	Study group			Coeliac controls		Non-coeliac controls		
	Probable coeliac disease without atrophy ($n = 20$)	Latent coeliac disease ($n = 17$)	Dermatitis herpetiformis ($n = 12$)	Untreated coeliac disease ($n = 22$)	Treated coeliac disease ($n = 20$)	Coeliac disease excluded ($n = 33$)	Dyspepsia controls ($n = 34$)	Intestinal disease controls ($n = 25$)
Vh/CrD; mean (95% CI)	3.1 (2.8–3.5)	3.1 (2.8–3.5)	2.3 (1.7–2.9)*	1.4 (1.3–1.6)	2.6 (2.4–2.7)	3.2 (3.0–3.4)	3.5 (3.3–3.7)	2.9 (2.5–3.2)
Serum EmA positive; *n* (%)	18/20 (90)	12/17 (71)	4/11 (36)	19/22 (86)	0/20 (0)	0/33 (0)	0/34 (0)	1/19 (5)
HLA DQ2 or DQ8 positive; *n* (%)	15/15 (100)	9/9 (100)	ND	4/4 (100)	11/11 (100)	0/33 (0)	17/34 (50)	3/5 (60)
Gluten response; *n* (%)								
Histological	17/17 (100)†	5/5 (100)†		16/16 (100)				
Clinical	20/20 (100)	17/17 (100)	12/12 (100)	21/22 (95)‡				

ND = no data.
*All had Vh/CrD over one, seven patients had normal villous structure and five partial villous atrophy.
†Decrease in inflammation.
‡One patient refused to adopt gluten-free diet.

with treated coeliac disease (Table III). Ninety percent of patients with probable coeliac disease without atrophy, 71% of patients with latent coeliac disease and 36% of those with dermatitis herpetiformis were EmA-positive. None of the patients in the study group was negative for both HLA DQ2 and DQ8.

The mean densities of tip IELs in the study group were lower than those in untreated classic coeliac controls, but statistically significantly higher than those in any control group (Table IV and Fig. 1). The densities of CD3+ and $\gamma\delta$+ cells were likewise increased in the study group, but there was some overlap between study group and controls in 95% confidence intervals.

The cut-off value for tip IELs was set at 4.2 IELs/20 enterocytes to obtain optimal sensitivity and specificity. The sensitivity to discover early coeliac disease was 0.84 for tip IELs, 0.84 for $\gamma\delta$+ cells and 0.61 for CD3+ cells (Table V). The specificities were 0.88, 0.70 and 0.73, respectively. The sensitivity of tip IELs in revealing classic coeliac disease was better than that of CD3+ and $\gamma\delta$+ cells, as shown in Table V.

A tip IEL count was available in 13 patients with probable coeliac disease without atrophy before and after 6–12 months on a gluten-free diet (Fig. 2). The decrease in cell numbers induced by the diet was evident in 9 out of 13 patients. The mean densities of CD3+ and $\gamma\delta$+ IELs were similarly lower in treated than in untreated coeliac disease (Table IV and Fig. 1). The densities of $\gamma\delta$+ IELs, and to a lesser degree also tip IELs, remained elevated after adoption of a gluten-free diet.

The correlation coefficients for tip IELs and CD3+ IELs and tip IELs and $\gamma\delta$+ IELs were 0.53 and 0.59 ($P < 0.0001$). The intra- and interobserver variations for tip IELs were determined to assess the value of this testing method in clinical practice. The variations were 0.89 for intraobserver and 0.87 for interobserver variation.

Eighty-five percent of patients with early-stage coeliac disease, 95% of patients with classic coeliac disease and 49% of non-coeliac controls had an even distribution of IELs along the sides and over the tips of the villi. The intra- and interobserver variations for this method were 0.73 and 0.41, respectively.

Discussion

Detecting early coeliac disease changes with normal villous architecture is something of a challenge. Preliminary data indicate that counting the villous tip IELs might be a feasible approach in clinical practice (14). We therefore tested this method in a study comprising a large series of patients.

A body of evidence indicated that patients in the study group did in fact have early-stage coeliac disease. The Vh/CrD values were comparable with those in controls, meaning that villous atrophy had not yet appeared. In patients with latent coeliac disease, the development of classic coeliac disease was subsequently demonstrated. Dermatitis herpetiformis is one form of coeliac disease, and in this skin condition the villous architecture is often indicative of early coeliac lesion (17, 18). The probable early coeliac disease in the remainder of patients in the study group was more challenging to define. However, gluten dependence of symptoms was shown in every case, and HLA DQ2 or DQ8 was found in all subjects examined. The presence of positive serum EmA and increased density of $\gamma\delta$+ IELs further supports the conception of early coeliac disease (Table II).

As also shown elsewhere, in immunohistochemical stain-

Table IV. Mean values, 95% confidence intervals (CI), ranges and numbers of abnormal values of intraepithelial lymphocytes (IELs)

	Study group			Coeliac controls		Non-coeliac controls		
	Probable coeliac disease without atrophy ($n = 20$)	Latent coeliac disease ($n = 17$)	Dermatitis herpetiformis ($n = 12$)	Untreated coeliac disease ($n = 22$)	Treated coeliac disease ($n = 20$)	Coeliac disease excluded ($n = 33$)	Dyspepsia controls ($n = 34$)	Intestinal disease controls ($n = 25$)
Tip IELs								
Mean (95% CI)	6.6 (5.6–7.7)	6.8 (5.6–8.0)	6.7 (4.7–8.6)	11.6 (9.7–13.6)	4.7 (3.9–5.6)	2.3 (1.9–2.8)	2.6 (2.3–2.9)	3.3 (2.8–3.8)
Range	2.8–11.8	2.5–11.1	1.5–12.4	2.1–21.0	2.1–11.1	0.5–5.8	1.3–5.1	1.6–6.3
N:o of abnormal values	18	14	9	21	12	4	1	6
CD3+ IELs								
Mean (95% CI)	54 (43–66)	46 (28–65)	50 (39–63)	64 (54–74)	28 (24–33)	28 (23–33)	33 (28–38)	29 (22–35)
Range	7–118	13–185	8–79	19–118	7–53	3–61	11–62	10–87
N:o of abnormal values	14	9	7	18	3	9	12	4
$\gamma\delta$+ IELs								
Mean (95% CI)	16.2 (11.7–20.7)	10.5 (5.5–15.4)	13.9 (6.9–20.9)	22.8 (17.4–28.2)	10.5 (8.1–13.0)	4.6 (3.2–5.9)	3.8 (2.0–5.6)	3.9 (1.7–6.1)
Range	0.5–37.1	0.0–34.8	0.0–42.0	3.3–45.8	1.3–26.1	0.0–15.8	0.0–24.8	0.0–23.2
N:o of abnormal values	19	12	10	20	18	14	7	7

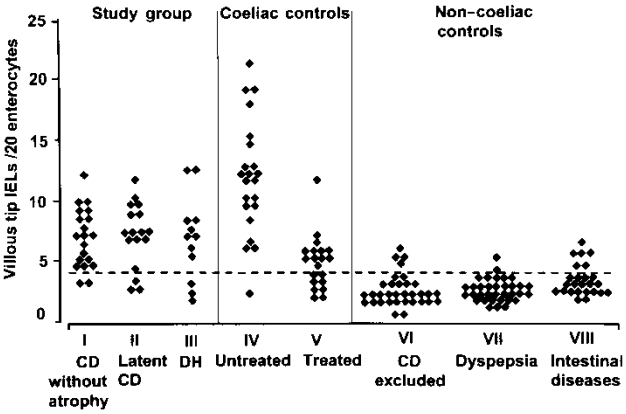

Fig. 1. Villous tip intraepithelial lymphocyte (IEL) count per 20 enterocytes in the study group consisting of patients with early coeliac disease (CD), in classic coeliac disease controls and in non-coeliac controls. The dotted line is set at 4.2, which is the cut-off value for tip IELs. DH = dermatitis herpetiformis.

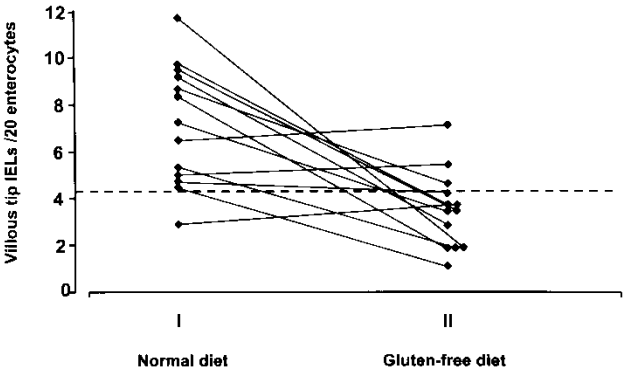

Fig. 2. Villous tip intraepithelial lymphocyte (IEL) count per 20 enterocytes in patients with probable coeliac disease without villous atrophy before (I) and after (II) 6–12 months on a gluten-free diet.

ings, analysis of $\gamma\delta$+ IELs worked better than analysis of CD3+ IELs (13), which again equals the total IEL count in haematoxylin-eosin specimens (11). In this study, counting the tip IELs was at least as sensitive and specific as the immunohistochemical analysis of $\gamma\delta$+ IELs; and the method was also as effective as $\gamma\delta$+ cells in distinguishing other intestinal disorders from coeliac disease. The current results

Table V. Sensitivity and specificity of intraepithelial lymphocytes (IELs)

	Sensitivity to detect untreated coeliac disease with normal villous architecture	Sensitivity to detect untreated coeliac disease with severe partial villous atrophy	Specificity*
Tip IELs	0.84	0.95	0.88
CD3+ IELs	0.61	0.82	0.73
$\gamma\delta$+ IELs	0.84	0.91	0.70

*Comparisons were made with all non-coeliac disease controls.

further showed the limitations of $\gamma\delta+$ cells in early and latent coeliac disease, an issue that has been controversial (12, 19). However, it must be pointed out that our study group was selected in favour of positive coeliac disease antibodies and elevated density of $\gamma\delta+$ cells, since these findings had in some cases been the reason for suspecting early coeliac disease.

Determination of the IEL distribution pattern along villi was not found to be a reliable indicator of early coeliac disease in our study. An even distribution considered to be a marker of gluten sensitivity in a previous study (14) was found in most patients with newly detected coeliac disease but also in almost half of the non-coeliac controls. Furthermore, the interpretation was highly observer-dependent, and we conclude that the density of IELs in villous tips is clearly a more reliable marker of gluten sensitivity than the density in other parts of the villi.

Finally, no single method can detect early coeliac disease cases reliably. Based on the results of this study we suggest that tip IEL analysis combined with determination of endomysial (or tissue transglutaminase) antibodies would be the first-line method in detecting early-stage coeliac disease without villous atrophy. This method can be performed on routine specimens and is inexpensive and relatively easy to administer: that is working by counting IELs per 20 tip epithelial cells instead of 100 epithelial cells, as the comparative methods have been performed. As a next step in borderline cases, the compatibility of HLA haplotypes for coeliac disease should be confirmed. Immunohistochemical analysis of $\gamma\delta+$ IELs lends further support to detecting suspected early-stage coeliac disease. After a careful survey, symptomatic patients with probable early coeliac disease should be treated and the demonstration of gluten dependence will confirm diagnosis of the condition.

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References

1. Walker-Smith JA, Guandalini S, Schmitz J, Shmerling DH, Visakorpi JK. Revised criteria for diagnosis of coeliac disease. Arch Dis Child 1990;65:909–11.
2. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity (‘celiac sprue’). Gastroenterology 1992;102:330–54.
3. Polvi A, Eland C, Koskimies S, Mäki M, Partanen J. HLA DQ and DP in Finnish families with coeliac disease. Eur J Immunogen 1996;23:221–34.
4. Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, et al. HLA types in celiac disease patients not carrying the DQA1*05–DQB1*02 (DQ2) heterodimer: results from the European genetics cluster on celiac disease. Hum Immunol 2003;64:469–77.
5. Michalski JP, McCombs CC, Arai T, Elston RC, Cao T, McCarthy CF, et al. HLA-DR, DQ genotypes of celiac disease patients and healthy subjects from the West of Ireland. Tissue Antigens 1996;47:127–33.
6. Kaukinen K, Mäki M, Partanen J, Sievänen H, Collin P. Celiac disease without villous atrophy. Revision of criteria called for. Dig Dis Sci 2001;46:879–87.
7. Wahab PJ, Crusius JBA, Meijer JWR, Mulder CJJ. Gluten challenge in borderline gluten-sensitive enteropathy. Am J Gastroenterol 2001;96:1464–9.
8. Tursi A, Brandimarte G. The symptomatic and histologic response to a gluten-free diet in patients with borderline enteropathy. J Clin Gastroenterol 2003;36:13–7.
9. Ferguson A, Murray D. Quantitation of intraepithelial lymphocytes in human jejunum. Gut 1971;12:988–94.
10. Montgomery RD, Shearer AC. The cell population of the upper jejunal mucosa in tropical sprue and post infective malabsorption. Gut 1974;15:387–91.
11. Arranz E, Bode J, Kingstone K, Ferguson A. Intestinal antibody pattern of coeliac disease: association with gamma/delta T cell receptor expression by intraepithelial lymphocytes, and other indices of potential coeliac disease. Gut 1994;35:476–82.
12. Mäki M, Holm K, Collin P, Savilahti E. Increase in gamma/delta T cell receptor bearing lymphocytes in normal small bowel mucosa in latent coeliac disease. Gut 1991;32:1412–4.
13. Järvinen TT, Kaukinen K, Laurila K, Kyrönpalo S, Rasmussen M, Mäki M, et al. Intraepithelial lymphocytes in celiac disease. Am J Gastroenterol 2003;98:1332–7.
14. Goldstein N, Underhill J. Morphologic features suggestive of gluten sensitivity in architecturally normal duodenal biopsy specimens. Am J Clin Pathol 2001;116:63–71.
15. Savilahti E, Örmälä T, Arato A, Hacek G, Holm K, Klemola T, et al. Density of gamma/delta T cells in jejunal epithelium of patients with coeliac disease and dermatitis herpetiformis is increased with age. Clin Exp Immunol 1997;109:464–7.
16. Sulkanen S, Collin P, Laurila K, Mäki M. IgA- and IgG-class antihuman umbilical cord antibody tests in adult coeliac disease. Scand J Gastroenterol 1998;33:251–4.
17. Weinstein WM. Latent celiac sprue. Gastroenterology 1974;66:489–93.
18. Reunala T. Dermatitis herpetiformis: coeliac disease of the skin. Ann Med 1998;30:416–8.
19. Iltanen S, Holm K, Ashorn M, Ruuska T, Laippala P, Mäki M. Changing jejunal gamma/delta T cell receptor (TCR)-bearing intraepithelial lymphocyte density in coeliac disease. Clin Exp Immunol 1999;117:51–5.

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Immunoglobulin A autoantibodies against transglutaminase 2 in the small intestinal mucosa predict forthcoming coeliac disease

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SUMMARY

Background
Reliable markers of early developing coeliac diseases are needed. Coeliac autoantibodies in the serum or Marsh I inflammation may be indicators of subsequent coeliac disease.

Aim
To investigate whether determination of intestinal transglutaminase 2-targeted autoantibody deposits would detect early developing coeliac disease better than previous methods.

Methods
The study investigated patients previously excluded for coeliac disease: 25 had positive serum coeliac autoantibodies (endomysial), 25 antibody-negative had Marsh I, and 25 antibody-negative had Marsh 0 finding. Seven (median) years after baseline investigation, new coeliac cases were recorded, and small bowel biopsy was offered to the rest of the patients. Serum and intestinal coeliac autoantibodies and intraepithelial lymphocytes were assessed as indicators of developing coeliac disease.

Results
Seventeen patients had developed coeliac disease: 13 in the autoantibody-positive group, three in the Marsh I group and one in the Marsh 0 group. At baseline, intestinal coeliac autoantibody deposits had a sensitivity and specificity of 93% and 93% in detecting subsequent coeliac disease, CD3+ 59% and 57%, $\gamma\delta$+ 76% and 60%, and villous tip intraepithelial lymphocytes 88% and 71%, respectively.

Conclusions
Endomysial antibodies with normal histology indicates early developing coeliac disease. Transglutaminase 2-targeted intestinal autoantibody deposits proved the best predictor of subsequent coeliac disease.

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INTRODUCTION

The diagnosis of coeliac disease requires the presence of small intestinal mucosal villous atrophy and crypt hyperplasia (Marsh III).[1, 2] However, mucosal intraepithelial lymphocytosis evincing normal villous architecture (Marsh I) precedes this lesion. Marsh I lesion may thus indicate early developing coeliac disease, but this finding is also associated with other disorders. Only 2–10% of patients are eventually shown to suffer from genetic gluten intolerance.[3, 4] Increased densities of $\gamma\delta$+[5] or villous tip intraepithelial lymphocytes (IELs)[6] have been of better positive predictive value than IELs in general in the diagnosis of early developing coeliac disease, but so far no single marker is considered a reliable indicator of this condition.

Serum immunoglobulin (Ig)A-class endomysial (EmA)[7] and transglutaminase 2 (TG2)[8, 9] antibodies are powerful tools in disclosing coeliac disease with overt villous atrophy. Some studies have suggested, however, that these autoantibodies might be less useful in the case of early developing coeliac disease,[10–12] as the antibodies are thought to enter the circulation only after severe villous atrophy has developed. In contrast, there are also cases where coeliac autoantibodies have appeared in the serum before the development of villous atrophy, implying that they might be valuable in identifying patients with early developing coeliac disease.[5, 13, 14]

Previous studies have demonstrated that TG2 is the antigen for *in vitro* binding of coeliac IgA-class antibodies to intestinal and extraintestinal tissues.[15, 16] Coeliac autoantibodies are produced in the small bowel mucosa,[17–19] and it has been recognized that the small intestinal epithelial basement membrane region contains deposited IgA in untreated coeliac disease, though the target of this deposition remained unknown for decades.[20, 21] We recently demonstrated *in vivo* that deposited extracellular IgA in the small intestinal mucosa targets TG2 in untreated coeliac disease patients.[22] Further, our previous findings have suggested that TG2-targeted intestinal autoantibody deposits might be detectable before the development of overt villous atrophy and precede the appearance of serum coeliac autoantibodies.[22, 23] These preliminary results raised the intriguing hypothesis that intestinal autoantibody deposits targeted against TG2 would precede manifest coeliac mucosal lesion, and further that this might furnish a method applicable in the diagnosis of early developing coeliac disease.

Recent evidence shows that coeliac disease is no longer restricted to severe enteropathy,[24] and that patients can suffer from gluten-dependent clinical symptoms and complications of coeliac disease even before small bowel mucosal villous atrophy has developed.[25, 26] Such patients benefit of gluten-free diet, and therefore, reliable markers of early developing coeliac disease are needed. However, previously published evidence of this condition has mainly consisted of case reports. This long-term follow-up study focused for the first time on early developing coeliac disease in a large, well-defined patient material. The aim was first to establish whether patients with normal villous architecture but positive serum coeliac autoantibodies, or Marsh I lesion in the small bowel mucosa, suffer from early developing coeliac disease. Secondly, we investigated whether intestinal TG2-targeted autoantibody deposits are superior in detecting early developing coeliac disease than the CD3+, $\gamma\delta$+ and villous tip IELs currently employed.

MATERIALS AND METHODS

Patients and study design

Patients who initially had normal villous architecture but were subsequently shown to develop manifest coeliac disease with villous atrophy and crypt hyperplasia are referred as early developing coeliac disease patients in this study. This condition has also been regarded as latent coeliac disease in the literature.

From 1995 to 1999 altogether 707 adult patients underwent small intestinal biopsy at the Department of Gastroenterology and Alimentary Tract Surgery in Tampere University Hospital due to suspicion of coeliac disease. Small bowel mucosal villous morphology and the densities of CD3+ and $\gamma\delta$+ IELs were determined in all. A coeliac disease diagnosis was established when villous atrophy and crypt hyperplasia (Marsh III lesion) were present in the biopsy specimens.[2] In 545 patients the villous architecture was normal and coeliac disease was hence excluded. However, 29 of them had positive coeliac autoantibodies in the serum and either Marsh 0 or Marsh I findings in the mucosa; in addition, 69 antibody-negative patients had Marsh I lesions in the small bowel mucosa.

According to power calculations the number of patients in each study group was set at 25. The study groups consisted of age- and sex-matched patients with normal villous architecture: a random sample of 25 of 29 patients with positive serum coeliac autoantibodies, 25 antibody-negative with Marsh I and 25 antibody-negative with Marsh 0 finding in the small bowel mucosa. The demographic data and primary reasons for coeliac disease suspicion in the 75 study patients are shown in Table 1.

The follow-up investigation took place in 2004. Altogether 69 of the original 75 subjects could be traced. The emergence of coeliac disease during the follow-up was recorded in patient files, and all patients without prior development of coeliac disease were invited for a follow-up visit a median of 7.3 years after baseline investigation. At the follow-up, clinical symptoms were recorded. The daily consumption of gluten was analysed by a dietician according to a 4-day record of food intake to ascertain that patients had not reduced their gluten intake. Patients were asked to undergo upper gastrointestinal endoscopy and small bowel biopsy.

Finally, baseline (Figure 1) histological and serological findings in patients who were shown to have developed mucosal lesion compatible with coeliac disease during the whole follow-up were compared with patients again excluded for the disease by small bowel biopsy.

Serum coeliac autoantibodies

EmA (primate-type reticulin) and reticulin antibodies (ARA, rodent-type reticulin) have been shown to be directed exclusively to TG2 in both rodent and primate tissues.[15, 16] During the study period, EmA replaced ARA in clinical practice. These two coeliac autoantibody tests have proved to be virtually identical in our laboratory,[27] and are thus termed here coeliac autoantibodies. At the follow-up visit EmA was used together with the TG2 antibody test.

Serum IgA-class ARA and EmA were determined by an indirect immunofluorescence (IF) method; a serum dilution of 1:≥5 was considered positive in both. In ARA, a typical R1 pattern was required in rat kidney and liver sections;[28] in EmA human umbilical cord was used as a substrate.[7] Assessment of serum IgA-class TG2 was carried out by enzyme-linked immunosorbent assay (ELISA; Celikey, Pharmacia Diagnostics, GmbH, Freiburg, Germany) using human recombinant TG2 as antigen, and the lower limit of positivity was a unit value of 5.[29]

Small bowel mucosal morphology and IELs

Upon endoscopy, seven forceps biopsy specimens were taken from the distal part of the duodenum. Five of these were processed, stained with haematoxylin and eosin (H & E) and studied under light

Table 1. Demographic data and primary reason for coeliac disease suspicion in study patients at baseline when coeliac disease was excluded upon normal small bowel biopsy finding

	Patients with positive coeliac autoantibodies in the serum ($n = 25$)	Patients with Marsh I finding in the small bowel mucosa* ($n = 25$)	Patients with Marsh 0 finding in the small bowel mucosa* ($n = 25$)
Female, *n* (%)	19 (76)	19 (76)	19 (76)
Age, median (range)	39 (18–67)	39 (17–73)	39 (18–68)
Primary reason for coeliac disease suspicion, *n* (%)			
Abdominal symptoms†	8 (32)	18 (72)	14 (56)
Anaemia or malabsorption	2 (8)	2 (8)	6 (24)
Extraintestinal symptoms‡	7 (28)	5 (20)	5 (20)
Coeliac disease at-risk groups§	8 (32)	–	–

* Age- and sex-matched to coeliac autoantibody-positive patients (patients had no measurable coeliac autoantibodies in the serum).
† Diarrhoea, flatulence, indigestion, abdominal distension or pain.
‡ Arthritis, skin symptoms, mouth ulcers, dental enamel defects, neurological symptoms, elevated liver enzymes, alopecia, gynaecological disorders.
§ Autoimmune thyroid disorders, Sjögren's disease, insulin-dependent diabetes mellitus, family history of coeliac disease.

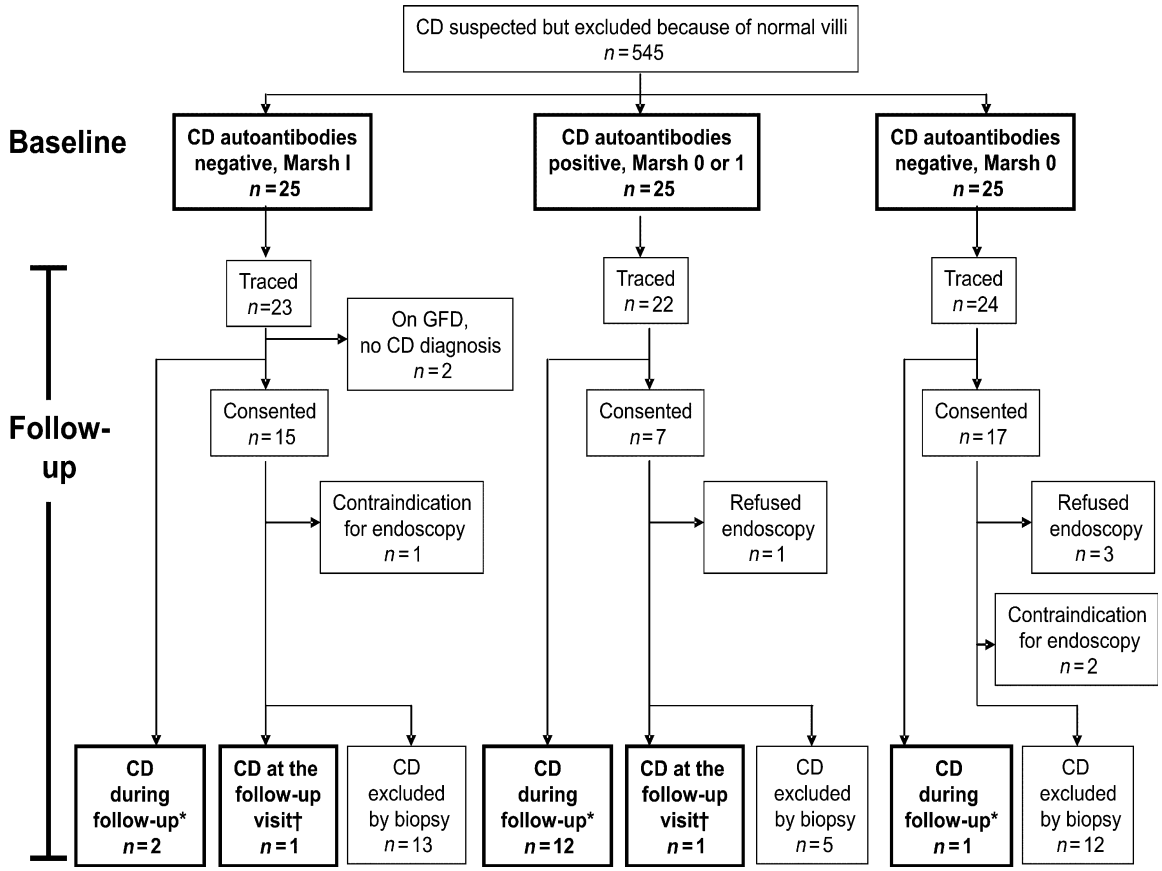

Figure 1. The development of coeliac disease (CD) in study patients with prior CD suspicion but normal villous architecture. Study groups consisted of 25 serum coeliac autoantibody-positive patients with Marsh 0 or with Marsh I finding in the small bowel mucosa and age- and sex-matched autoantibody-negative patients; 25 with Marsh I finding and 25 with Marsh 0 finding in the small bowel mucosa. Baseline indicates the time when CD was first suspected but excluded based on normal small bowel biopsy finding in all study patients; all of the statistical calculations in this study were carried out from baseline findings (GFD = gluten-free diet, * CD diagnosed during the follow-up on clinical grounds, † CD diagnosed at the follow-up visit).

microscopy. The villous height–crypt depth ratios (Vh/CrD) were determined from several well-oriented biopsy samples from multiple sites in order to detect patchy forms of villous atrophy, as described previously.[30] Vh/CrD < 2 was considered compatible with coeliac disease (Marsh III).

The villous tip IEL score/20 enterocytes was calculated from the mean value of five random villi. The reference value was set at 4.2 IELs/20 enterocytes.[31] The correlation coefficients for intraobserver variation for villous tip IELs were 0.89 and for interobserver variation 0.87 in our laboratory.

Two small bowel biopsy specimens were freshly embedded in optimal cutting temperature compound (OCT; Tissue-Tec, Miles Inc, Elkhart, IN, USA), snap-frozen in liquid nitrogen and stored at −70 °C. Immunohistochemical stainings were carried out on 5-μm-thick frozen sections. CD3+ IELs were stained with monoclonal antibody Leu-4 (Becton Dickinson, San Jose, CA, USA) and $\gamma\delta$+ IELs with T-cell receptor (TCR)-γ antibody (Endogen, Woburn, MA, USA). Positive IELs were counted with a ×100 flat field light microscope objective throughout the surface epithelium; at least 30 fields measuring 1.6 mm in epithelial length were counted and IEL density expressed as cells/mm of epithelium.[32] For comparison, in a case report the values of IELs are also given as IELs/100 enterocytes. CD3+ IELs correlated well with the total IEL density in the small bowel mucosa[32] and thus a normal density of these cells indicated a Marsh 0 and increased density of a Marsh I finding.[2] The reference values were set at

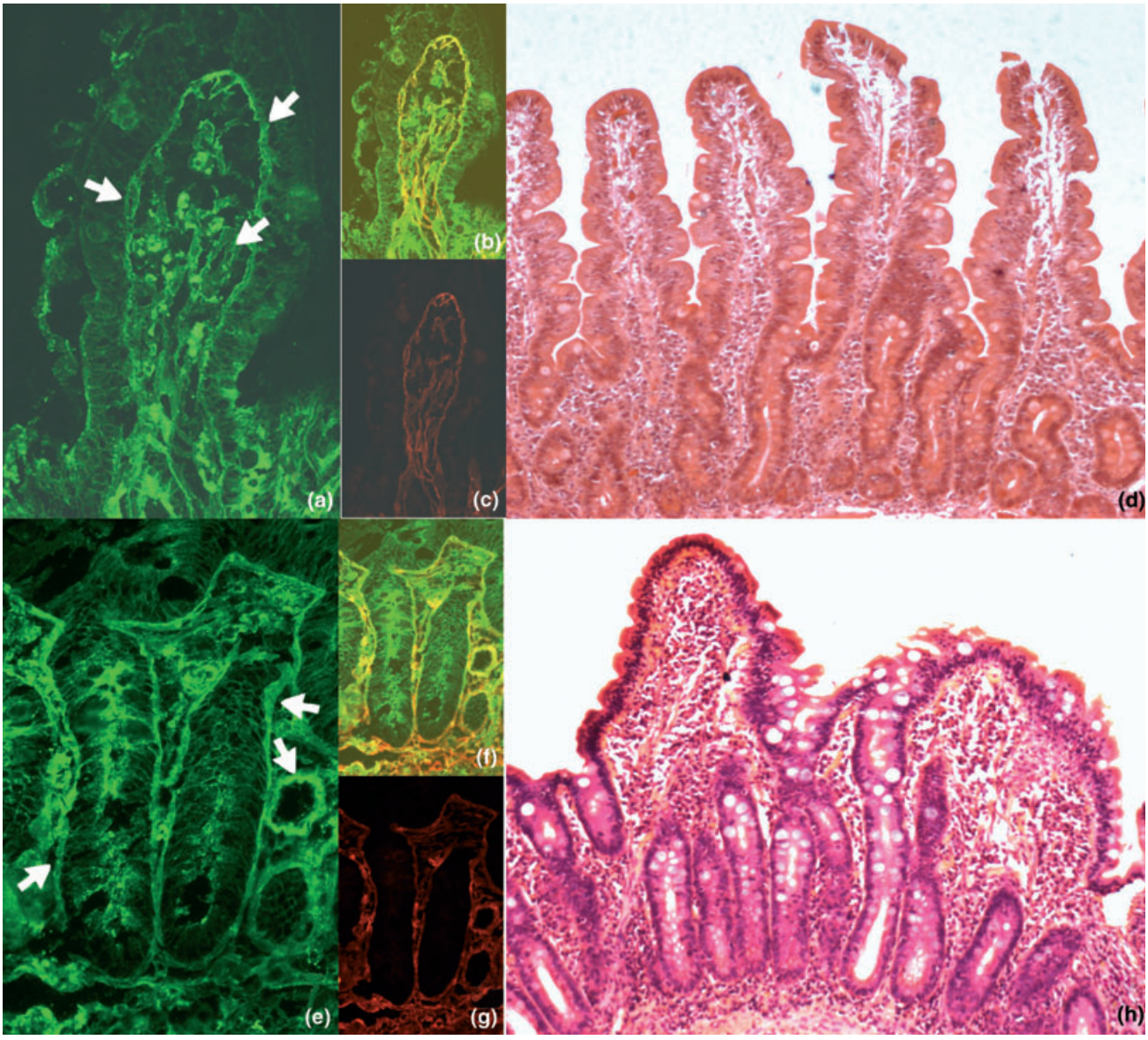

Figure 2. Patient with early developing coeliac disease having positive coeliac autoantibodies in the serum at baseline when coeliac disease was excluded by biopsy. At baseline the density of CD3+ intraepithelial lymphocytes (IELs) was 12 cells/mm (9 IELs/100 enterocytes) and the density of $\gamma\delta$+ IELs was 0.0 cells/mm (0 IELs/100 enterocytes). Subepithelial coeliac-type small bowel mucosal immunoglobulin (Ig)A deposits (a, green, arrow) and transglutaminase 2 (TG2; c, red) in the early developing stage of coeliac disease and after villous atrophy has developed (e and g respectively). Intestinal IgA deposits were detected both at baseline and at the time coeliac disease was diagnosed. Yellow colour in composite pictures (b and f) indicates co-localization of coeliac-type IgA deposits and TG2. In haematoxylin and eosin (H & E)-stained sections normal villous architecture at baseline (d) followed by villous atrophy compatible with coeliac disease 5.2 years later (h) is shown. The density of CD3+ IELs was 76 cells/mm (52 IELs/100 enterocytes) and the density of $\gamma\delta$+ IELs was 26.0 cells/mm (18 IELs/100 enterocytes) at the time coeliac disease was diagnosed.

37 cells/mm for CD3+ IELs and 4.3 for $\gamma\delta$+ IELs.[33] In our laboratory the correlation coefficients for intraobserver variation for CD3+ and $\gamma\delta$+ IELs were 0.95 and 0.98, and those for interobserver variation were 0.92 and 0.98 respectively. Evaluations of the specimens and calculations of the IELs were carried out by one investigator and without prior knowledge of disease history or laboratory findings.

Small bowel mucosal TG2-specific IgA deposits

In earlier studies we have shown that untreated coeliac disease patients have *in vivo in situ* IgA deposits on TG2 in their small bowel mucosa. When this IgA was eluted from the tissues, it targeted purified TG2 both in ELISA and Western blot. In addition, when TG2 binding to fibronectin was disrupted by chloroacetic acid, disappearance of extracellular IgA deposits was demonstrated in coeliac small bowel samples.[22] The method used here was based on our previous experiments to detect TG2-specific antibodies *in situ* in tissue sections by their co-localization with TG2 when double-labelled by IF.

In the present study, small bowel mucosal TG2-specific IgA deposits were investigated in each patient with an available frozen small bowel specimen at baseline. From each of these cases altogether six unfixed, 5-μm-thick sections from frozen small bowel specimens were processed, three for investigating IgA deposits and three for double-colour labelling for both IgA and TG2 by direct IF. IgA was detected using fluorescein isothiocyanate-labelled rabbit antibody against human IgA (Dako AS, Glostrup, Denmark) at a dilution of 1:40 in phosphate-buffered saline (PBS, pH 7.4). In coeliac disease a clear subepithelial IgA deposition can be found below the basement membrane along the villous and crypt epithelium and around mucosal vessels; in contrast in normal small bowel samples IgA is detected inside the plasma and epithelial cells only.[22, 23] The evaluation was carried out blindly without knowledge of the disease history or laboratory findings. For the double labelling, sections were stained for human IgA (green, as above) and for TG2 (red) using monoclonal mouse antibodies against TG2 (CUB7402, NeoMarkers, Fremont, CA, USA) followed by rhodamine-conjugated antimouse immunoglobulin antibodies (Dako), both diluted 1:200 in PBS. More than 500 small bowel specimens have been investigated for IgA deposits in our laboratory so far, and intraobserver and interobserver variations have both been 98% in the detection of the presence or the absence of TG2-targeted IgA deposits between five investigators.

HLA typing

The study patients were genotyped for human leucocyte antigen (HLA)-DQB1*02, DQB1*0302 and DQA1*05 alleles using the DELFIA Coeliac Disease Hybridization Assay (Perkin-Elmer Life and Analytic Sciences, Wallac Oy, Turku, Finland). DQB1*02 and DQA1*05 are associate alleles for HLA DQ2 and DQB1*0302 for HLA DQ8, and these haplotypes are found in 96–100% of coeliac disease patients.[34, 35]

Statistics

The size of the study groups was determined by power calculations: the assumption was that at least 50% of patients with positive coeliac autoantibodies in the serum and at most 10% of patients with negative autoantibodies having a Marsh I or a Marsh 0 finding at baseline would develop overt coeliac disease during the study. α was given the value of 0.05 and the power was 90%. In order to elicit significant differences between the study groups the number of patients in each group had to be at least 23, and was finally set at 25.

Quantitative data were expressed as medians and ranges. Statistical differences between study groups were evaluated using the Pearson chi-square test, Fisher's exact test or Mann–Whitney *U*-test, as appropriate. $P < 0.05$ was considered statistically significant. Sensitivities and specificities of different markers in detecting early developing coeliac disease were calculated. All calculations were performed with SPSS (version 12.0.1).

Ethical considerations

The study protocol was approved by the Ethics Committee of Tampere University Hospital. Informed consent was obtained from all study subjects.

RESULTS

Coeliac disease diagnosed on clinical grounds during the follow-up

In 2004, altogether 15 with initially normal villous architecture had developed small bowel mucosal villous atrophy and crypt hyperplasia, and hence were diagnosed with coeliac disease by routine clinical follow-up before study enrolment. Twelve were in the autoantibody-positive group, two in Marsh I and one in Marsh 0 group (Figure 1, Table 2). In addition, two patients from the original study group had adopted a complete gluten-free diet during the follow-up without proper histological verification of the disease. They were excluded from further evaluations and considered not to have coeliac disease.

Table 2. Clinical, histological and serological findings in patients who later developed villous atrophy compatible with CD; investigations were carried out at baseline when CD was excluded

Number and study group	Gender	Age (years)	Primary reason for CD suspicion	HLA DQ2/ DQ8	Serum IgA-class coeliac autoantibodies	Intestinal TG2-specific IgA deposits	CD3+ IELs (Marsh I)	$\gamma\delta$+ IELs	Villous tip IELs	Follow-up time before the development of villous atrophy (years)	Clinical manifestations at the time of the CD diagnosis
1. Ab+	F	64	Mouth ulcers	ND	+	+	+	+	+	0.3	Mouth ulcers
2. Ab+	F	36	Family history	ND	+	+	+	+	+	1.0	None
3. Ab+	F	27	Family history	+/−	+	+	−	+	+	1.0	Abdominal pain
4. Ab+	M	44	Neurological symptoms	+/−	+	+	+	+	ND	1.4	Neurological symptoms
5. Ab+	F	62	Abdominal distension	ND	+	+	+	−	+	1.5	Abdominal distension
6. Ab+	F	47	Abdominal distension	+/−	+	+	−	−	+	2.0	Abdominal distension
7. Ab+	F	42	Autoimmune thyroid disease	+/−	+	+	+	+	+	2.2	Fatigue
8. Ab+	F	50	Abdominal pain	ND	+	ND	−	+	+	2.6	Abdominal pain
9. Ab+	M	32	Diarrhoea	+/−	+	+	−	+	+	4.0	Abdominal pain
10. Ab+	F	39	Loose stools	+/−	+	+	+	+	+	4.3	Loose stools
11. Ab+	F	67	Mouth ulcers	ND	+	+	−	+	+	4.3	Loose stools
12. Ab+	F	26	Elevated liver enzymes	+/−	+	+	+	+	−	4.7	Abdominal pain
13. Ab+	F	66	Type 1 diabetes	+/−	+	+	−	−	+	5.2*	Mouth ulcers
14. Marsh I	F	67	Loose stools	+/−	−	+	+	+	−	1.7	Loose stools
15. Marsh I	M	32	Arthritis	+/−	−	ND	+	+	+	3.4	Arthritis
16. Marsh I	M	34	Diarrhoea	+/+	−	−	+	−	+	7.4*	Diarrhoea
17. Marsh 0	F	68	Abdominal pain	+/−	−	+	−	+	+	1.3	Abdominal pain

Ab, serum coeliac autoantibodies; ND, no data; TG2, transglutaminase; IEL, intraepithelial lymphocyte; IgA, immunoglobulin A; CD, coeliac disease.
+ = positive, present or increased density; − = negative, absent or normal density.
* Coeliac disease diagnosed at the follow-up visit.

Follow-up investigation

At the follow-up visit two new coeliac disease patients with typical small bowel histological findings were detected: one in the autoantibody-positive group and one in the Marsh I group (Table 2, Figures 1 and 2). At follow-up, both new coeliac disease patients had positive serum EmA and TG2 antibodies. During the follow-up, three patients had developed severe heart disease and endoscopy was considered unethical (Figure 1).

All patients except those with histologically proven coeliac disease were consuming a normal, gluten-containing diet throughout the study period. The remaining 30 patients who underwent endoscopy were again excluded for coeliac disease; all showed normal villous architecture in the follow-up biopsy specimens and were negative for serum EmA and TG2 antibodies. This means that five patients who were autoantibody-positive at baseline had undergone negative seroconversion; at baseline, one of three with a frozen sample available had detectable intestinal IgA deposits, while at the end of the follow-up it was one of four. All five patients undergoing negative seroconversion had clinical symptoms suggesting coeliac disease at baseline, and all had Marsh 0 finding in the small bowel mucosa.

Characteristics of early developing coeliac disease patients

Baseline symptoms, serological and histological findings in the 17 patients with established early developing coeliac disease (at the time coeliac disease was excluded) are shown in Table 2. Fifty-two percentage (13 of 25) of patients with baseline-positive autoantibodies in the serum, 12% (three of 25) of patients with Marsh I and 4% (one of 25) of patients with Marsh 0 at baseline were later shown to have developed villous atrophy compatible with coeliac disease ($P < 0.001$). Of the autoantibody-positive patients with early developing coeliac disease, seven had Marsh I and six had Marsh 0 findings in the small bowel mucosa at baseline. The presence of intestinal IgA deposits in patients with early developing coeliac disease and in patients again excluded for coeliac disease at baseline and at the end of the follow-up is shown in Table 3. Baseline serum TG2 antibody results were available in five patients developing coeliac disease during the follow-up. Two of these were positive for these antibodies, and also for EmA; three were negative for TG2 antibodies (one of whom had positive EmA).

There were no differences in median age or gender distribution between patients with early developing coeliac disease and patients excluded for coeliac disease. Forty-four percentage of patients with early developing coeliac disease and 10% of those excluded for coeliac disease had first-degree relatives affected by coeliac disease ($P = 0.021$). HLA DQ2 or DQ8 was detected in every early developing coeliac disease patient with an available sample ($n = 12$), and in 10 of 29 (35%) patients excluded for coeliac disease ($P < 0.001$).

Counting of total IELs (CD3+) at baseline failed to detect early developing coeliac disease (Table 4). Intestinal TG2-specific IgA deposits had the best sensitivity

Table 3. The presence of intestinal transglutaminase 2-specific IgA deposits in study groups at baseline when CD was excluded and at the end of the follow-up period

	Baseline, *n* (%)	End of the follow-up, *n* (%)
Early developing CD patients, all	14/15 (93)*	15/15 (100)*†
CD autoantibody-positive group	12/12 (100)	12/12 (100)
Marsh I group	1/2 (50)	2/2 (100)
Marsh 0 group	1/1 (100)	1/1 (100)
Patients excluded for CD by biopsy, all	1/14 (7)*	1/28 (4)*‡
CD autoantibody-positive group	1/3 (33)	1/4 (25)
Marsh I group	0/8 (0)	0/12 (0)
Marsh 0 group	0/3 (0)	0/12 (0)

* All available intestinal deposit results are shown.
† Small bowel mucosal villous atrophy and crypt hyperplasia was demonstrated in all patients.
‡ Normal small bowel mucosal morphology.
IgA, immunoglobulin A; CD, coeliac disease.

Table 4. Histological findings in patients with prior coeliac disease suspicion at baseline when coeliac disease was excluded upon normal small bowel biopsy finding

	Early developing coeliac disease ($n = 17$)	Coeliac disease excluded by biopsy ($n = 30$)	P-value
Vh/CrD, median (range)	2.7 (2.0–5.0)	3.1 (2.4–4.3)	0.063
Increased density of CD3+ IELs (Marsh I), n (%)	10/17 (59)	13/30 (43)	0.371
Increased density of $\gamma\delta$+ IELs, n (%)	13/17 (76)	12/30 (40)	0.032
Increased density of villous tip IELs, n (%)	14/16 (88)	8/28 (29)	<0.001
Intestinal TG2-specific IgA deposits present	14/15 (93)	1/14 (7)	<0.001

Vh/CrD, villous height–crypt depth ratio; TG2, transglutaminase; IEL, intraepithelial lymphocyte; IgA, immunoglobulin A.

and specificity for the condition, 93% in both. The percentages of $\gamma\delta$+ and villous tip IELs were lower: the sensitivity of $\gamma\delta$+ IELs was 76% and specificity was 60%, and the corresponding values for villous tip IELs were 88% and 71% respectively (Table 5). Nonetheless, even $\gamma\delta$+ and villous tip IELs were statistically significantly more often elevated in patients with early developing coeliac disease than in patients excluded for the disease (Table 4). We also tested different combinations of the tests shown in Table 5. The combination of HLA DQ2/DQ8 and intestinal TG2-specific IgA deposits achieved the sensitivity of 91% and the specificity of 100% and HLA and serum autoantibody testing gave a 67% sensitivity and a 97% specificity. In other combinations, the maximal sensitivity was with villous tip IELs and HLA DQ2/DQ8, 82%, with the specificity of 89%. Some other combinations yielded the 100% specificity, but the sensitivity was at its best only 79% (villous tip IELs and intestinal TG2-specific IgA deposits). Thus, the combination did not provide any additional benefit.

DISCUSSION

This study elucidated the natural history of coeliac disease. Altogether 17 patients with normal villous architecture at baseline, while consuming a gluten-containing diet, subsequently developed mucosal damage diagnostic for coeliac disease. Majority of cases had initially positive coeliac autoantibodies in the serum. When these TG2-targeted autoantibodies were examined where they are produced, in the small bowel

Table 5. Sensitivities and specificities of different markers in detecting coeliac disease before the development of villous atrophy

	Sensitivity (95% CI)	Specificity (95% CI)
Intestinal TG2-specific IgA deposits present	0.93 (0.70–0.99)	0.93 (0.69–0.99)
Serum IgA-class coeliac autoantibodies present	0.76 (0.53–0.90)	0.83 (0.66–0.93)
Increased density of villous tip IELs	0.88 (0.64–0.97)	0.71 (0.53–0.85)
Increased density of $\gamma\delta$+ IELs	0.76 (0.53–0.90)	0.60 (0.42–0.75)
Increased density of CD3+ IELs (Marsh I)	0.59 (0.36–0.78)	0.57 (0.39–0.73)
HLA DQ2/DQ8 present	1.0 (0.76–1.0)	0.66 (0.47–0.80)

Patients excluded for coeliac disease at re-evaluation served as controls.
CI, confidence interval; TG2, transglutaminase; IEL, intraepithelial lymphocyte; IgA, immunoglobulin A.

mucosa, 93% of all patients with early developing coeliac disease were identified in the absence of villous atrophy.

Occasionally coeliac disease diagnosis can be problematic; small bowel biopsy is considered the gold standard in coeliac disease diagnosis despite involving some obvious difficulties in interpretation. Even in the diagnostics of overt coeliac disease, a false diagnosis can be made, because at least 10% of biopsy specimens are of poor quality[36] and further, villous atrophy may occasionally be patchy.[37] In this study multiple small bowel biopsies were taken from several sites in order to detect also patients with patchy mucosal lesion. There is still always a possibility that a patchy form of villous atrophy is missed, and hence new methods are needed.

The diagnosis of early developing coeliac disease is even more challenging; minor histological abnormalities are unspecific, subjective and difficult to interpret. According to the recent studies focusing on early developing coeliac disease it has become evident that the recognition of coeliac disease without villous atrophy is important,[24–26] especially because the patients benefit of dietary treatment. In agreement with this also our patients with early developing coeliac disease suffered from various symptoms typical of coeliac disease before the development of overt villous atrophy (Table 2). However, we demonstrated that Marsh classification could not be utilized reliably in the detection of early developing coeliac disease. Therefore, patients with Marsh I lesion in the small bowel mucosa should not be advised to adhere to a gluten-free diet in the absence of additional evidence.

The value of villous tip IELs in discovering patients with early developing coeliac disease was superior to Marsh I lesion, as has also previously been shown in smaller series.[6, 31, 38] The clear advantage of villous tip IELs is that they can be studied in routine H & E-stained sections, whereas other histological methods applied here required frozen samples. In this study increased densities of $\gamma\delta$+ IELs were not only indicative of early developing coeliac disease, but also 40% of patients excluded for coeliac disease also on re-evaluation had increased densities of these cells at baseline when coeliac disease was first suspected. False-positive $\gamma\delta$+ cells have similarly been reported elsewhere.[13, 23]

The diagnosis of both overt and early developing coeliac disease should be based on reliable evidence. Histology has its limitations, as stated above. Nor are coeliac autoantibodies 100% specific. Five autoantibody-positive patients in this study did not proceed to villous atrophy during the follow-up, though it is possible that a longer follow-up might reveal progression of the disease in these individuals. Antibody testing was changing during the study period, and TG2 antibody test was not available at the time of baseline investigations in most patients. However, EmA and TG2 antibody tests correlate closely,[8, 29] and the sensitivity and specificity values of these tests have been equal,[39] even though some occasional patients remain negative for EmA despite positive for TG2 antibodies, and vice versa. DQ is mandatory in the identification of patients with genetic gluten intolerance, and patients with HLA DQ2 or DQ8 having positive coeliac autoantibodies in the serum are highly likely to suffer from coeliac disease. However, HLA DQ2 and DQ8 alone can only be used when patients are excluded for coeliac disease, because they are common in the population in general.[40]

We consider that intestinal IgA deposits targeted against TG2 are currently the best method in revealing early developing coeliac disease. Although serological tests predicted the development of villous atrophy relatively well, four (24%) of 17 patients with histologically confirmed coeliac disease had negative serology at baseline. Furthermore, there were five patients who underwent spontaneous negative seroconversion. Only one of them had intestinal IgA deposits at baseline, again showing that the method is more specific than serology. Investigation of intestinal IgA deposits is a special method requiring frozen small bowel biopsy specimens, which limits its utility. This method should be available at least in special centres, because it is clearly beneficial in cases where the conventional histology is ambiguous, especially in cases where a second investigation and biopsy is considered to confirm the diagnosis of coeliac disease. By investigating these deposits in small bowel biopsy specimens, when coeliac disease was first suspected, we were able in the majority of cases to diagnose early developing coeliac disease before the development of forthcoming villous atrophy. However, patients with early developing coeliac disease do not yet fulfil the traditional European Society for Pediatric Gastroenterology and Nutrition diagnostic criteria for coeliac disease. We have now shown that the criteria are no longer valid; many patients suffer from gluten-dependent symptoms before the development of villous atrophy, and the criteria should thus be revised.

In conclusion, the detection of intestinal TG2-specific IgA deposits proved a powerful diagnostic tool in coeliac disease without villous atrophy. We recommend that these deposits be invariably investigated when early developing coeliac disease is suspected. The presence of these intestinal TG2-targeted deposits strengthens the diagnosis of coeliac disease and dietary treatment should be considered at least in symptomatic patients. Further, patients with coeliac disease suspicion but normal villous architecture having positive coeliac autoantibodies in the serum or increased density of $\gamma\delta$+ or villous tip IELs in the small bowel mucosa should be followed up, whereas patients with Marsh I alone do not require routine surveillance. The detection of intestinal TG2-specific IgA deposits provides a reliable means to discover patients with early developing coeliac disease, and thus the results of this study should be taken into consideration when the diagnostic criteria for coeliac disease are revised.

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REFERENCES

1 Walker-Smith JA, Guandalini S, Schmitz J, Shmerling DH, Visakorpi JK. Revised criteria for diagnosis of coeliac disease. *Arch Dis Child* 1990; **65**: 909–11.
2 Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 1992; **102**: 330–54.
3 Lähdeaho ML, Kaukinen K, Collin P, *et al*. Celiac disease: from inflammation to atrophy: a long-term follow-up study. *J Pediatr Gastroenterol Nutr* 2005; **41**: 44–8.
4 Kakar S, Nehra V, Murray JA, Dayharsh GA, Burgart LJ. Significance of intraepithelial lymphocytosis in small bowel biopsy samples with normal mucosal architecture. *Am J Gastroenterol* 2003; **98**: 2027–33.
5 Iltanen S, Holm K, Ashorn M, Ruuska T, Laippala P, Mäki M. Changing jejunal gamma/delta T cell receptor (TCR)-bearing intraepithelial lymphocyte density in coeliac disease. *Clin Exp Immunol* 1999; **117**: 51–5.
6 Goldstein NS, Underhill J. Morphologic features suggestive of gluten sensitivity in architecturally normal duodenal biopsy specimens. *Am J Clin Pathol* 2001; **116**: 63–71.
7 Ladinser B, Rossipal E, Pittschieler K. Endomysium antibodies in coeliac disease: an improved method. *Gut* 1994; **35**: 776–8.
8 Sulkanen S, Halttunen T, Laurila K, *et al*. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* 1998; **115**: 1322–8.
9 Dieterich W, Laag E, Schöpper H, *et al*. Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 1998; **115**: 1317–21.
10 Rostami K, Kerckhaert J, Tiemessen R, von Blomberg ME, Meijer JWR, Mulder CJJ. Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. *Am J Gastroenterol* 1999; **94**: 888–94.
11 Tursi A, Brandimarte G, Giorgetti G, Gigliobianco A, Lombardi D, Gasbarrini G. Low prevalence of antigliadin and anti-endomysium antibodies in subclinical/silent celiac disease. *Am J Gastroenterol* 2001; **96**: 1507–10.
12 Abrams JA, Diamond B, Rotterdam H, Green PHR. Seronegative celiac disease: increased prevalence with lesser degrees of villous atrophy. *Dig Dis Sci* 2004; **49**: 546–50.
13 Iltanen S, Holm K, Partanen J, Laippala P, Mäki M. Increased density of jejunal gamma/delta+ T cells in patients having normal mucosa – marker of operative autoimmune mechanisms? *Autoimmunity* 1999; **29**: 179–87.
14 Collin P, Helin H, Mäki M, Hällström O, Karvonen AL. Follow-up of patients positive in reticulin and gliadin antibody tests with normal small bowel biopsy findings. *Scand J Gastroenterol* 1993; **28**: 595–8.
15 Korponay-Szabo IR, Sulkanen S, Halttunen T, *et al*. Tissue transglutaminase is the target in both rodent and primate tissues for celiac disease-specific autoantibodies. *J Pediatr Gastroenterol Nutr* 2000; **31**: 520–7.
16 Korponay-Szabo IR, Laurila K, Szondy Z, *et al*. Missing endomysial and reticulin binding of coeliac antibodies in transglutaminase 2 knockout tissues. *Gut* 2003; **52**: 199–204.
17 Marzari R, Sblattero D, Florian F, *et al*. Molecular dissection of tissue transglutaminase autoantibody response in celiac disease. *J Immunol* 2001; **166**: 4170–6.
18 Picarelli A, Maiuri L, Frate A, Greco M, Auricchio S, Londei M. Production of antiendomysial antibodies after in-vitro gliadin challenge of small intestine biopsy samples from patients with coeliac disease. *Lancet* 1996; **348**: 1065–7.
19 Sblattero D, Ventura A, Tommasini A, *et al*. Cryptic gluten intolerance in type 1 diabetes: identifying suitable candidates for a gluten free diet. *Gut* 2006; **55**: 133–4.
20 Shiner M, Ballard J. Antigen-antibody reactions in jejunal mucosa in childhood coeliac disease after gluten challenge. *Lancet* 1972; **1**: 1202–5.
21 Karpati S, Kosnai I, Török E, Kovacs J. Immunoglobulin A deposition in jejunal mucosa of children with dermatitis herpetiformis. *J Invest Dermatol* 1988; **91**: 336–9.

22 Korponay-Szabo IR, Halttunen T, Szalai Z, *et al.* In vivo targeting of intestinal and extraintestinal transglutaminase 2 by coeliac autoantibodies. *Gut* 2004; **53**: 641–8.
23 Kaukinen K, Peräaho M, Collin P, *et al.* Small-bowel mucosal transglutaminase 2-specific IgA deposits in coeliac disease without villous atrophy: a prospective and randomized clinical study. *Scand J Gastroenterol* 2005; **40**: 564–72.
24 Paparo F, Petrone E, Tosco A, *et al.* Clinical, HLA, and small bowel immunohistochemical features of children with positive serum antiendomysium antibodies and architecturally normal small intestinal mucosa. *Am J Gastroenterol* 2005; **100**: 2294–8.
25 Kaukinen K, Mäki M, Partanen J, Sievänen H, Collin P. Celiac disease without villous atrophy: revision of criteria called for. *Dig Dis Sci* 2001; **46**: 879–87.
26 Tursi A, Brandimarte G. The symptomatic and histologic response to a gluten-free diet in patients with borderline enteropathy. *J Clin Gastroenterol* 2003; **36**: 13–7.
27 Mäki M. The humoral immune system in coeliac disease. *Baillieres Clin Gastroenterol* 1995; **9**: 231–49.
28 Hällström O. Comparison of IgA-class reticulin and endomysium antibodies in coeliac disease and dermatitis herpetiformis. *Gut* 1989; **30**: 1225–32.
29 Mäki M, Mustalahti K, Kokkonen J, *et al.* Prevalence of celiac disease among children in Finland. *N Engl J Med* 2003; **348**: 2517–24.
30 Kuitunen P, Kosnai I, Savilahti E. Morphometric study of the jejunal mucosa in various childhood enteropathies with special reference to intraepithelial lymphocytes. *J Pediatr Gastroenterol Nutr* 1982; **1**: 525–31.
31 Järvinen TT, Collin P, Rasmussen M, *et al.* Villous tip intraepithelial lymphocytes as markers of early-stage coeliac disease. *Scand J Gastroenterol* 2004; **39**: 428–33.
32 Arranz E, Bode J, Kingstone K, Ferguson A. Intestinal antibody pattern of coeliac disease: association with gamma/delta T cell receptor expression by intraepithelial lymphocytes, and other indices of potential coeliac disease. *Gut* 1994; **35**: 476–82.
33 Järvinen TT, Kaukinen K, Laurila K, *et al.* Intraepithelial lymphocytes in celiac disease. *Am J Gastroenterol* 2003; **98**: 1332–7.
34 Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for a primary association of celiac disease to a particular HLA-DQ α/β heterodimer. *J Exp Med* 1989; **169**: 345–50.
35 Polvi A, Arranz E, Fernandez-Arquero M, *et al.* HLA-DQ2-negative celiac disease in Finland and Spain. *Hum Immunol* 1998; **59**: 169–75.
36 Collin P, Kaukinen K, Vogelsang H, *et al.* Anti-endomysial and anti-human recombinant tissue transglutaminase antibodies in the diagnosis of coeliac disease: a biopsy-proven European multicentre study. *Eur J Gastroenterol Hepatol* 2005; **17**: 85–91.
37 Scott BB, Losowsky MS. Patchiness and duodenal-jejunal variation of the mucosal abnormality in coeliac disease and dermatitis herpetiformis. *Gut* 1976; **17**: 984–92.
38 Biagi F, Luinetti O, Campanella J, *et al.* Intraepithelial lymphocytes in the villous tip: do they indicate potential coeliac disease? *J Clin Pathol* 2004; **57**: 835–9.
39 Hill ID. What are the sensitivity and specificity of serological tests for celiac disease? Do sensitivity and specificity vary in different populations? *Gastroenterology* 2005; **128**: S25–32.
40 Karell K, Louka AS, Moodie SJ, *et al.* HLA types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the European genetics cluster on celiac disease. *Hum Immunol* 2003; **64**: 469–77.