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ORIGINAL PAPER

The Absence of a Mucosal Lesion on Standard Histological Examination Does Not Exclude Diagnosis of Celiac Disease

Bashir M. Mohamed · Conleth Feighery · Christian Coates · Una O'Shea · David Delaney · Seán O'Briain · Jacinta Kelly · Mohamed Abuzakouk

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Abstract Some patients with undiagnosed celiac disease have minor mucosal lesions that may not be apparent during routine histological analysis. Twenty-five such patients of our institution were discharged to their primary-care physicians despite having positive endomysial antibody serology. To re-evaluate diagnosis for these patients, immunohistological staining with antibodies to CD2, CD3, CD7, CD8, CD69, and Ki67 was conducted on original biopsies from twenty patients. Clinical, serological, and histological investigations were offered to all fourteen patients who attended for review. We observed a significantly greater ($P < 0.0001$) numbers of intraepithelial lymphocytes and Ki67-positive enterocytes in sections from these twenty patients than for normal controls. Of the fourteen patients who attended for further review, firm diagnosis of celiac disease was made for seven patients and diagnosis was likely for another two. Our study clearly revealed that over-reliance on standard histological findings results in failure to diagnose celiac disease.

Keywords Celiac disease · Endomysial antibodies · Tissue transglutaminase antibodies · Intraepithelial lymphocytes · Enterocytes

B. M. Mohamed · C. Feighery (✉) · C. Coates · U. O'Shea · M. Abuzakouk
Department of Immunology, St. James's Hospital and Trinity College Dublin, Dublin 8, Ireland
e-mail: con.feighery@tcd.ie

B. M. Mohamed
e-mail: mohamebm@tcd.ie

C. Coates
e-mail: coatesc@eircom.net

U. O'Shea
e-mail: unadoshea@hotmail.com

M. Abuzakouk
e-mail: abuzakm@tcd.ie

S. O'Briain
Department of Histopathology, St. James's Hospital and Trinity College Dublin, Dublin 8, Ireland
e-mail: dobriain@tcd.ie

B. M. Mohamed · C. Feighery · C. Coates · U. O'Shea · M. Abuzakouk
Dublin Institute of Molecular Medicine Dublin, Dublin 8, Ireland

J. Kelly
Dublin Institute of Technology, Dublin, Ireland
e-mail: jacinta.kelly@dit.ie

D. Delaney
Department of Histopathology, St. James's Hospital, Dublin 8, Ireland
e-mail: davidwdelaney@eircom.net

Introduction

Celiac disease is an inflammatory disorder of the small intestine caused by the ingestion of gluten by genetically susceptible individuals [1, 2]. Although a common clinical disorder, perhaps affecting 0.5–1% of the population, for many the disorder is not diagnosed because they have few symptoms suggestive of celiac disease [3–6]. Evidence of the presence of celiac disease is often suggested by the finding of antibodies to tissue transglutaminase [7] and to endomysium [8]. Several publications confirm the high specificity and sensitivity of these serological assays [8–13]. Confirmation of the presence of enteropathy, revealed by histological examination of small-intestinal biopsies, remains the traditional method of making the diagnosis, however [14–16].

With increased use of serological tests for celiac disease it has become evident that some patients with positive celiac autoantibodies have apparently normal small-intestinal histology [17–19]. For many such patients diagnosis of celiac disease was rejected, because of the absence of an histological lesion. For some of these patients, however, more detailed histological analysis may reveal subtle histological abnormalities, for example a larger number of intraepithelial lymphocytes [17, 19] with the increase sometimes being confined to the villous tip [20, 21].

In our institution over a ten-year period (1991–2001) twenty-five patients were identified with a positive endomysial antibody test in whom routine small intestinal histology was reported to show no evidence of enteropathy. These patients had been discharged from hospital to their primary-care physicians. Despite initial rejection of the diagnosis of celiac disease in these patients, it was hypothesized that re-evaluation of the original biopsies, using detailed immunohistological staining, would reveal subtle abnormalities suggestive of this condition. The current clinical status of these patients was assessed by inviting them to return for complete clinical review.

Materials and methods

Patients

Between 1991 and 2001 twenty-five patients with a positive endomysial antibody test but normal duodenal mucosal histology were identified in our institution. These patients are referred to throughout the manuscript as the study-group subjects. They included eleven males (age range 22–75 years) and fourteen females (age range 20–86 years). Most of the patients presented with symptoms consistent with malabsorption, including iron deficiency anaemia, weight loss, chronic diarrhoea, dyspepsia, abdominal pain, fatigue, and bloating. Demographic, clinical, and laboratory features of the patients are presented in Table 1. On the basis of normal duodenal histological findings none of these patients was regarded as having celiac disease and they were discharged to their primary-care physicians. At the time of this study all these patients were invited to attend for review, and fourteen returned. Further clinical and laboratory assessments were made, including a small-intestinal biopsy for nine of the patients (Fig. 1).

Ethics approval

Ethical approval for this study was granted by the Joint Ethics Committee of St James's Hospital, Dublin, Ireland.

Celiac serological tests

The IgA endomysial antibody assay was performed using an indirect immunofluorescence technique as described elsewhere [12]. Briefly, patient serum (diluted 1:5) was added to commercial monkey oesophagus slides (Binding Site, UK) and reactivity was detected by use of FITC-labelled rabbit anti-human IgA (Dako). IgA anti-tissue transglutaminase and IgA anti-gliadin antibodies were measured using commercial ELISA kits (Celikey, Pharmacia Diagnostics, Sweden).

Small intestine biopsy specimens

Biopsies taken from the second stage of the duodenum of twenty of the twenty-five study-group subjects were available for further histological evaluation. For each patient approximately four to six biopsies were obtained. Repeat routine histological evaluation of the biopsies from these twenty patients was performed by two histopathologists (DD, SO'B), and detailed immunohistological staining of these biopsies was also performed (Fig. 1). Of the fourteen patients who returned for review, duodenal biopsies were obtained in nine and these biopsies were investigated similarly.

Duodenal biopsies from two control groups were also examined. These included celiac patients and a group of non-celiac individuals. The celiac disease group consisted of sixteen untreated patients with partial villous atrophy and crypt hyperplasia and thirteen treated celiac patients on a gluten-free diet. Endomysial and tissue transglutaminase antibodies were positive for all patients with untreated celiac disease and for seven of the thirteen treated patients. The non-celiac control group included twenty subjects who had been investigated because of symptoms of dyspepsia but had normal duodenal villous architecture and negative tTG and EMA antibodies.

Routine histology and immunohistochemistry

Haematoxylin and eosin-stained duodenal sections from all patients were examined in detail by a trained pathologist for features suggestive of celiac disease including villous atrophy, increased intraepithelial lymphocytes, enterocyte nuclear disarray, crypt hyperplasia, and increased lamina propria cellular infiltrate.

Immunohistochemical investigation was conducted on 5 μm thick, formalin fixed, paraffin-embedded duodenal sections using the avidin–biotin–peroxidase complex detection procedure (Vector Laboratories, USA; ABC technique). Deparaffinised and rehydrated sections were heated in a domestic microwave oven for at least 20 min in either 0.1 mol L^{-1} citrate buffer (pH 6.0) or EDTA

Table 1 Age, sex, clinical symptoms and laboratory results for the study-group subjects

Patient	Age (years)	Sex	Symptoms	EMA	tTG	Hb	Ferritin
1	20	F	Anaemia	+	+	4.9	
2	49	F	Bloating, dyspepsia, weight loss	+		13	3.9
3	67	F	Bloating, diarrhoea, dyspepsia	+	+		8.7
4	54	M	Fatigue	+	+		
5	34	F	Anaemia, diarrhoea, fatigue	+	+	11	3.9
6	67	F	Weight loss	+	+	12	
7	31	M	Abdominal pain, weight loss	+			
8	62	M	Anaemia, dyspepsia	+		10	89
9	22	M	Abdominal pain, weight loss	+		14	40
10	75	M	Loose bowel motion	+		14	
11	73	M	Abdominal pain, fatigue, weight loss	+			
12	41	M	Anaemia, diarrhoea, mouth ulcer	+		14	
13	55	F	Abdominal pain	+			71
14	59	F	Abdominal pain, fatigue, weight loss	+			
15	22	F	Abdominal pain, weight loss	+		12	
16	27	F	Abdominal pain, fatigue	+	+		3.9
17	81	F	Diarrhoea, fatigue, weight loss	+	+	11	5.7
18	22	F	Abdominal pain	+	+		
19	31	F	Abdominal pain	+			
20	35	F	Anaemia, fatigue, weight loss	+			7.5
21	29	M	Diarrhoea, fatigue	+		13	
22	54	M	Abdominal pain, dyspepsia	+			280
23	86	F	Anaemia, vomiting	+		12	
24	47	M	Diarrhoea, weight loss	+	+		72
25	26	M	Dyspepsia	+	+		60

Ferritin (normal range = 20–300 μg L^{-1}); haemoglobin (Hb; normal range = 11–18 g dL^{-1})

EMA: anti-endomysial antibody; tTG: anti-tissue transglutaminase antibody

(pH 8.0) as appropriate, to unmask the antigens. To block endogenous peroxidase activity sections were then immersed in 0.3% hydrogen peroxide in 100% methanol for 10 min. After incubation in normal horse serum for 20 min sections were incubated with anti-CD2, anti-CD3, anti-CD7, anti-CD8, anti-CD69 antibodies, or Ki67 antibody (NCL-Ki67p) (Novocastra, UK) at the optimised dilution in Tris-buffered saline (TBS; pH 7.6). Sections were then incubated with biotinylated rabbit anti-mouse IgG (secondary antibody) for 30 min then with peroxidase-conjugated streptavidin (Vector Laboratories ABC technique) for 30 min at room temperature. After application of each antibody, sections were washed in TBS (pH 7.6) containing 0.05% Tween. DAB (Sigma) was used for specific colour development and slides were counterstained with haematoxylin. In the immunohistochemical studies both TBS and an irrelevant, isotype control were used.

Quantification of T cell markers on intraepithelial lymphocytes and Ki67 staining of crypt cells

Intraepithelial lymphocytes expressing specific surface markers were determined by counting the number of positive cells per 500 enterocytes at high power magnification (×40) using a light microscope (Olympus Bx41). Results were expressed as the percentage of positive intraepithelial lymphocytes per 500 enterocytes.

Ki67-positive crypt cells were counted in five fields at high power magnification (×40) using an eyepiece graticule. The number of positive crypt cells was expressed as a percentage of total crypt cells. All sections were coded and the characteristics were assessed without prior knowledge of clinical and serological information.

Statistical analysis

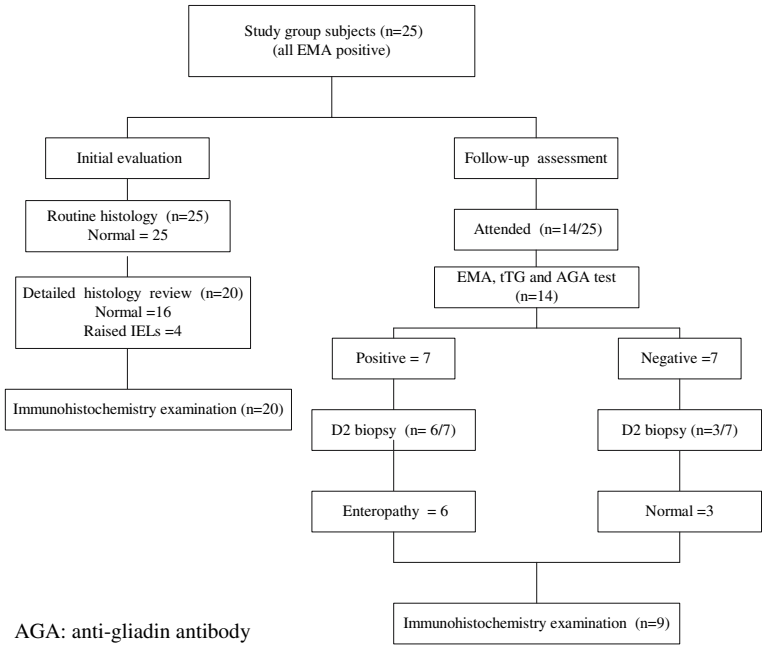
The 95% confidence intervals (CI) of intraepithelial lymphocyte counts and the level of Ki67 expression for the study group subjects and the three control groups were calculated. The Mann–Whitney *U*-test was then used to assess the statistical significance of differences. A *P*-value of <0.05 was considered to be statistically significant.

Results

Clinical review

The medical charts of the twenty-five patients were examined to ascertain whether additional features consis-

Fig. 1 Patient flow diagram



tent with a diagnosis of celiac disease were present (Table 1). For two patients prior evidence of gluten-sensitive disease was noted—patient number 7 had a history of childhood celiac disease, and for patient number 4 dermatitis herpetiformis had been diagnosed ten years earlier. Six of twelve patients for whom serum ferritin results were available had a reduced level. Ten patients had increased anti-tTG antibodies; in seven of these patients this finding was based on stored serum samples.

Re-evaluation of the original duodenal biopsies

Haematoxylin and eosin-stained slides for twenty of the twenty-five individuals were available for review by light microscopy. The number of intraepithelial lymphocytes per 100 enterocytes was counted and values in excess of 25%, regarded as elevated by others [22, 23], was found in four patients (number 3, 15, 16 and 17, Table 1) with values of 37%, 32%, 34%, and 43% respectively. All these patients were regarded as having normal villous architecture. No abnormality was observed in the other sixteen biopsies.

Immunohistochemistry studies of original duodenal biopsies

Immunohistochemical staining of the twenty duodenal biopsies was performed; the findings are illustrated in Fig. 2. Findings for untreated celiac and treated celiac patients and for the normal control group are also given. The number of intraepithelial lymphocytes expressing CD2, CD3, CD7, CD8, and CD69 molecules was significantly ($P < 0.0001$) increased in the study-group subjects than in the normal control group (Table 2). These cell populations were also significantly larger in the two celiac patient groups. Patients with untreated celiac disease had significantly greater numbers of positively stained intraepithelial lymphocytes than those in the study group, with the exception of CD69-stained cells. In contrast, the results for the study-group subjects did not differ significantly from those for treated celiac patients.

The percentage of crypt cells expressing Ki67 was significantly higher ($P < 0.0001$) in the study-group subjects and in the study and treated celiac patients than for the normal control group (Table 2). Expression of Ki67 was also significantly higher ($P < 0.0005$) in untreated celiac patients than in the study-group subjects but no difference was detected between the latter group and the treated celiac patients (Fig. 2F). Photomicrographs of intraepithelial lymphocytes expressing CD3 and crypt cells expressing Ki67 in a study-group subject and in a normal control are shown in Fig. 3 (A–D).

Follow-up data for fourteen patients who attended for reassessment

All study-group subjects were invited to attend for reassessment. Of the twenty-five patients, fourteen responded and agreed to further evaluation including clinical review and laboratory tests. Details of their current symptoms, haematology, celiac serology, and biopsy findings are

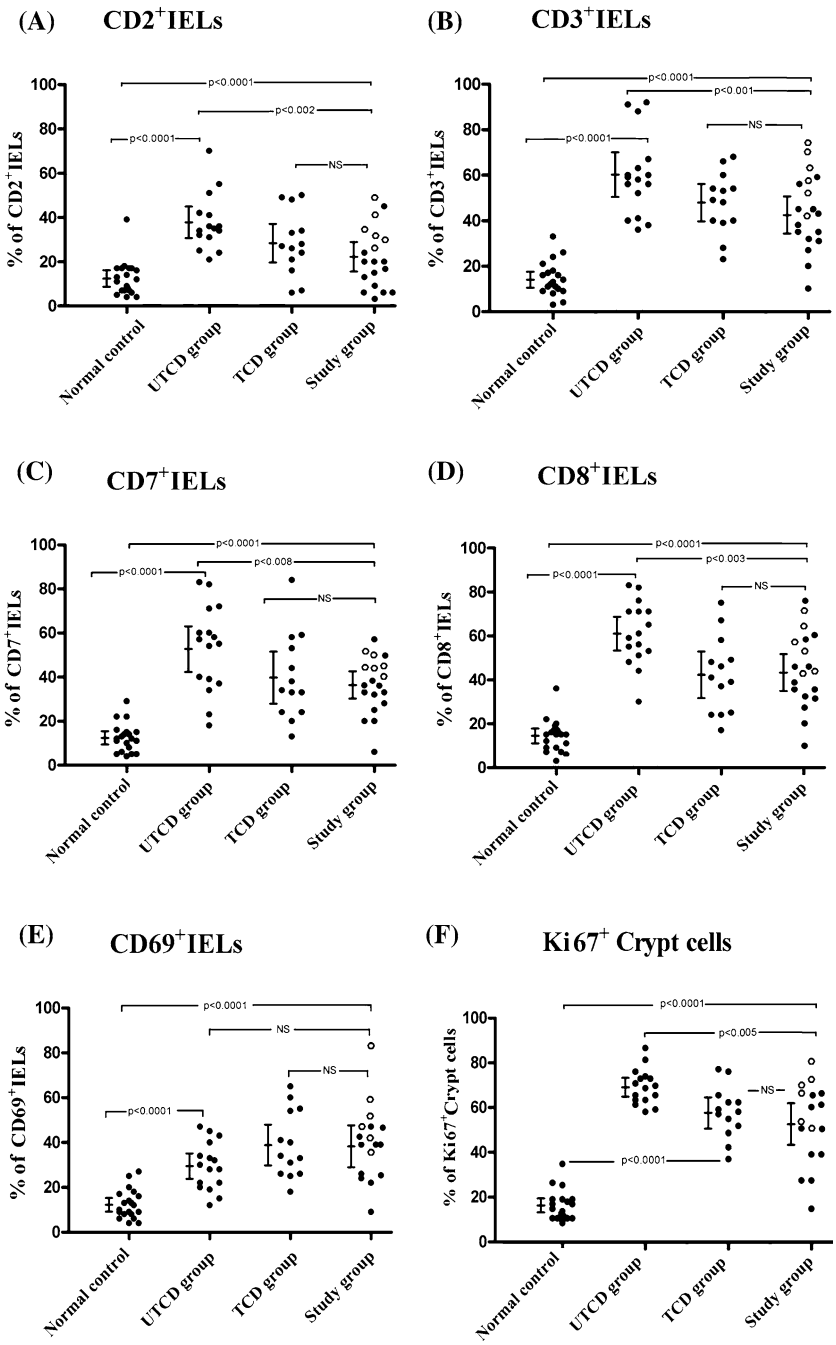


Fig. 2 (**A**–**F**) Percentages of intraepithelial lymphocytes expressing CD2, CD3, CD7, CD8, and CD69, and percentages of crypt cells expressing Ki67 in study-group subjects, untreated celiac-disease patients (UTCD), treated celiac-disease patients (TCD), and normal controls. *Open circles* denote patients who later developed a classical celiac lesion. Differences between groups were calculated by use of the Mann–Whitney *U*-test. *Bars* represent means and 95% confidence intervals (CI)

Table 2 Mean percentages and 95% confidence intervals of the examined intraepithelial lymphocyte markers and the proliferation marker (Ki67) for study-group subjects, untreated and treated celiac-disease patients, and the normal control group

Markers	Study-group subjects	Untreated celiac-disease patients	Treated celiac-disease patients	Normal controls
CD2	22% (15–29%)	38% (31–45%)	28% (20–37%)	12% (7–16%)
CD3	43% (35–51%)	60% (50–70%)	48% (40–56%)	14% (11–18%)
CD7	36% (30–44%)	53% (42–63%)	40% (28–52%)	13% (9–16%)
CD8	43% (35–52%)	61% (53–69%)	42% (32–53%)	14% (11–19%)
CD69	38% (29–48%)	29% (24–35%)	39% (30–48%)	13% (9–15%)
Ki67	50% (41–59%)	66% (62–70%)	55% (48–61%)	15% (13–15%)

summarised in Table 3 (in which patients are given the same identifying number (1, 2, 3, etc.) as in Table 1). All patients had continuing symptoms consistent with diagnosis of celiac disease. Interestingly, of the fourteen patients only seven continued to have positive endomysial, tissue transglutaminase, and anti-gliadin antibody tests. The remaining seven patients had negative antibody tests.

Nine of the fourteen patients agreed to undergo upper gastrointestinal endoscopy and duodenal biopsy. Six of the nine patients (numbers 1–6) had persistently positive celiac serology tests and all had histological changes consistent with celiac disease—total villous atrophy in two and partial villous atrophy in four. The duodenal biopsies of the remaining three patients (numbers 8, 9, 10; Table 3) who had negative tTG, EMA, and AGA antibodies on re-evaluation all had normal intestinal mucosa. Interestingly, patient number10 was discovered to have ulcerative colitis.

Immunohistochemistry on repeat biopsies

Immunohistochemical studies were performed on the repeat biopsies obtained from the nine patients. The immunohistochemical data from both original and current biopsies of the six patients who developed an histological lesion were compared with data from twenty normal controls. The number of intraepithelial lymphocytes expressing CD2, CD3, CD7, CD8, and CD69 molecules, and crypt cells expressing the Ki67 proliferation marker was significantly higher ($P < 0.0001$) for the original and current biopsies of the six patients with histological changes than for those of the normal control group. The level of expression of these markers was unchanged from the original to the repeat biopsy for the six patients, except for CD7, expression of which in the repeat biopsy was significantly higher ($P < 0.01$). For the remaining three patients, all now with negative celiac serology, immunohistochemical findings were similar to those for the control group in two patients (numbers 8 and 9) whereas an increase in all six markers was observed for the patient with ulcerative colitis (number 10).

Further testing of serum samples

The original, stored serum samples from twelve of the twenty-five patients were available for repeat analysis. For seven patients the positive EMA test finding was confirmed and, moreover, all of these patients were shown to have elevated anti-tTG antibodies. For five patients originally reported to have a positive EMA test, however, both the EMA and tTG antibody tests were negative on re-analysis. Four of these patients were included in the re-assessment group and serological tests were negative at this time also.

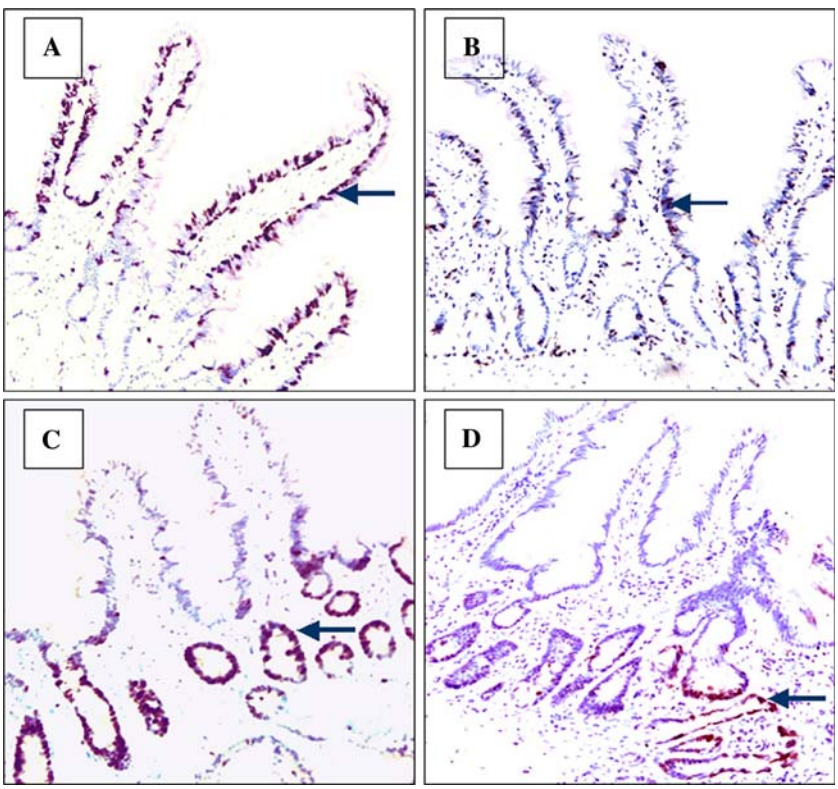


Fig. 3 Expression of CD3 and Ki67 in the gut mucosa for a study-group subject and for a normal control, revealed by immunohistochemistry. Stained intraepithelial lymphocytes expressing CD3 in the duodenal mucosa of the study-group subject (**A**) and the normal control (**B**). Stained crypt cells expressing proliferation marker Ki67 in the duodenal mucosa of the study-group subject (**C**) and the normal control (**D**). *Arrows* indicate the stained intraepithelial lymphocytes (magnification ×10)

Table 3 Age, sex, clinical and laboratory data of the fourteen patients who attended for reassessment

Patient	Age (years)	Sex	Symptoms	Follow-up interval (years)	EMA	TTG	AGA	Hb	Ferritin	Histology
1	27	F	Anaemia, bloating, fatigue	8	+	+	+	14	19	TVA
2	53	F	Abdominal pain, dyspepsia, weight loss	5	+	+	+	13		TVA
3	70	F	Anaemia, depression, skin rash	4	+	+	+	11	3.4	PVA
4	59	M	Fatigue	6	+	+	+	15	9	TVA
5	40	F	Anaemia, fatigue	7	+	+	+	9	3.9	TVA
6	77	F	Bloating, dyspepsia	12	+	+	+			PVA
7	34	M	Bloating[a]	4	+	+	+	15	160	
8	63	M	Diarrhoea, dyspepsia	6	–	–	–	10	32	Normal
9	25	M	Abdominal pain, vomiting, weight loss	4	–	–	–	15	40	Normal
10	78	M	Diarrhoea, weight loss	4	–	–	–	14	77	Normal[b]
11	75	M	Diarrhoea	3	–	–	–	14	377	
12	45	M	Mouth ulcer	5	–	–	–	12		
13	57	F	Abdominal pain	3	–	–	–	14	71	
14	64	F	Diarrhoea	7	–	–	–	13	293	

TVA: total villous atrophy; PVA: partial villous atrophy

[a] History of childhood celiac disease

[b] Normal duodenal tissue but ulcerative colitis found in colonic biopsies; ferritin (normal range = 20–300 μg L^{-1}); haemoglobin (Hb) (normal range = 11–18 g dL^{-1}); EMA: anti-endomysial antibody; tTG: anti-tissue transglutaminase antibody; AGA: anti-gliadin antibody

Discussion

Diagnosis of celiac disease is currently based on the finding of an inflammatory histological lesion in the small-intestinal mucosa [1, 14–16]. Some celiac patients have only a minimal mucosal lesion, however, and this may not be observed during routine histological analysis [17–19]. The presence of on-going gluten-sensitive enteropathy in these patients may be indicated by the finding of celiac-related antibodies [17, 24, 25]. The diagnostic status of patients presenting in this manner was addressed in this study. Twenty-five patients with a positive endomysial antibody test but who were believed not to have celiac disease on the basis of normal duodenal histological findings were further evaluated. These patients had presented over a ten-year period. Detailed analysis of the original biopsies, repeat serology, and further clinical evaluation revealed that six of these patients had developed clear-cut evidence of celiac disease. A diagnosis of childhood celiac disease was discovered for a seventh patient and celiac disease was also believed very likely for at least two more patients (patients 16 and 17 in Table 1).

The original duodenal biopsy samples from 20 individuals were still available. Further studies were performed on these biopsies to see if there was evidence of gluten-sensitive pathology. Repeat examination of haematoxylin and eosin-stained tissue sections revealed that intraepithelial lymphocyte count was elevated in four subjects but no other morphological abnormalities were noted. In contrast, when immunohistochemical staining of the sections was performed a significantly greater number of intraepithelial T cells expressing CD2, CD3, CD7, CD8, and CD69 antigens was observed (Fig. 2). On the basis of Ki67 staining, a significant increase in enterocyte proliferation was also found in these patients. Thus, despite the initial finding that no histological lesion was evident in these patients, more detailed investigation did reveal the presence of abnormalities.

Follow-up clinical evaluation and investigation was performed on fourteen of the twenty-five patients, three to twelve years after their initial presentation. Duodenal biopsies showed that six of these patients had developed the classic small-intestinal enteropathy characteristic of celiac disease. In these patients the finding of positive celiac serological tests (anti-endomysial, anti-tissue transglutaminase, and anti-gliadin antibodies) with low serum ferritin levels was entirely consistent with this diagnosis [4, 8–10]. In a seventh patient, who failed to have a repeat biopsy, a history of childhood celiac disease was revealed. This patient continued to have positive celiac serology. Thus, of the fourteen patients available for further clinical review, seven had celiac disease. It is unlikely that any of the other seven reviewed patients had celiac disease—for all of these celiac serology was now negative and for the three patients who were biopsied duodenal histology was normal.

The current gold standard investigation for gluten-sensitive enteropathy is small-intestinal biopsy [14–16]. The

sensitivity of this investigation has rarely, if ever, been formally investigated, however [12], and when minor, subtle, or patchy histological lesions are present these may not be noted by the histopathologist [17–21]. Some such patients are, nonetheless, symptomatic and have gluten-sensitive malabsorption. Indeed, several of the patients in this study were likely to have established celiac disease when they initially presented. These patients had typical symptoms, positive celiac antibodies, and reduced serum ferritin. Because no histological lesion was identified, however, they were believed not to have celiac disease and discharged from the hospital.

An increase in intraepithelial lymphocytes, called a Marsh grade 1 lesion, is the most common mild histological lesion found in celiac disease [14]. In recent publications it has, moreover, been reported that increased numbers of intraepithelial lymphocytes may only be evident at the villous tip [20, 21]. This abnormality may be missed on routine histological examination of biopsies unless a formal count of these cells is performed. In this study, to enhance recognition of intraepithelial lymphocytes, tissue sections were stained with a range of monoclonal antibodies, including antibodies to CD2, CD3, CD7, CD8, and CD69. A significant increase in intraepithelial lymphocytes was noted in the study population with each of these antibodies. Furthermore, those patients ($n = 6$) who developed definite evidence of celiac disease had the highest intraepithelial lymphocyte counts (Figs. 2A–2E). Earlier publications have emphasised the greater number of intraepithelial T cells expressing the gamma delta T cell receptor, and this was considered a specific finding in celiac disease [26–28]. Currently available monoclonal antibodies to the gamma delta T cell receptor do not react satisfactorily on formalin-fixed tissue, however, and frozen tissue is not routinely available for examination [27].

The findings reported in this study are in keeping with earlier reports of raised intraepithelial lymphocyte counts in celiac mucosa when biopsies were stained with anti-CD3 or anti-CD8 monoclonal antibodies [29–31]. There are few reports of immunohistochemical studies examining intraepithelial lymphocyte staining with antibodies to CD2, CD7, and CD69. In one study numbers of CD3-negative/CD7-positive cells were found to be either unchanged or reduced in celiac disease [32] and a marked reduction in this subset of cells has also been reported in a study using flow cytometry [33]. The results of the current study suggest that investigation of small intestinal tissue with any of these monoclonal antibody reagents may be of value in identifying an increase in the intraepithelial lymphocyte population in gluten-sensitive mucosa.

Enterocyte proliferation is a reported finding in celiac disease [34–36] and assessment of this characteristic might be useful in the detection of evidence of a minimal inflammatory lesion in this disorder. In this study the Ki67 antibody, which detects a nuclear antigen associated with cell proliferation, was used for this purpose [37]. In the study group subjects, expression of Ki67 was increased, and this was also found in patients with untreated and treated celiac disease. These findings concur with a recent study which reported a higher Ki67 index for individuals with positive celiac antibodies and normal small-intestinal mucosa [19].

In the early years of the study (1991–1997) the anti-tissue transglutaminase test had yet to be developed. Patients who presented in those years had anti-endomysial antibody investigations only performed. Stored samples were available for twelve patients, however, enabling repeat serology to be performed, including investigation of levels of anti-tissue transglutaminase antibodies. In seven of these patients repeat serology testing confirmed the presence of anti-endomysial antibodies and higher levels of anti-tissue transglutaminase antibodies. This group included five patients for whom later biopsies showed the presence of enteropathy. The two additional sero-positive patients (Table 1, numbers 16 and 17) had low serum ferritin levels and symptoms indicative of celiac disease. In contrast, the stored serum samples from the remaining five patients were negative for both anti-tissue transglutaminase and endomysial antibodies, and results remained negative when these patients returned for review in the follow-up study. This would suggest the original serological findings for these latter patients were false-positive results. Such a finding is, perhaps, not surprising when it is considered that in excess of 50,000 samples were tested over this decade, so the approximate false-positive rate of 0.014%. It is probable this false-positive rate is even lower now, because positive samples are first detected by use of the anti-tissue transglutaminase assay and then re-examined with the anti-endomysial antibody test.

In conclusion, the findings of this study emphasise the value of serological tests in the diagnosis of celiac disease. Absolute reliance on finding a mucosal lesion when standard histological examination of biopsy tissue is performed is likely to under-diagnose gluten-sensitive enteropathy. If more subtle abnormalities are present, including increased numbers of intraepithelial lymphocytes and increased enterocyte proliferation, these can be detected by immunohistological staining of tissue sections. When diagnosis of celiac disease is being considered in a patient with positive celiac antibodies but apparently normal small-intestinal mucosa it is important that continued clinical follow-up be performed.

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