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ABSTRACT

Objective An aberrant immunophenotype and monoclonality of intraepithelial lymphocytes (IELs) are frequently found in refractory coeliac disease (RCD). However, the utility of continual monitoring of IEL immunophenotype and clonality in the surveillance of RCD remains to be studied.

Design The diagnostic and follow-up biopsies from 33 patients with CD, 7 with suspected RCD, 41 with RCD and 20 with enteropathy-associated T cell lymphoma (EATL) (including 11 evolved from RCD) were investigated by CD3ε/CD8 double immunohistochemistry and PCR-based clonality analysis of the rearranged T cell receptor (TCR) genes.

Results An aberrant immunophenotype ($CD3\varepsilon^+CD8^-$ IELs ≥40%) and monoclonality were detected occasionally in CD biopsies, either transiently in patients with CD not compliant with a gluten-free diet or in those who subsequently developed suspected RCD, RCD or EATL. In contrast, the aberrant immunophenotype and monoclonality were found in 30 of 41 (73%) and 24 of 37 (65%) biopsies, respectively, at the time of RCD diagnosis. Among the patients with RCD who did not show these abnormalities in their diagnostic biopsies, 8 of 10 (80%) and 5 of 11 (45%) cases gained an aberrant immunophenotype and monoclonality, respectively, during follow-up. Irrespective of whether detected in diagnostic or follow-up biopsies, persistence of both abnormalities was characteristic of RCD. Importantly, the presence of concurrent persistent monoclonality and aberrant immunophenotype, especially ≥80% $CD3\varepsilon^+CD8^-$ IELs, was a strong predictor of EATL development in patients with RCD (p=0.001).

Conclusions Continual monitoring of both immunophenotype and clonality of IELs is more important than snapshot analysis for RCD diagnosis and follow-up, and could provide a useful tool for surveillance of patients at risk of EATL.

Significance of this study

What is already known about this subject?

- An aberrant immunophenotype (cytoplasmic CD3ε positive but surface CD3ε, CD8 and TCR antigens negative) and monoclonality of IELs are frequently found in RCD
- RCD patients with a clonal population of aberrant IELs (RCD II) have a more severe clinical presentation and worse outcome than those without such abnormalities (RCD I) due to EATL development
- The aberrant immunophenotype of IELs can be demonstrated by CD3ε/CD8 double immunohistochemistry for loss of CD8 expression ($CD3\varepsilon^+CD8^-$) on formalin-fixed specimens, and monoclonality can be detected by PCR for TCR gene rearrangements.

What are the new findings?

- The presence of an aberrant immunophenotype and monoclonality of IELs is not specific to RCD as they are also seen during the CD stage, although often being transient and associated with GFD non-compliance
- An aberrant immunophenotype and monoclonality in RCD are nearly always persistent, and both abnormalities are often concurrent
- The presence of persistent concurrent aberrant IEL immunophenotype, especially ≥80% $CD3\varepsilon^+CD8^-$ IELs, and monoclonality is associated with patients who subsequently developed EATL
- A high proportion of RCD cases showing normal IELs at diagnosis gained the aberrant immunophenotype and monoclonality during follow-up, thus representing a transition from RCD I to RCD II.

How might it impact on clinical practice in the foreseeable future?

- Continual monitoring of both IEL immunophenotype and clonality is more important than snapshot analysis in diagnosis and surveillance of RCD.

INTRODUCTION

Coeliac disease (CD) is a gluten-sensitive enteropathy characterised histologically by villous atrophy, crypt hyperplasia and increased intraepithelial lymphocytes (IELs) in the small intestine.[1 2] It is a common disorder affecting ~1% of Northern European populations and is usually effectively controlled by a gluten-free diet (GFD).[2] However, as many as 5% of adult patients develop refractory coeliac disease (RCD), defined by a lack of clinical and histological response to a strict GFD.[1–4]

The prognosis of RCD is usually poor, but variable among cases. Apart from the uncontrolled malabsorption, up to 50% of patients with RCD

develop an enteropathy-associated T cell lymphoma (EATL)[5–8] which carries a 2-year survival of <30%.[8–10] Clearly, early diagnosis and risk stratification of RCD are clinically important, but these cannot be reliably achieved on clinical and histological grounds.[2 4] Recent studies have shown that RCD may be subdivided into type I (RCD I), in which IELs are polyclonal and have a normal phenotype, or type II (RCD II), in which there is a clonal expansion of an aberrant IEL population, expressing cytoplasmic CD3ε but not surface CD3ε, CD8 and T cell receptor (TCR) antigens.[2 5 6 11–13] This aberrant monoclonal IEL population is found in small intestinal biopsies prior to the development of EATL and in the intervening enteropathic mucosa uninvolved by the tumour, and more importantly shares the same aberrant immunophenotype and TCR gene rearrangement as the tumour clone.[5 8 11 12 14] These findings suggest that such IELs constitute a neoplastic population and RCD II is an early manifestation of EATL, also known as cryptic T cell lymphoma.[1 15–17] There is now mounting evidence indicating that patients with RCD II have a more severe clinical presentation and a poorer prognosis than those without such abnormalities.[7 8] Therefore, identification of an immunophenotypically aberrant clonal IEL population is very important for prognosis and management of RCD.[18 19]

CD3ε/CD8 double immunohistochemistry and PCR-based clonality analysis of TCR gene rearrangements are commonly used to determine the phenotype and clonality of IELs, respectively, on routine formalin-fixed paraffin-embedded intestinal biopsies. However, both 'false negativity' and 'false positivity' are frequently encountered by both methodologies, making it difficult to distinguish two types of RCD accurately. Although the percentage of $CD3\varepsilon^{+}CD8^{-}$ IELs in RCD is significantly higher than that in uncomplicated CD, there is a considerable overlap between the two conditions. In addition, there is no consensus on the cut-off value to be used for diagnosis of an aberrant immunophenotype.[5 12 13 20] Similarly, T cell clonality analysis also has its limitations due to the oligoclonal nature of the T cell repertoire in the normal gut mucosa[21] and use of non-standardised PCR primers and protocols in previous studies before the BIOMED-2 era.[22] This may potentially account for the variable incidences of monoclonality and also the variable correlations between monoclonality and the aberrant immunophenotype in both CD and RCD groups from previous studies.[6 7 11 20 23] Furthermore, most of the previous studies were a snapshot analysis of biopsies taken at a single time point or within a short period, and the natural course of IEL immunophenotypic and clonality changes during the disease progression from CD to RCD and EATL is poorly understood. The true value of aberrant IEL immunophenotype and monoclonality in diagnosis, follow-up and risk stratification of patients with RCD has not yet been fully explored. We therefore conducted a detailed parallel analysis of immunophenotype and clonality of IELs in multiple sequential intestinal biopsies of a large series of patients with CD with and without complications.

Materials and methods

Patients

Ninety patients with CD with or without complications were reviewed and followed up from 2004 to 2008 in the authors' institutions. These included 33 patients with uncomplicated CD, 7 patients with suspected RCD, 41 patients with RCD including 11 who developed EATL, and further 9 patients with EATL in whom a history of CD was documented but only tumour specimens were available for study (table 1). The selection of patients was biased towards those with RCD and those with RCD that evolved to EATL.

The diagnosis of CD, RCD and EATL was based on internationally accepted criteria as summarised in table 2.[1 4 24–26] Suspected RCD was analysed as a separate group as not all criteria for RCD were fulfilled in these patients—that is, patients showed persistent villous atrophy despite adhering to a strict GFD and showing negative serology and no obvious clinical symptoms for >2 years (table 2).

Specimens

A total of 220 duodenal or intestinal biopsies taken at diagnosis and during follow-up of the disease were studied. These included 104 (47%) specimens retrieved retrospectively and 116 (53%) specimens collected prospectively during the study period. Formalin-fixed paraffin-embedded specimens were available in each case. In addition, fresh/frozen materials were available in 33 biopsies from 19 patients. The use of these pathological materials for research was approved by the local research ethics committee.

Immunohistochemistry

Double immunohistochemistry for CD3ε and CD8 was performed essentially as described previously.[12] Briefly, deparaffinised tissue sections were pressure-cooked for antigen retrieval in citrate buffer, pH 6.0, for 2 min, and then sequentially stained for CD8 and CD3ε. CD8 was detected with a mouse

Table 1 Characteristics of patients and specimens

Patient groups	No. of patients (M:F)	Median age (range, years) at diagnosis of			No. of patients followed up (M:F)	Median months of follow-up (range)	Median no. of sequential biopsies (range)	Median interval of sequential biopsies (range, months')
		CD	RCD	EATL				
CD	33 (11:22)	46 (16–77)			24 (6:18)	29 (3–360)	2 (2–4)	10 (3–57)
Suspected RCD	7 (1:6)	52 (47–69)			7 (1:6)	53 (28–141)	5 (2–7)	11 (3–77)
RCD	41 (19:22)	52 (27–79)	63 (38–80)		31 (12:19)	37 (10–194)	3 (2–8)	12 (1–84)
RCD only	30 (14:16)	52 (27–75)	63 (38–78)		20 (6:14)	40 (14–194)	3 (2–8)	12 (4–84)
RCD evolved to EATL	11 (6:5)	54 (27–79)	56 (38–80)	59 (40–81)	11 (6:5)	32 (10–101)	4 (2–7)	11 (1–84)
EATL	9 (7:2)			61 (38–80)				
Subtotal	90 (38:52) *	50 (16–79)	63 (38–80)†	61 (38–81)†	62 (19:43)	40 (3–360)	3 (2–8)‡	12 (1–84)

Patients with CD: 14 studied retrospectively, 6 prospectively and 13 both retrospectively and prospectively. Eleven were poorly compliant with a GFD. Patients with suspected RCD: 2 prospectively and 5 both retrospectively and prospectively. Patients with RCD: 15 retrospectively, 14 prospectively and 12 both retrospectively and prospectively.
*$p=0.045$ for gender difference (CD vs EATL, including EATL evolved from RCD).
†$p<0.01$ for age difference (CD vs RCD or EATL).
‡$p<0.01$ for difference in number of biopsies (CD vs suspected RCD or RCD).
CD, coeliac disease; EATL, enteropathy-associated T cell lymphoma; F, female; GFD, gluten-free diet; M, male; RCD, refractory coeliac disease.

Table 2 Diagnostic criteria of CD, suspected RCD, RCD and EATL

Patient groups	Diagnostic criteria
CD	Patients presented with clinical symptoms of malabsorption, circulating antigliadin, antitissue transglutaminase and/or antiendomysium antibodies, histological evidence of villous atrophy, crypt hyperplasia and increased number of IELs in duodenal biopsy, and prompt improvement of these clinicopathological presentations following a strict GFD.[1]
RCD	Patients showed persistent symptoms and villous atrophy or deterioration on biopsies despite being on a GFD for ≥12 months, or symptoms recurred after a former period of response on a strict GFD. The compliance with the diet was checked by a dietician and confirmed by negative serology. Other causes of villous atrophy were excluded.[4 24 25]
Suspected RCD	Not all criteria for RCD were fulfilled. Notably, although adhering strictly to a GFD, as confirmed by a dietician, and having negative serology and no obvious clinical symptoms, patients continued to show histological evidence of villous atrophy for >2 years.
EATL	'WHO classification of tumours of hematopoietic and lymphoid tissues'.[26]

CD, coeliac disease; EATL, enteropathy-associated T cell lymphoma; GFD, gluten-free diet; IELs intraepithelial lymphocytes; RCD, refractory coeliac disease.

monoclonal antihuman CD8 antibody (Dako UK, Ely, UK) using an avidin–biotin peroxidase/DAB kit (Sigma-Aldrich Company, Poole, UK), whilst CD3ε was identified with a rabbit polyclonal antihuman CD3 antibody (Dako) using the alkaline phosphatase/fast blue reaction reagents (Sigma). The percentage of $CD3\varepsilon^+CD8^-$ IELs was obtained by counting the number of $CD3\varepsilon^+CD8^-$ cells in at least 100 labelled IELs. Two histopathologists performed the counting, and a high degree of agreement between them was achieved (κ=0.772).

Clonality analysis

DNA was prepared from paraffin-embedded specimens and frozen tissues as described previously.[27] Clonality analysis was performed using the standard BIOMED-2 multiplex PCR primer mixes (InVivoScribe Technologies, San Diego, California, USA) and heteroduplex analysis of PCR products[22] with modifications as described previously.[27] Each sample was analysed in duplicate for both TCRG and TCRB gene rearrangements. In each case where clonal TCR gene rearrangements were detected in multiple sequential biopsies, the PCR products from different specimens were analysed on the same gel to determine their identity.

Statistical analysis

Non-parametric tests including the Kruskall–Wallis test were used for quantitative variables. Fisher exact test, the χ^2 test, McNemar test, mixed model analysis and logistic regression were applied for association analysis and comparison of categorical variables. The receiver operating characteristic curves (ROCs) plot analysis was used to assess the cut-off value of the percentage of $CD3\varepsilon^+CD8^-$ IELs. Two-sided p values <0.05 were considered to be statistically significant. Statistical packages SPSS and MedCalc were used for statistical analyses.

Results

Characteristics of patients and specimens

Patients were female predominant in the CD and suspected RCD groups, nearly equal in both sexes in the RCD group, but male predominant in the EATL group (p=0.045, table 1). The median age was younger in patients with CD than those with RCD and EATL at the time of their diagnosis (all p<0.01). The length of follow-up and interval of biopsies taken were not statistically different among different patient groups. The median number of follow-up biopsies was higher in patients with RCD and suspected RCD than in those with uncomplicated CD (all p<0.01).

Establishment of the cut-off value of $CD3\varepsilon^+CD8^-$ IELs to define aberrant immunophenotype

To avoid any bias, only the initial diagnostic biopsies of well-characterised uncomplicated CD, RCD and EATL were used for establishment of the cut-off value for diagnosis of aberrant IEL immunophenotype. As shown in figure 1, the median percentage of $CD3\varepsilon^+CD8^-$ T cells was significantly higher in both the RCD and EATL groups than in the CD group (p<0.01). The ROC plot analysis showed that 40% was the optimal value to separate both the RCD and EATL groups from the CD group (p<0.01), with a sensitivity of 70% for RCD and 86% for EATL, and a specificity of 100% for both groups. This percentage (≥40%) was thus used as the cut-off value to diagnose aberrant immunophenotype in the present study.

Aberrant immunophenotype and monoclonality in diagnostic biopsies of CD, RCD and EATL

Using the above cut-off value, an aberrant immunophenotype was found in 1 of 30 (3%) diagnostic CD biopsies but in 73% of diagnostic RCD biopsies and 89% of EATL specimens (p<0.01, table 3). The only CD diagnostic biopsy with an aberrant immunophenotype was from a patient who subsequently developed RCD and then EATL (Case 26 in figure 2 and left panel in figure 3). Among the diagnostic RCD biopsies, the aberrant immunophenotype was more frequent in patients with RCD with subsequent development of EATL (90%) than in those without evidence of EATL (67%), but the difference was not statistically significant (p=0.23).

Similarly, monoclonality was far less frequent in diagnostic CD biopsies than in diagnostic RCD biopsies and EATL specimens (p<0.01, table 3). Where detected in the diagnostic CD biopsies, it was mostly seen in patients with subsequent development of suspected RCD, RCD or EATL. Among the diagnostic RCD biopsies, monoclonality was more frequent in patients who subsequently developed EATL (88%) than in cases without EATL

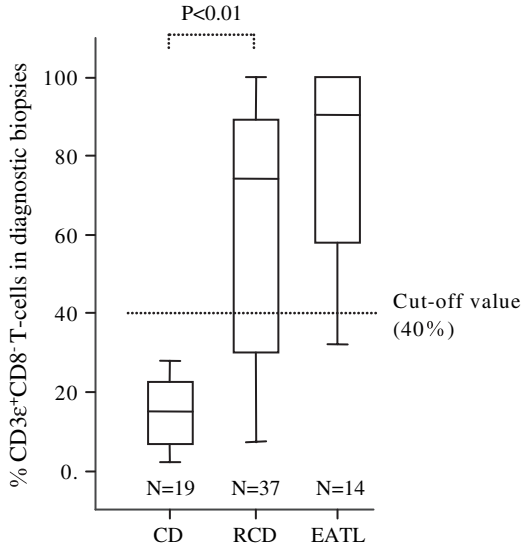

Figure 1 Percentage of $CD3\varepsilon^+CD8^-$ T cells in diagnostic biopsies of coeliac disease (CD), refractory coeliac disease (RCD) and enteropathy-associated T cell lymphoma (EATL). The median percentage of $CD3\varepsilon^+CD8^-$ intraepithelial lymphocytes (IELs) in diagnostic CD biopsies is significantly lower than that in diagnostic RCD biopsies and $CD3\varepsilon^+CD8^-$ T cells in EATL specimens (p<0.01). Using receiver operating characteristic (ROC) plot analysis, a cut-off value of 40% was found to be optimal to define aberrant IEL immunophenotype ($CD3\varepsilon^+CD8^-$ IELs ≥40%) (p<0.01).

Table 3 Immunophenotype and clonality of IELs in diagnostic biopsies of CD, RCD and EATL

	Aberrant immunophenotype	Monoclonality	Concurrent aberrant immunophenotype and monoclonality
Diagnostic CD biopsies (n=38 patients)	1/30 (3%)	6/37 (16%)	1/29 (3%)
In patients with uncomplicated CD (n=24)	0/19 (0%)	2*/24 (8%)	0/19 (0%)
In patients with subsequent suspected RCD (n=4)	0/2 (0%)	1/4 (25%)	0/2 (0%)
In patients with subsequent RCD (n=8)	0/7 (0%)	2/7 (29%)	0/6 (0%)
In patients with subsequent RCD evolved to EATL (n=2)	1/2 (50%)	1/2 (50%)	1/2 (50%)
Diagnostic RCD biopsies (n=41 patients)	30/41 (73%)†	24/37 (65%)†	21/37 (57%)†
In patients with RCD (n=30)	20/30 (67%)	17/29 (59%)	14/29 (48%)
In patients with RCD evolved to EATL (n=11)	10/11 (90%)	7/8 (88%)	7/8 (88%)
EATL specimens (n=18 patients)	16/18 (89%)†	17/17 (100%)†	15/17 (88%)†

*Both patients were not GFD compliant;
†All p<0.01, CD vs RCD or EATL.
CD, coeliac disease; EATL, enteropathy-associated T cell lymphoma; IELs intraepithelial lymphocytes; RCD, refractory coeliac disease.

(59%), although the difference was not statistically significant (p=0.21).

Immunophenotype and clonality were correlated in each group. In the group of diagnostic CD biopsies, the only biopsy showing an aberrant immunophenotype as described above was also clonal. In the group of diagnostic RCD biopsies, the aberrant immunophenotype and monoclonality were concurrent in the majority of the patients and the concurrent abnormalities were more frequent in patients with RCD who subsequently developed EATL (88%) than in those without evidence of EATL (48%), although the difference was not statistically significant (p=0.10). In the EATL group, the majority of patients showed concurrent abnormalities (88%).

Aberrant immunophenotype and monoclonality during follow-up of CD, suspected RCD and RCD

Sequential follow-up biopsies of CD, suspected RCD and RCD were analysed and the results were presented according to the phase of the disease progression.

CD phase

Thirty-six patients had follow-up biopsies during the CD phase (table 4 and figure 2). Among the 24 patients with uncomplicated CD, the aberrant immunophenotype was found in four patients, of whom three were GFD non-compliant. Interestingly, the aberrant immunophenotype was only seen in one intermediate biopsy, thus was transient, and the percentage of $CD3\varepsilon^{+}CD8^{-}$ IELs in these biopsies was only marginally higher than the 40% cut-off value (all <50%). Similarly, monoclonality was detected in three patients who were also GFD non-compliant, and the abnormality was transient in two patients but present in two consecutive biopsies, with an 8 month interval in the third patient. None of the patients with uncomplicated CD showed concurrent aberrant immunophenotype and monoclonality that persisted in two or more consecutive biopsies.

In contrast, among the 12 patients with CD who subsequently developed RCD or EATL, four showed monoclonality during the CD phase. In each case the monoclonality was persistent and identical in all follow-up biopsies. Of these cases, only one case (Case 26 in figure 2 and left panel in figure 3) showed concurrent aberrant immunophenotype and monoclonality, and both the abnormalities persisted in all CD, RCD and EATL specimens.

Suspected RCD phase

All seven patients with suspected RCD had follow-up biopsies (table 4). By the last follow-up, six patients showed an aberrant IEL immunophenotype and in five of them this persisted in two or more biopsies including the last biopsy. Monoclonality was seen in two patients and was persistent in both, with one of them also showing persistent aberrant IEL immunophenotype.

RCD phase

Twenty-nine patients with RCD, including 11 who subsequently developed EATL, had follow-up biopsies available for study (table 4, figure 2). Among them, 19 showed an aberrant IEL immunophenotype in the diagnostic RCD biopsy and this persisted in all follow-up biopsies in 18. The other 10 patients showed a normal IEL immunophenotype in the diagnostic RCD biopsy and eight of them gained the aberrant immunophenotype during follow-up, seen in two or more consecutive biopsies including the last follow-up biopsy in four, and in only one follow-up biopsy (often the last) in the remaining four patients.

Similarly, of the 15 patients showing monoclonality in the diagnostic RCD biopsy, each displayed persistent identical monoclonality in all the subsequent biopsies. Of the 14 cases with polyclonality (n=11) or without clonality data (n=3) in the diagnostic RCD biopsy, eight showed monoclonality during follow-up, seen in two or more consecutive biopsies including the last follow-up biopsy in five cases, and in the last biopsy in the remaining three cases.

When immunophenotype and clonality data were correlated, 11 of the 12 patients with concurrent aberrant immunophenotype and monoclonality in the diagnostic RCD biopsy had the abnormalities persisting in all subsequent biopsies. Of the 17 cases showing no or either abnormality (n=14) or lacking concurrent data (n=3) in the diagnostic RCD biopsy, 11 gained concurrent abnormalities during follow-up, seen in two or more consecutive biopsies in four cases, and in only one follow-up biopsy (often the last) in the remaining seven cases.

By the end of follow-up, the rates of the aberrant immunophenotype, monoclonality and concurrent abnormalities detected in the last follow-up biopsies were significantly higher

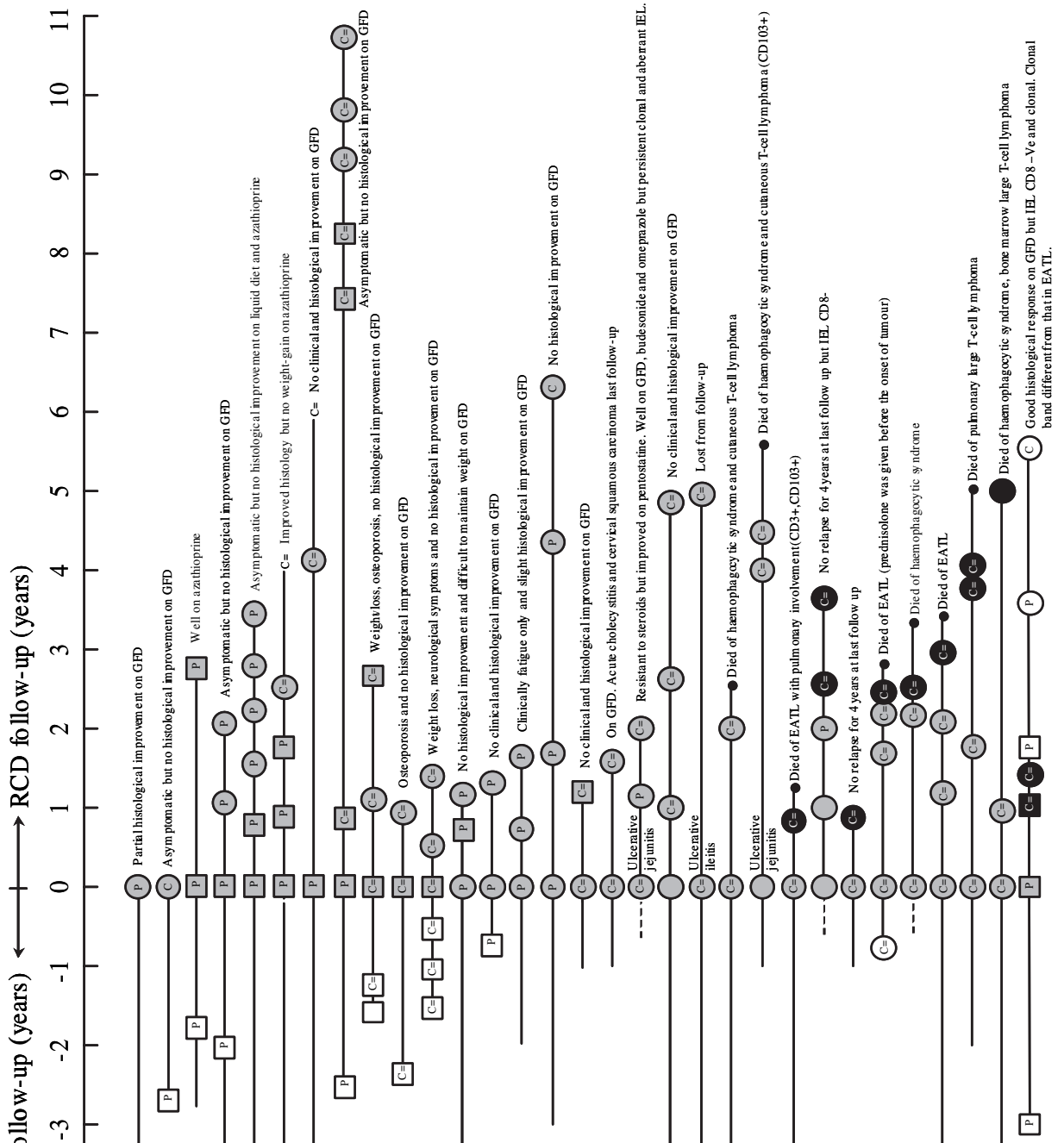

Figure 2 Follow-up of immunophenotype and clonality of intreaepithelial lymphocytes (IELs)/T cells in patients with refractory coeliac disease (RCD). Squares, biopsies with normal immunophenotype; circles, biopsies with aberrant immunophenotype; P, biopsies with polyclonal T cell receptor (TCR) gene rearrangements; C, biopsies with clonal TCR gene rearrangements; C=, clonal pattern identical. Open squares and circles, biopsies from CD phase; grey squares and circles, biopsies from RCD phases; black squares and circles: enteropathy-associated T cell lymphoma (EATL) specimens; small black dots, patients died but specimens not available for study.

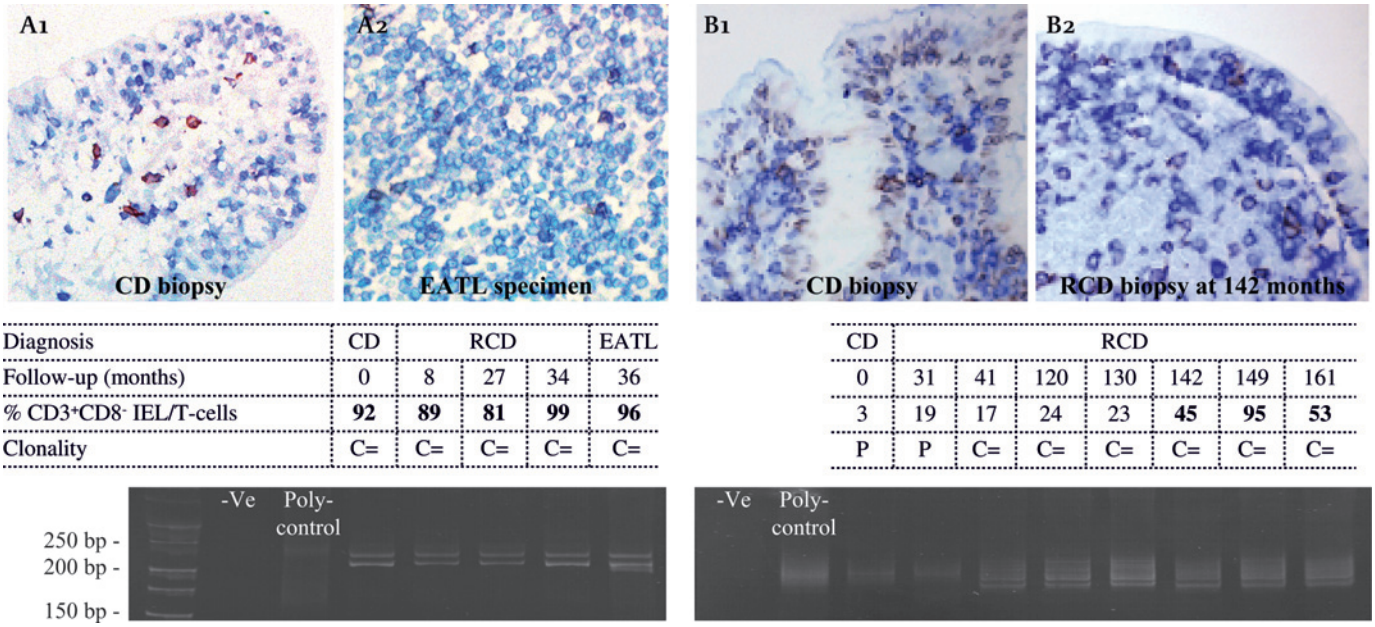

Figure 3 Left panel: a patient with refractory coeliac disease (RCD) (Case 26 in figure 2) with subsequent development of enteropathy-associated T cell lymphoma (EATL). The coeliac disease (CD) biopsy showed a marked increase of intraepithelial lymphocytes (IELs) (A1). The IELs were CD3ε$^+$ (blue) and rarely CD8$^+$ (brown) and were clonal for T cell receptor (TCR) G gene rearrangement. The aberrant immunophenotype and identical clonality were maintained in all three follow-up RCD biopsies and in the EATL specimen (A2). Right panel: a patient with RCD (Case 8 in figure 2) showing progression of IELs from normal to aberrant immunophenotype and from polyclonality to monoclonality. The diagnostic CD biopsy showed a marked increase of IELs (B1) that were predominantly CD3ε$^+$ and CD8$^+$ and were polyclonal for TCRG gene rearrangement (P). During follow-up, monoclonality was gained 41 months later albeit accompanied by a polyclonal background, and was persistent in all subsequent biopsies. Meanwhile, the percentage of CD3ε$^+$CD8$^-$ IELs increased progressively from the 130th month, surpassing the 40% cut-off value for aberrant immunophenotype at the 142nd month, and reached 95% at the 149th month (B2). C=, clonal pattern identical among consecutive biopsies; bold figures, percentage of CD3ε$^+$CD8$^-$ IELs >40% (immunophenotype aberrant).

than those detected in the diagnostic biopsies (61% vs 87%, n=31; 54% vs 75%, n=26; 46% vs 73%, n=26, respectively. All p<0.05).

Rate of increase in CD3ε$^+$CD8$^-$ IELs during RCD follow-up

Eleven patients with RCD and three with suspected RCD with normal IEL immunophenotype at their diagnosis showed a progressive increase in CD3ε$^+$CD8$^-$ IELs during a median follow-up of 27 (4–58) months (with a median 3 (2–4) biopsies), reaching or surpassing the 40% cut-off value for an aberrant immunophenotype by the last biopsy (right panel in figure 3). The median percentage of CD3ε$^+$CD8$^-$ IELs in these cases was 18% (range 4–38%) at the initial and 58% (range 35–95%) at the last follow-up biopsy. The median rate of the increase was 1.8% (mean 1.9%, range 0.5–4.5%) per month, equivalent to an increase of 11% for a 6-month period (p<0.01). Of these 14 cases, the gain of the aberrant IEL immunophenotype was

Table 4 Immunophenotype and clonality of IELs in follow-up biopsies during CD and RCD phases

	Median months of follow-up	Persistent* aberrant immunophenotype	Persistent* monoclonality	Persistent* concurrent aberrant immunophenotype and monoclonality
Follow-up biopsies during CD phase (n=36 patients)	22 (3–360)	1/35 (3%)	5/36 (14%)	1/36 (3%)
In patients with uncomplicated CD (n=24†)	29 (3–360)	0/24	1‡/24 (4%)	0/24
In patients with subsequent RCD or EATL (n=12)	24 (7–144)	1/11 (9%)	4/12 (33%)	1/11 (9%)
Follow-up biopsies during suspected RCD phase (n=7 patients)	53 (28–141)	5/7 (71%)§	2/7 (30%)	1/7 (14%)
Follow-up biopsies during RCD phase (29 patients)	25 (10–130)	22/29 (76%)§	20/29 (69%)§	15/29 (52%)§
In patients without EATL (n=18)	25 (11–130)	12/18 (67%)	11/18 (61%)	6/18 (33%)
In patients with subsequent EATL (n=11)	26 (10–44)	10/11 (91%)	9/11 (82%)	9/11 (82%)¶

*Aberrant immunophenotype and/or monoclonality maintained in two or more consecutive biopsies. The two patients with aberrant immunophenotype and monoclonality shown in the last RCD biopsies and persisted in EATL specimens were included.
†Including 11 GFD non-compliant as indicated by persistent or intermittent positive serology and confirmed by dietary assessment.
‡A GFD-non-compliant patient with identical clonal TCR rearrangement detected in diagnostic biopsy and follow-up biopsy taken 8 months later.
§All p<0.01, CD vs suspected RCD or RCD.
¶p=0.02, patients with RCD without EATL vs patients RCD with subsequent EATL.
CD, coeliac disease; EATL, enteropathy-associated T cell lymphoma; GFD, gluten-free diet; IELs intraepithelial lymphocytes; RCD, refractory coeliac disease; TCR, T cell receptor.

accompanied by persistent monoclonality in three patients and progression from polyclonality to monoclonality in a further six cases.

Predictive factors of EATL development in patients with RCD

Eleven of 29 patients with RCD developed EATL and there was no difference in the age, sex and follow-up period between the patients with RCD with and without subsequent EATL development (table 5). Neither persistent aberrant immunophenotype nor persistent monoclonality (seen in two or more consecutive biopsies) alone was associated with the development of EATL. The presence of concurrent persistent aberrant immunophenotype and persistent monoclonality was a predictive risk factor for EATL development (p=0.02). However, a high proportion (33%) of patients with RCD also showed the concurrent persistent abnormalities and this restricted its practical use as a reliable predictor of EATL development. In view of this, we investigated further whether the quantitative change of aberrant IEL immunophenotype was valuable in prediction of EATL development.

Using a mixed model analysis, the percentage of $CD3\varepsilon^+CD8^-$ IELs in all RCD biopsies was compared between the patients with and without EATL development and was shown to be significantly higher in patients with EATL development ($p<0.01$). ROC plot analysis showed that 80% of $CD3\varepsilon^+CD8^-$ IELs was the optimal cut-off value to separate RCD biopsies in patients with EATL development from those without (sensitivity 73%, specificity 94%, $p<0.01$, figure 4). When this quantitative threshold of IEL immunophenotype was added as a variable to the logistic regression, the presence of persistent ≥80% $CD3\varepsilon^+CD8^-$ IELs in RCD biopsies was found to be a stronger predictor of EATL development (p=0.002, table 5). Furthermore, the presence of concurrent persistent ≥80% $CD3\varepsilon^+CD8^-$ IELs and monoclonality was the strongest and also the only independent predictor of EATL development (p=0.001, OR 45 with 95% CI of 4 to 506).

DISCUSSION

In the present study, we undertook a detailed parallel analysis of clonality and immunophenotype of IELs on both diagnostic and follow-up biopsies in a large series of patients with CD with and without complications. The clonality was analysed by a standardised BIOMED-2 PCR protocol for both TCRG and TCRB gene rearrangements.[22 27 28] The immunophenotype was investigated by a well-established CD3ε/CD8 double immunohistochemistry,[12 29] and was defined as aberrant when $CD3\varepsilon^+CD8^-$ counts of IELs were ≥40%. An alternative flow cytometry-based method has determined aberrant IEL phenotype with ≥20% of $CD7^+$, surface $CD3\varepsilon^-$ and cytoplasmic $CD3\varepsilon^+$ IELs[7 30]; however, this method requires fresh tissue biopsies and thus cannot be applied to cases where only paraffin-embedded tissues are available.

Using these methods, we showed several novel findings. First, the presence of aberrant immunophenotype and monoclonality of IELs is not specific to RCD as they are also seen during the CD stage, although often being transient and associated with GFD non-compliance. Secondly, the aberrant immunophenotype and monoclonality in RCD are nearly always persistent, and both abnormalities are often concurrent. Thirdly, the presence of persistent concurrent aberrant IEL immunophenotype, especially ≥80% $CD3\varepsilon^+CD8^-$ IELs, and monoclonality in RCD biopsies is associated with patients who subsequently developed EATL. Fourthly, the IEL alteration is a progressive and accumulative process and a high proportion of cases of RCD showing normal IEL immunophenotype and polyclonality at the time of diagnosis gained the aberrant immunophenotype and monoclonality during follow-up.

As generally acknowledged, both aberrant immunophenotype and monoclonality of IELs are not a feature of CD. Nonetheless, in the present study, these abnormalities were occasionally detected in both the diagnostic and follow-up CD biopsies. When detected in patients with uncomplicated CD, both aberrant immunophenotype and monoclonality were essentially associated with cases who were not compliant with a GFD. For example, the aberrant immunophenotype seen in three of four uncomplicated cases of CD was associated with GFD non-compliance. Similarly, monoclonality detected in all three uncomplicated patients with CD was associated with GFD non-compliance. Nevertheless, such abnormality was nearly always transient, being detected in only one of the earlier sequential biopsies, and the aberrant immunophenotype and monoclonality were rarely associated with each other.

In contrast, the aberrant immunophenotype and/or monoclonality seen in the CD phase of patients who subsequently developed suspected RCD, RCD or EATL were persistent in two or more sequential follow-up biopsies including the last biopsy, and these patients were GFD compliant. In one case, the initial CD biopsy showed concurrent aberrant IEL immunophenotype and monoclonality, and both abnormalities were persistent in subsequent RCD and EATL specimens. Retrospectively, this case may be regarded as de novo RCD.[1 26] Similarly, in each of the other three cases showing monoclonality in the CD biopsies, an identical clonal pattern was seen in the biopsies following the development of suspected RCD or RCD, and was also accompanied by a gain of the aberrant immunophenotype. These

Table 5 Predictive factors of development of EATL in patients with RCD

	RCD patients		Logistic regression	
Variables	Without EATL (n=18)	With EATL (n=11)	OR (95% CI)	p Value
Age at diagnosis, median (range)	62 (36–72)	54 (38–80)	0.97 (0.92 to 1.04)	0.44
Gender, male/female	5/13 (38%)	5/6 (83%)	2.17 (0.45 to 10.44)	0.34
Follow-up months, median (range)	25 (11–130)	26 (10–44)	1.00 (0.97 to 1.02)	0.87
Persistent monoclonality	11 (61%)	9 (82%)	3.60 (0.60 to 21.60)	0.16
Persistent aberrant immunophenotype	12 (67%)	10 (91%)	5.00 (0.51 to 48.75)	0.17
Persistent concurrent aberrant immunophenotype and monoclonality	6 (33%)	9 (82%)	9.00 (1.46 to 55.48)	0.02
Persistent ≥80% $CD3\varepsilon^+CD8^-$ IELs	2 (11%)	8 (73%)	21.33 (2.94 to 154.56)	0.002
Persistent concurrent monoclonality and ≥80% $CD3\varepsilon^+CD8^-$ IELs*	1 (6%)	8 (73%)	45.33 (4.05 to 506.86)	0.001

*The only independent variant by multivariant analysis (p=0.02).
CD, coeliac disease; EATL, enteropathy-associated T cell lymphoma; IELs intraepithelial lymphocytes; RCD, refractory coeliac disease.

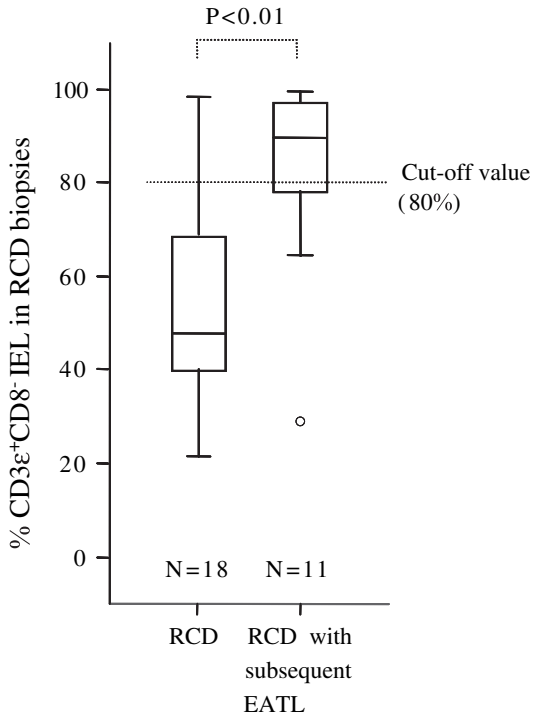

Figure 4 Percentage of CD3ε$^+$CD8$^-$ intraepithelial lymphocytes (IELs) in refractory coeliac disease (RCD) biopsies of patients with and without enteropathy-associated T cell lymphoma (EATL) development. The median percentage of CD3ε$^+$CD8$^-$ IELs in patients with subsequent EATL development is significantly higher than that in patients without EATL development ($p<0.01$ by mixed model analysis). Receiver operating characteristic (ROC) plot analysis showed that 80% was the optimal cut-off value to separate RCD biopsies in patients with subsequent EATL development from those without the lymphoma development ($p<0.01$).

findings strongly suggest that the aberrant IEL immunophenotype and monoclonality seen in patients with CD should be interpreted closely in conjunction with dietary assessment. Upon exclusion of GFD non-compliance, detection of these abnormalities, particularly both, in patients with CD should raise alarm of emergence of RCD or EATL.[1]

Unlike CD, RCD showed highly frequent aberrant immunophenotype and monoclonality of IELs at the time of RCD diagnosis, or gained these abnormalities during the RCD follow-up. Once acquired in RCD, both the abnormalities were nearly always persistent in the subsequent follow-up biopsies and were often concurrent. These findings, together with those in CD biopsies as discussed above, highlight the importance of continual monitoring of both IEL immunophenotype and clonality in differential diagnosis between CD and RCD.

Continual monitoring of IEL immunophenotype and clonality in RCD is also valuable in the prediction of EATL development. EATL is a devastating complication and is the major factor determining the prognosis of RCD. The presence of concurrent persistent aberrant immunophenotype and monoclonality was associated with patients with RCD who subsequently developed EATL. More significantly, the percentage of CD3ε$^+$CD8$^-$ IELs was consistently higher in RCD biopsies of patients with subsequent EATL development than in those without the lymphoma complication, and the presence of concurrent persistent ≥80% CD3ε$^+$CD8$^-$ IELs and monoclonality in RCD biopsies was the strongest and independent predictive factor for EATL development.

RCD can be classified as RCD I or RCD II based on the absence or presence of an immunophenotypically aberrant and/or clonal IEL.[1 2] In this study, we did not assign RCD to type I or type II at diagnosis and during follow-up as our study was designed to investigate the natural course of IEL changes during RCD development and progression, and to assess the practical utility of the current definition for RCD subtyping. We confirmed that patients with RCD with an immunophenotypically aberrant and clonal IEL, or RCD II, had a much worse outcome due to EATL development. Our results, therefore, support use of the above definition of RCD subtypes for patient prognosis and management. Our findings also suggest that patients in whom aberrant IELs are demonstrated may benefit from continual surveillance, as neither the aberrant immunophenotype nor the monoclonality of IELs is a definite diagnostic marker for RCD II except where these changes are persistent and concurrent.

Furthermore, patients with RCD without aberrant IELs—that is, RCD I—should also benefit from continual monitoring of IEL changes. The clinical follow-up data on patients with RCD I are well documented in several studies. Nonetheless, the data on IEL immunophenotype and clonality during RCD I follow-up are deficient. Using 40% CD3ε$^+$CD8$^-$ IELs as the threshold to define the aberrant IEL immunophenotype, we demonstrated for the first time that a high proportion of patients with RCD who initially showed no IEL abnormalities gained the aberrant immunophenotype (8/10 cases) during follow-up, thus transition from RCD I to RCD II. Although it could be argued that 40% may not be the best threshold to use, application of either a lower (30%) or higher (50%) threshold still demonstrated gain of the aberrant immunophenotype during RCD follow-up in 8/9 and 5/9 cases, respectively. In addition, the transition from RCD I to RCD II is strongly supported by T cell clonality analysis. Of the 11 patients with RCD who showed polyclonality at diagnosis, five gained monoclonality and all these cases also gained the aberrant immunophenotype during the follow-up. These findings, although contrary to two previous reports in which none of the patients with RCD I developed RCD II or EATL,[8 31] are consistent with recent studies in which a minority of patients with RCD I progressed to RCD II[19] or developed an overt lymphoma.[18 32] Our current observations also support the suggestion that patients with RCD I may represent an earlier stage of the disease than RCD II but the risk of developing an overt lymphoma exists.[4] The findings that a high proportion of RCD cases progressed from RCD I to RCD II in the present study are most probably attributed to the investigation of multiple consecutive biopsies over a long period of follow-up. In view of the poor survival of patients with RCD II, our observations underpinned the need to monitor the IEL changes in patients with RCD I.

The present longitudinal follow-up study allowed us to depict the natural course of IEL changes during RCD development and progression. Although the percentage of CD3ε$^+$CD8$^-$ IELs varied among different biopsies in each patient, a high proportion of cases showed a steady increase during follow-up, with a median 11% increase over a period of 6 months. In many cases, such progression, although not yet reaching the diagnostic cut-off value, was clearly seen in biopsies prior to RCD diagnosis. Thus, the trend of CD3ε$^+$CD8$^-$ IEL changes, in addition to its absolute level, is also potentially important for prediction of RCD development and progression.

In general, there was a good correlation between the results of immunohistochemistry and clonality analysis (tables 3 and 4, figure 2) and the two methods did not differ in sensitivity and specificity in detection of aberrant IELs (data not shown). However, in a small proportion of biopsies, only one of the abnormalities was detected. Such inconsistency has been described.[5 13 20 23] The lack of 100% sensitivity and specificity of these methodologies might be a cause for the inconsistency, but true non-concordance between IEL immunophenotype and clonality probably exists. Goerres *et al* reported that three of nine patients with RCD showed a monoclonal T cell population but no evidence of aberrant IEL phenotype, and two of seven patients with RCD had polyclonal T cells but aberrant IEL phenotype by flow cytometry.[7] Similarly, in a recent study by Verbeek *et al*, polyclonality was seen in 2 of 16 patients with RCD II with an aberrant IEL phenotype by flow cytometry.[30] The underlying reason for the non-concordance is unclear, although rare cases of clonal RCD showing an aberrant IEL immunophenotype other than loss of $CD8^+$ have been reported.[23 30] Nevertheless, the presence of non-concordance between clonality and immunophenotype also highlights the importance of combined application of both analyses in monitoring IEL changes in RCD diagnosis and follow-up.

There are a number of implications of these findings for clinical practice. First, we have shown that neither aberrant IEL immunophenotype nor monoclonality alone is diagnostic for RCD, except where these changes persist and are concurrent. Secondly, there was a group of patients, referred to as suspected RCD in this study, showing persistent villous atrophy despite good dietary compliance, negative serology and lack of apparent symptoms. The presence of concurrent aberrant IEL immunophenotype and monoclonality in these patients favours a diagnosis of RCD. Together, these findings challenge the requirement for ongoing malabsorption for diagnosis of RCD, which is also not consistent with our current knowledge that the extent of histological inflammation does not correlate with symptoms and many patients with total loss of intestinal villi are entirely asymptomatic.[6] Thus, in this context, the definition of RCD could be widened. Thirdly, RCD I is not static and may progress to RCD II, and careful clinical as well as laboratory follow-up are required. Finally, persistent aberrant immunophenotype and monoclonality are characteristic of RCD, and the presence of persistent concurrent abnormalities, especially when the $CD3\varepsilon^+CD8^-$ IELs were $\geq$80%, is a significant risk factor of EATL development. Although these findings will need further validation in a prospective cohort with a larger number of patients and longer follow-up, we believe that our results showed strong evidence that continual monitoring of both IEL immunophenotype and clonality is more important than snapshot analysis in diagnosis and surveillance of RCD.

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