CAT
Critically Appraised Topic

Laboratorium analyses op darmbiopten in kader van de diagnostiek voor "refractaire coeliakie type I of II"

Author: Dr Antoine Mairesse
Supervisor: Prof. Dr. Boeckx Nancy
Search/methodology verified by: Prof. Dr. Boeckx Nancy
Date: 07/06/2022

CLINICAL BOTTOM LINE

The refractory celiac disease (RCD) is a rare but potentially fatal complication of celiac disease (CD). Two types of RCD are recognized: RCD type I with a good prognosis and RCD type II, considered a premalignant condition with a poor prognosis. To establish this diagnosis, collaboration between different specialist is paramount. We reviewed the duodenal biopsies of 16 patients with a suspected diagnosis of RCD, referred to our laboratory for flow cytometry (FCM) analysis between 2015-2021. Our first step was to confirm CD diagnoses, we found that only 62% of patients had a clear CD diagnosis. To diagnose RCD, other causes of malabsorption should be excluded. We found that one third of the cohort as being suspected of poor compliance to the diet. Nevertheless, investigations to exclude RCD type II should be performed given its potentially severe consequences. Afterwards, we compared the results of pathology, molecular analysis and FCM analyses and discussed the discrepancies. We observed a 100% concordance in the 2 patients with type RCD type II with the 3 methods. According to our data, TCR rearrangement analysis and pathology could be used to exclude the diagnosis of RCD type II. We also discussed the perspectives in the diagnosis and treatment. To conclude, management and diagnosis of RCD patients is challenging and requires a multidisciplinary approach.

CLINICAL/DIAGNOSTIC SCENARIO

CD is defined as "a chronic immune-mediated enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals" (1,2). In addition to environmental factors, genetic susceptibility plays an important role in the pathogenesis of CD and is highly associated with HLA-DQ 2 and/or HLA-DQ8 haplotypes (1). The worldwide prevalence of CD is 1.4% based on serology and 0.7% using biopsies, although the prevalence of biopsy-confirmed CD in some populations is as high as 4.3%. The incidence of CD might to be underestimated due to a considerable number of patients who are never diagnosed or diagnosed late, due to its broad

clinical manifestations: a patient may be asymptomatic or presenting with osteoporosis, vitamin deficiencies, neuropathy or even severe malabsorption (3). The diagnosis of CD is based on serology and duodenal biopsies in patients who are on a normal gluten-containing diet (4). There are several antibodies of interest: IgA anti-tissue transglutaminase antibodies (TG2-IgA), endomysial antibodies (EMAs), and/or deamidated antigliadin antibodies (DGP). The TG2-IgA test will be positive in about 98% of patients with CD without gluten free diet (GFD) (4). An elevated TG2-IgA level (>10 times the upper limit of normal) is a reliable and accurate test for the diagnosis of active CD (5). IgA deficiency occurs in 2-3% of patients with CD (6) and affects the performance of the TG2-IgA assay. So, it is recommended to measure IgA and IgG-DGP in patients with suspected IgA deficiency (7). Current guidelines recommend duodenal biopsies to confirm the diagnosis: a bulb biopsy is mandatory in case of ultra-short CD (5,8). Histological examination of duodenal mucosa biopsies is the gold standard for CD diagnosis in adult, based on a villous atrophy (VA), an increase in intraepithelial lymphocytes (IELs) and crypt hyperplasia. Biopsies of the duodenal mucosa are taken during an endoscopic procedure, the practitioner must take several biopsies to avoid missing any VA. However, the new guidelines for CD from the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition propose to omit duodenal biopsies in the diagnosis of CD in some cases for children (9).

Initially, biopsies can be falsely negative and showing only an increase in IELs (in the presence of positive antibodies) (10). More, VA is not 100% specific for coeliac disorders, as it can be found in other pathologies such as infections (Giardiasis, HIV), autoimmune enteropathy, drugs-enduced enteropathy (11). As follow-up of patients undergoing gluten-free diet (GFD), duodenal biopsies should be performed 12 months after the start of the diet (11), recovery to a normal histology is the only way to confirm CD. Histopathological changes in the duodenal mucosa are classified according to the modified Marsh classification (**Figure 1**)(12).

RCD is a rare complication of CD, with an estimated prevalence of <1% of patients with CD (13,14). It is defined by the persistence (or recurrence) of gastrointestinal (GI) symptoms and VA despite adherence to a strict GFD for more than 12 months (2,6,15). Two subtypes of RCD are recognized, namely RCD type I and RCD type II, depending on the clonal proliferation of the IELs (16). Generally, RCD type I is less severe while RCD type II presents with more symptoms and can occur with ulcerative jejunitis. Diagnosis of RCD requires negative celiac serology and the confirmation of a GFD diet by a dietician (17). Studies have established a relationship between the presence of clonal IELs and the development of enteropathy associated T-cell lymphoma (EATL) (13,15,17). Patients with RCD type I have a low risk of developing EATL (3-14% within 5 years) (13,15,17), while patients with RCD type II have a high risk of developing EATL (40%-50% within 5 years (13,15,17)). Currently, FCM analysis is the gold standard for the analysis of the IELs compartment (18). Stratification of patients with RCD into

type I or II is based on the levels of cells lacking surface CD3 (sCD3) but with expression of intracellular CD3 (iCD3) IELs (18,19). These cells can be present in the normal intestine but represent <10% of total lymphocytes. The cut-off in RCD patients is set at 20%. In RCD type I, aberrant IELs are < 20% of total lymphocytes. While in RCD type II, they represent > 20% of lymphocytes (7,8).

To understand the perspectives, it is essential to consider the pathophysiology of CD and the transformation to RCD.

A link is demonstrated between the main environmental stimulus found in wheat, barley, or rye (gluten), and the main genetic risk factor: the major histocompatibility complex (MHC) class II molecules. Indeed, 90% of CD patients express HLA-DQ2.5 (DQA1*05-DQB1*02) and most of the remaining patients express HLA-DQ8 (DQA1*03-DQB1*03:02) or HLA-DQ2.2 (DQA1*02:01-DQB1*02) (22). When CD patients ingest gluten, proteins are incompletely digested in the lumen of the intestine and release peptides. These gluten peptides are modified by transglutaminase-2 (TG2) and presented by the HLA system of the dendritic cells. These cells activate CD4+ T cells with the modified gluten peptides. Once activated, gluten-specific CD4+ T cells secrete cytokines: interleukin-2 (IL-2), IL-21 and interferon gamma (IFNγ) leading to activation of cytotoxic CD8+TCRαβ+ IELs, in cooperation with IL-15 (produced by epithelial cells). The massive expansion of IELs leads to epithelial cell destruction and tissue damage characteristic of CD. The mechanism of activation and malignant transformation of IELs in RCD type II has recently been described (23). A somatic gain-of-function mutation in JAK1 or STAT3, which confer hyperresponsiveness to IL-15, IL-2 and IL-21 allow a clone of innate T-IELs to progressively supplant normal T-IELs and invade the epithelium. During their expansion into the gut epithelium, IELs in RCD type II can acquire additional mutations, which promote their transformation into aggressive EATL (24) **(Figure 2)**.

QUESTIONS

I. Determine which laboratory analytical techniques are used to diagnose RCD.

II. Determine which analytical laboratory techniques (FCM analysis, molecular analysis, pathology) are used to make the diagnosis of RCD type I versus RCD type II.

III. Determine the contribution of each analytical laboratory technique (FCM analysis, molecular analysis, pathology) to the final diagnosis of RCD type I/II (concordances/discordances)?

IV. Discuss the clinical course of patients diagnosed with RCD type I and RCD type II.

V. Determine which analytical laboratory technique(s) (FCM analysis, molecular analysis, pathology) best predicts the clinical course of patients with RCD type I and type II.

Question I: Determine which laboratory analytical techniques are used to diagnose RCD.

A considerable proportion of CD patients still have persistent symptoms or laboratory abnormalities despite a GFD for at least 6-12 months (14,15). Anamnesis and differential diagnosis is crucial to identify and treat a potential specific cause. The management of these patients is illustrated in **Figure 3**.

The first step is to confirm the diagnosis of CD, by reviewing the celiac serology and the biopsies at the time of diagnosis. If the diagnosis of CD is not confirmed, alternative causes of malabsorption should be considered. If the diagnostic data are consistent with CD the clinician should check the dietary compliance. Gluten ingestion is the most common cause of slow response and is identified or suspected in 35-50% of cases (15,25). Further food intolerances, for example lactose intolerance, should also be excluded. Celiac serology is useful in this context, if it is positive, the most probable cause of the poor response is a persistent gluten exposure (26). Many physicians therefore rely on follow-up serology to monitor adherence. Patient self-assessments are known to be unreliable with respect to adherence to a GFD. Evidence of strict adherence remains the normalization of the small intestine architecture. After excluding gluten exposure, the duodenal biopsies should be repeated. The presence of a pathological biopsy with VA should suggest RCD, or other causes of atrophy. For example, CD enteropathy has been reported in association with certain drugs, such as olmesartan, mycophenolate and losartan (27). A normal histology (Marsh 0-1) suggests other etiology such as irritable bowel syndrome (IBS), microscopic colitis, food intolerances, eating disorders, etc. (6). When other causes are excluded, the diagnosis of RCD is established. It is crucial to go further. IHC (immunohistochemistry), TCR rearrangement analysis and FCM analysis on duodenal biopsies should be performed to exclude RCD II. The possibility of development of the EATL should be explored when indicated.

Between 2015-2021, 16 patients with a suspected diagnosis of RCD were referred to our laboratory for FCM analysis of their duodenal biopsies. The celiac serology and the biopsies at the time of initial diagnosis were reviewed. These data are presented in **Table 1**.

Ten patients had a clear diagnosis of CD (62%). Two patients (12%) were unclear and in 4 patients (26%) the initial diagnose of CD could not be confirmed.

For all patient with confirmed CD and those with possible alternative diagnosis, the dietary compliance was checked by dietary review and serology (**Table 1**).

Although the 16 patients were advised to follow a strict GFD, 5 patients (31%) were identified as being suspected of poor compliance to the diet based on anamneses or/and serology. This is consistent with the literature. Finally, 6 patients were considered to be patients with possible (positive serology but GFD anamnesis) or probable RCD (negative serology and GFD

anamnesis) according to the algorithm (**Figure 3**). To conclude, the diagnosis of RCD can be difficult to establish, it is still a diagnosis of exclusion. Clearly, in case of any doubt and despite the evidence of another cause, examinations to exclude RCD type II must be carried out in view of the potentially serious consequences.

Question II: Determine which analytical laboratory techniques are used to make the diagnosis of RCD type I versus RCD type II.

As specified above, RCD is defined as persistent or recurrent symptoms and signs of malabsorption with VA despite strict GFD for more than 12 months and in the absence of other causes (2,6,28). Two types of RCD are recognized depending on the presence or absence of aberrant IELs sCD3- and generally CD8 but expressing iCD3 (6,25). The recognition of RCD type II is essential because this pathology is considered as a low-grade lymphoma, which can potentially evolve to EATL in +-50% of cases (17,29,30). Below we review the different diagnostic tests to differentiate between these two types of RCD.

1. Flow Cytometry

CD is characterized by specific changes of IELs in the duodenum (31,32). Active CD is characterized by an increased number of IELs (33), mainly T cells with TCRαβ or TCRγδ (31,34). When the patient strictly adheres to GFD, TCRαβ returns to normal while TCRγδ declines more slowly and may remain elevated for longer periods of time (35).

FCM analysis is currently the recommended method for the analysis of IELs and for the classification of patients into RCD type I or RCD type II based on aberrant levels of sCD3- and iCD3+ in IELs (18,36–38). These cells (sCD3-, iCD3+) can be found in the normal gut. For this reason, authors have found a cut-off to differentiate RCD type I (<20% of total lymphocytes) from RCD type II (>20% of total lymphocytes) (18,37).

The classic phenotype of aberrant IELs in RCD type II is iCD3+, sCD3- CD4-/CD8-. This is variable depending on the series, it is estimated that there is a high proportion of CD8+ samples (53% to 61%). Occasionally sCD3+ and sCD3-/iCD3- cases have also been described (39).

In the study of van Wanrooij *et al.* (38) duodenal biopsies were devised into three groups: control patients (n=5), the population with a moderate increase of IELs (20-50%: n=14) and RCD patients with a high number of aberrant IELs (>50%: n=5). Diagnosis was performed by FCM analysis, IHC analysis and TCR rearrangement analysis. They found that IHC and TCR rearrangement analysis are sensitive in identifying patients with high number of aberrant IELs but miss half of the patients with a moderate increase of aberrant IELs.

Technique used in the UZ Leuven laboratory:

In our laboratory we isolate IELs from duodenal biopsies for FCM analysis according to the following procedure. The sample (4-8 biopsies/individual) is collected in PBS and stored at 0-4°C . After removing the supernatant dissociation solution (0.5 M DTT, 0.5 M EDTA) is added and the biopsies are incubated for 60 minutes at 37°C in a water bath with agitation.

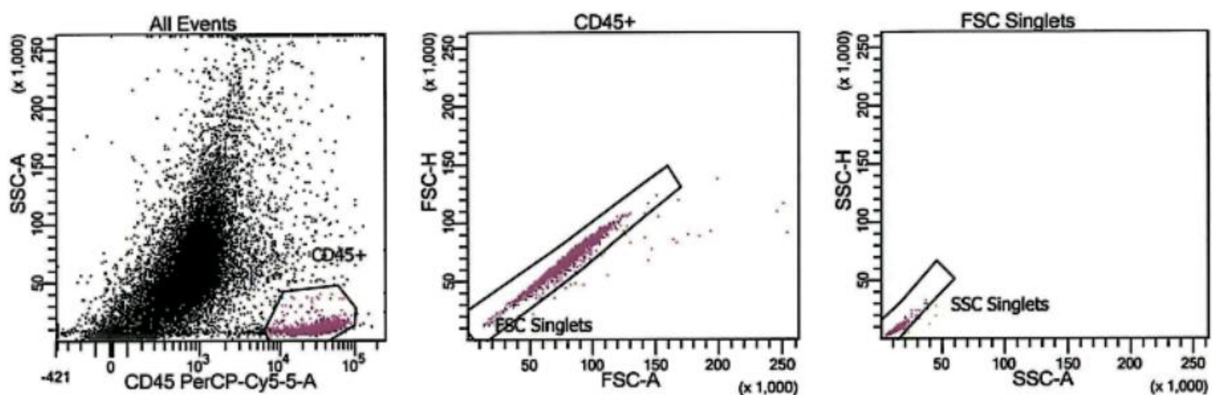
The supernatant containing the IELs are collected and washed twice.

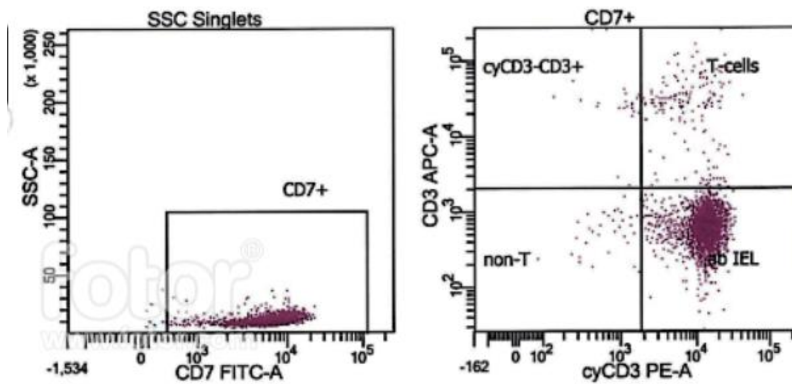
Finally, the suspension is divided into 3 tubes. The suspension is stained with fluorochrome-conjugated monoclonal antibodies* and analysed on the flowcytometer.

	Monoclonal antibodies*		
Fluorochrome	**Tube 1**	**Tube 2**	**Tube 3**
FITC	CD3	CD7	
PE	CD16/56	cyt Isotype	iCD3
PerCP	CD45	CD45	CD45
APC	CD19	sCD3	sCD3
PE-Cy7	CD4		
APC-H7	CD8		

<u>Gating strategy:</u>

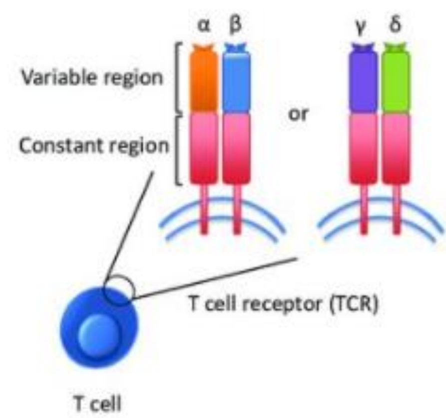
IELs are selected based on their CD45 cells compared to SSC (side scatter) characteristics and doublets are excluded. Subsequently, the aberrant T lymphocytes are identified as CD7+, sCD3- and iCD3+. Normal T cells are CD7+, sCD3+ and iCD3+. Below is an example of the gating strategy (tube 2 and 3).





2. Molecular analysis

The structure of TCR gene rearrangements in RCD type II is currently under investigation. The TCR is a heterodimeric receptor composed of α and β chains in 85% of IELs and of γ and δ chains in 15% (7). Antigen binding of TCR is determined by three hypervariable complementarity-determining regions (CDR)1–3, where CDR3 is currently responsible for recognizing processed antigens (7).



These CDR are composed through somatic recombination between the between variable (V), diversity (D only present in TCR-β) and junctional (J) segments, globally generating a spectrum of >2.5×10^7 unique TCR receptors (8). Clonal extension of mucosal T cells expressing the same CDR3 region are an important risk factor characteristic of RCD type II (7,9). Such a condition may be considered pre-malignant requiring oncology-like treatment rather than simple immunosuppressive therapy (10,11). However, the great diversity of TCR receptor in the duodenal mucosa and the coexistence of clones in the healthy duodenum as well as in active CD and other pathologies complicates the diagnosis. Assessing the clonality of IELs by TCR-γ and/or TCR-β gene rearrangement analysis is known to be important but not sufficient for the diagnosis of RCD type II. These assays can vary in sensitivity and/or specificity depending on the method used (21,44). Standardisation of multiplex PCR assays and optimisation of primers for TCR gene rearrangement has resulted in greater sensitivity of T clonal populations in T cell neoplasms (44). The sensitivity of TCR-γ gene rearrangement analysis to identify patients with RCD type II who subsequently developed EATL is reported to be between 67-78% (18,45). In some cases, the TCR-γ gene may not be rearranged (23,46). Additional analysis of TCR-β (21,40) or TCR-δ chain (7) may improve sensitivity. This test also suffers from a lack of specificity, clonal peak is seen in a significant fraction of RCD type I and GFD cases (17% and 6% respectively), but not in active CD and non-CD cases (20).

This highlights the need of complementary technique: FCM analysis and IHC.

Technique used in the UZ Leuven laboratory:

First, DNA is isolated from a tissue sample (preferably freshly frozen) and the quality of the isolated DNA is checked. If suitable, Ig and TCR rearrangements are checked using multiplex PCR with Biomed-2 developed primers with two fluorochromes (44).

For the T-cell receptor, the TCR-γ and TCR-β gene are analysed. The PCR product is visualised by means of a fluorescence-labelled DNA fragment analysis or 'GeneScanning', using automatic sequence analysis equipment, whereby the amplified PCR products are separated according to length.

3. Immunohistochemistry

Distinguishing between groups of aberrant lymphocytes and reactive lymphoid aggregates is morphologically difficult. Prior to the emergence of FCM analysis, the widely available method for diagnosing RCD type II was CD3/CD8 staining on IHC (14). IHC can differentiate abnormal IELs, which express CD3 but generally lack CD8, from normal CD8+ T-IELs.

For the pathologist, the defining feature of RCD type II is the loss of CD8 expression in more than 50% of IELs. IHC is sensitive in identifying patients with high number (38) of aberrant IELs but FCM remains necessary to detect low percentages of aberrant IELs (17). It is also useful for identifying the rare cases where abnormal IELs express CD8. Moreover, studies have shown that RCD type II is associated with a high frequency (78 to 92%) of severe VA (Marsh 3b/3c) (17), these proportion varies between cohorts, with one recent study showing a proportion of 55%, similar to other observations (48). One of the limitations of this technique is the high inter-observer variability (19) but IHC is widely available and readily applicable, whereas FCM analysis requires fresh duodenal biopsies and trained technicians.

Technique used in the UZ Leuven laboratory:

The analysis is performed on formalin-fixed paraffin-embedded mucosa (FFPE) samples.

The pathologist evaluates the density of lymphocytosis by counting at ×400 magnification the surface intraepithelial lymphocytes (IEL) per 100 enterocytes.

The mucosal healing by GFD induces a complete recovery with normal villous architecture and a decrease of IEL (normal IEL count <30 IEL/100 epithelial cells in duodenum and <40 IEL/100 epithelial cells in jejunum (Marsh type 0)). If VA and surface lymphocytosis persist,

immunohistochemistry on FFPE sections is mandatory using the CD103, CD3, CD8, CD4, CD30 and granzyme B antibodies. The percentage of IEL labelled by each antibody has to be assessed to conclude to a normal IEL phenotype defined by CD3 and CD8 coexpression in more than 60% of IEL with expression of CD103 and without expression of CD30 and CD4 (IEL expressing CD4 < 10%).

4. Perspectives

Chromosome Microarray Analysis

Trisomy of chromosome 1q is the only known recurrent chromosome abnormality in RCD type II (24,50). In the study of Verkarre *et al.*(50), a recurrent structural chromosomal aberration leading to partial trisomy of the long arm of chromosome 1 was found in 6 of 7 cell lines from RCD type II patients. Malamut *et al.*(17) detected a 1q trisomy in 93.8% of the RCD type II patients (n=16). However, in a recent study (48), this specific abnormality was found in only 1 patients with RCD type II (n=10) but 50% cases of RCD type II had chromosome copy number changes. Discerning which genes are relevant to the disease and their roles in the pathogenesis of RCD type II requires a more comprehensive knowledge of the pathogenesis of RCD type II and further research.

Next-generation sequencing Analysis (NGS)

Recently, Soderquist *et al.* (48) performed NGS of 465 cancer-related genes (Columbia Combined Cancer Panel) on DNA extracted from 11 RCD type II patients. Three of these patients have developed EATL and their biopsies were also analysed. 75 unique mutations, including 48 pathologic variants and 27 variants of uncertain significance were detected in RCD type II patients. No data was found for patients with RCD type I.

In RCD type II, cytokines bind to receptors on the surface of IELs and activate various downstream signaling pathways, including the *JAK-STAT* pathway. Activating *JAK1* and *STAT3* mutations led to hyperresponsiveness of IELs to cytokines allowing a clone of innate T-IELs to progressively supplant normal T-IELs and invade the epithelium. In this cohort, NGS detected recurrent genetic alterations in the *JAK-STAT* pathway in 9/11 (82%) of RCD type II patients. Mutations in the *JAK-STAT* pathway are common in a variety of lymphomas including EATL (51), Mutations in epigenetic modifiers is also described in RCD type II patients, in particular *TET2* and *KMT2D*. Sequencing led to the discovery of mutations in RCD type II similar to those reported in EATL and provides a better understanding of the physiopathology. This could lead to the development of new therapeutic strategies for these lymphoproliferative disorders.

NKp46 antibody

It is documented that lymphomas complicating CD arise from innate-like lymphocytes, which may carry NK receptors (NKRs). A recent study by the CELAC group (54) has compared the expression of NKRs by FCM analysis in IELs from CD, RCD type I and RCD type II patients. Moreover, they assessed NKp46 by IHC in paraffin-embedded biopsies from 204 patients with CD, RCD type I, RCD type II or GI T-cell lymphomas and a validation cohort of 61 patients. The results showed that NKp46 was significantly more expressed by malignant RCD type II IELs than by normal IELs in CD and RCD type I. In paraffin biopsies, the detection of >25 NKp46+ IELs per 100 epithelial cells distinguished RCD type II from CD and RCD type I. NKp46 was also detected in EATL (24/29) and monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL, 4/4) but not in indolent primary GI T-cell lymphoproliferative disease (T-LPD, 0/15). NKp46 could be become a novel biomarker useful for the diagnosis and therapeutic stratification of RCD.

Question III: Determine the contribution of each analytical laboratory technique (FCM analysis, molecular analysis, pathology) to the final diagnosis of RCD type I/II (concordances/discordances)?

The diagnosis of RCD type II involves a multidisciplinary approach.

Results of the FCM analysis (16 patients), TCR rearrangement analysis (8 patients) and IHC (16 patients) on the duodenal biopsies of the 16 patients are shown in **Table 2.** After review of diagnostic tests, in only 6 cases RCD was confirmed (case 7, case 8, case 9, case 14, case 15, case 16). IHC and FCM were performed in all 6 patients and TCR rearrangement analysis only in 4. Two patients had >50% aberrant IELs on IHC (case 14 and 15), three patients had a monoclonal TCR rearrangement (case 8, 14 and 15) and two patients had >20% aberrant IELs on FCM (case 14 and 15). Classically, the classification of patients with RCD into type I or type II is based on FCM (18,19). Therefore, we used FCM as the gold standard method and the performance of IHC and molecular TCR rearrangement analysis were compared to the results of FCM. In the 4 RCD type I patients, discordances were seen in 1 patients: case 8 where TCR rearrangement analysis was discordant. In all other RCD type I patients IHC findings were consistent with FCM. For the 2 RCD type II patients, based on the cut-off of >20% aberrant IELs on FCM, a 100% concordance of the results of the 3 methods was observed.

Both IHC and TCR rearrangement analysis showed a 100% sensitivity, while the specificity was 100% and 50% respectively. The negative predictive value of both test was 100% and the positive predictive values was 100% and 66% respectively.

According to our data, TCR rearrangement analysis and pathology/IHC could be used as 'rule-out' tests to exclude the diagnosis of RCD type II. However, a positive test should be interpreted with caution and compared with the FCM analysis.

One explanation for the monoclonal peak in non-RCD patients could be the presence of a prominent clonal peak on a polyclonal background, observed in all phases of CD, which can be misinterpreted (20). This pattern does not necessarily indicate the presence of a monoclonal T cell population. Furthermore, it is known that transient monoclonal peaks may appear in patients with low compliant GFD, without clear explanation (20). We concluded that TCR rearrangement analysis are reserved for patients meeting strict RCD criteria to avoid unnecessary treatment and anxiety.

Question IV: Discuss the clinical course of patients diagnosed with RCD type I and RCD type II.

The management of RCD patients is challenging and requires a multidisciplinary approach in tertiary centers. Treatments depend on the type of RCD, the **Figure 4** summarizes the currently available therapeutic options for RCD patients.

Budesonide is the first-line treatment in RCD type I and type II. In steroid-dependent RCD type I patients, immunosuppressive drugs should be considered. Autologous stem cell transplantation can be proposed in RCD type II patients before 65 years. Anti-IL-15 is currently tested in RCD type I and RCD type II patients. While JAK1 inhibitor may be considered in RCD type II patients (24).

We summarized the treatment, clinical course and survival of our cohort **(Table 3)**. One patient (case 14) with RCD type II died but not disease related (previous intraepithelial T-cell lymphoma). The other patient with RCD type II is still alive (case 15) and the follow-up consultation in 2021 indicates that the patient is still in remission; IHC analysis of duodenal biopsies doesn't show aberrant lymphocytes (no follow-up by FCM analysis or TCR rearrangement analysis has been performed). None of the patients with RCD developed EATL yet in our cohort.

Question V. Determine which analytical laboratory technique(s) best predicts the clinical course of patients with RCD type I and type II.

The most effective techniques for identifying complications will be discussed here. After the diagnosis of RCD, follow-up of patients is mandatory.

First, at the time of diagnosis, capsule endoscopy, devise-assisted enteroscopy, and magnetic resonance (MR) enterography should be used to identify eventual complications such as lymphoma (11). After, it is currently recommended that duodenal biopsies with only IHC analysis have to be repeated 3 months after the treatment starting (11). Then, annual duodenal

biopsies with IHC and FCM analysis are recommended (11). Patients with RCD type II celiac disease should be monitored for EATL with capsule endoscopy and CT or MR enterography.

Attachments

Figure 1

Figure 1: Classification of histologic findings in celiac disease (12).

Modified Marsh Classification of histologic findings in celiac disease (Oberhuber)

Marsh Type	IEL / 100 enterocytes – jejunum	IEL / 100 enterocytes – duodenum	Crypt hyperplasia	Villi
0	<40	<30	Normal	Normal
1	>40	>30	Normal	Normal
2	>40	>30	Increased	Normal
3a	>40	>30	Increased	Mild atrophy
3b	>40	>30	Increased	Marked atrophy
3c	>40	>30	Increased	Complete atrophy

IEL/100 enterocytes, intraepithelial lymphocytes per 100 enterocytes

- Type 0: Normal; celiac disease highly unlikely.
- Type 1: Seen in patients on gluten free diet (suggesting minimal amounts of gluten or gliadin are being ingested); patients with dermatitis herpetiformis; family members of celiac disease patients, not specific, may be seen in infections.
- Type 2: Very rare, seen occasionally in dermatitis herpetiformis.
- Type 3: Spectrum of changes seen in symptomatic celiac disease.

Figure 2

Figure 2: Mechanisms driving activation and malignant transformation of intraepithelial lymphocytes in celiac disease and type II refractory celiac disease (24).

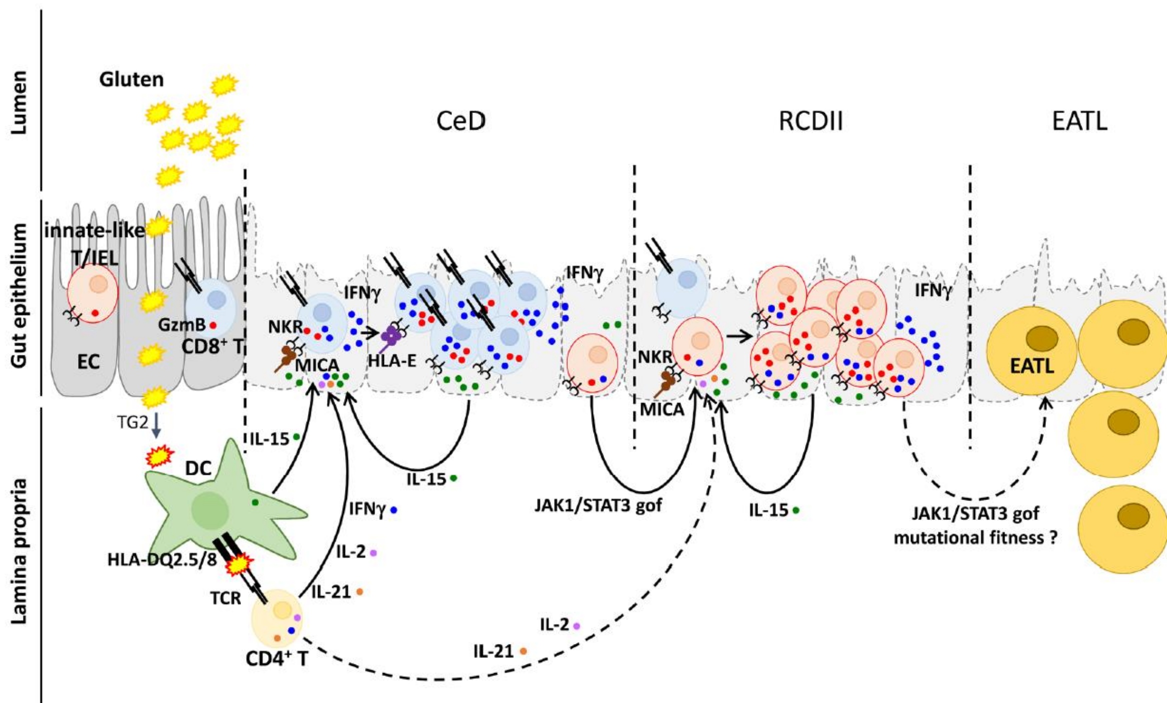


Figure 3

Figure 3: Diagnostic approach to symptomatic CD or laboratory abnormalities despite GFD (11).

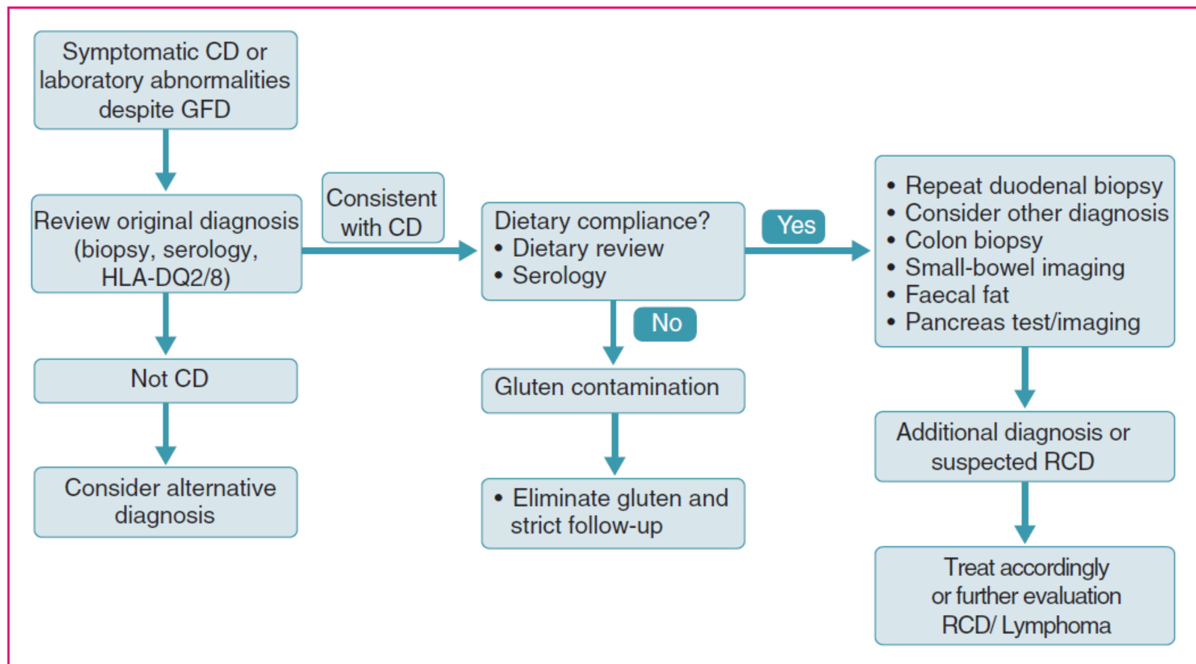


Figure 4

Figure 4: Therapeutic strategies in RCD (24) .

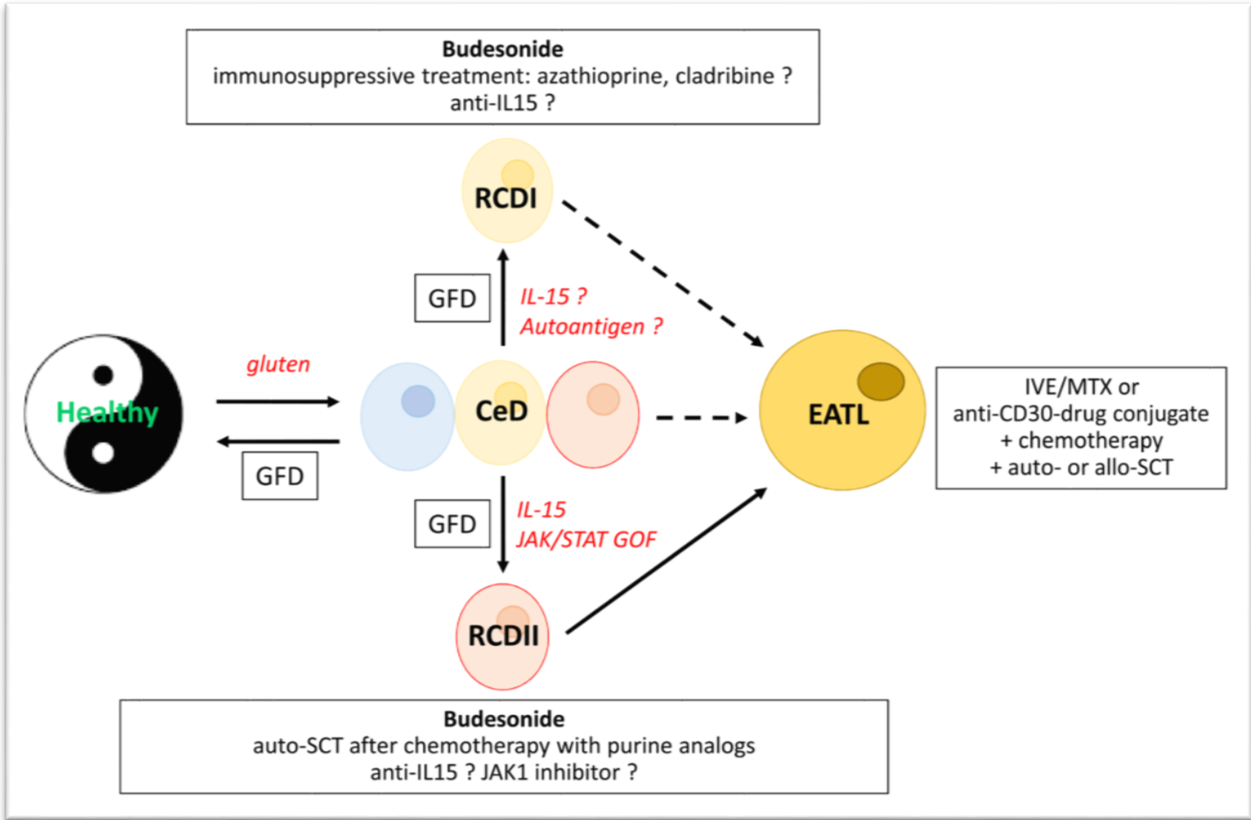

Budesonide
immunosuppressive treatment: azathioprine, cladribine ?
anti-IL15 ?
RCDI
GFD
IL-15 ?
Autoantigen ?
Healthy
gluten
GFD
CeD
EATL
IVE/MTX or
anti-CD30-drug conjugate
+ chemotherapy
+ auto- or allo-SCT
GFD
IL-15
JAK/STAT GOF
RCDII
Budesonide
auto-SCT after chemotherapy with purine analogs
anti-IL15 ? JAK1 inhibitor ?

Table 1

Table 1	Review of original diagnosis				Dietary compliance			Possible cause of malabsorption
Patients	Serology[1]	HLA testing	Histology	CD confirmed	Review dietary	Serology after GFD	Conclusion compliance	
1	negative	negative	no abnormality	No	GFD	negative	-	No cause found
2	positive	-	CD	Yes	Poor compliance with GFD	increased	No	Possible gluten contamination
3	positive	-	-	Yes	Poor compliance with GFD	negative	No	Possible gluten contamination
4	positive	-	CD	Yes	Poor compliance with GFD	increased	No	Possible gluten contamination
5	negative	-	CD	No	Suspect for poor compliance with GFD	negative	-	Crohn's disease
6	negative	negative	CD	No	-	-	-	Possible auto-immune enteropathy
7	positive	-	CD	Yes	GFD	increased	Yes	Possible RCD
8	positive	-	CD	Yes	GFD	negative	Yes	Probable RCD
9	positive	-	CD	Yes	GFD	increased	Yes	Possible RCD
10	negative	positive[3]	-	No	GFD	negative	-	Possible auto-immune enteropathy
11	positive	-	CD	Yes	Poor compliance with GFD	increased	No	Possible gluten contamination
12	positive	-	CD	Yes	Poor compliance with GFD	increased	No	Possible gluten contamination
13	positive	HLA DQ7.5 cis, very low risk[2]	CD	Yes/No[2]	GFD	increased	-	Sartan-induced enteropathy
14	negative	-	CD	Yes/No[2]	GFD	negative	Yes	Probable RCD
15	positive	-	CD	Yes	GFD	negative	Yes	Probable RCD
16	positive	-	CD	Yes	GFD	-	Yes	Probable RCD

[1] TG2-IgA were performed and IgG-DGP in patients with IgA deficiency. [2] The diagnostic doubt persists in this patient. He is a carrier of HLA DQ7.5 cis which is associated with a very low probability of the CD (52). Despite positive serology and histological CD, a sartan-induced enteropathy cannot be excluded. [3] HLA-DQ 2 haplotype.

Table 2

Figure 2: Results of the FCM analysis (16 patients), TCR rearrangement analysis (8 patients) and IHC (16 patients) on the duodenal biopsies of the 16 patients.

Table 2	Pathology (n=16)	TCR rearrangement analysis (n=8)	FCM, % aberrant IELs (n=16)
Patients	***iCD3+CD8- IELs>50%***		
1	no	Monoclonal	0,6
2	no	Monoclonal	1,2
3	yes	Monoclonal	0,8
4	no	Not done	1,2
5	no	Not done	0,12
6	yes	Monoclonal	1,5
7*	no	Not done	4,1
8*	no	Monoclonal	0,9
9*	no	Polyclonal	0,2
10	no	Not done	0,2
11	no	Not done	<0,2
12	no	Not done	0,3
13	no	Not done	0,1
14*	yes	Monoclonal	73
15*	yes	Monoclonal	96,5
16*	no	Not done	0,6

* Patients with confirmed RCD

Table 3

Patient	Treatment	Outcome	Follow-up time
7	Budesonide	Indicates alive	-
8	Budesonide	Indicates alive	No abnormalities on follow-up histology in 2018 after treatment
9	Budesonide/medrol	Indicates alive	No abnormalities on follow-up histology in 2020 after treatment
14	Cladribine	Dead not disease related	-
15	Cladribine + everolimus	Indicates alive	Histological remission at the consultation in 2021
16	CHOP[2]	Dead not disease related	-

- = data not found

[1] = mycophenolate mofetil

[2] = cyclophosphamide, doxorubicine, vincristine and prednisone

References

1. Pitchumoni C.S. (2020) Celiac Disease. In: Pitchumoni C.S., Dharmarajan T. (eds) Geriatric Gastroenterology. Springer, Cham. https://doi.org/10.1007/978-3-319-90761-1_69-1
2. Ludvigsson JF, Leffler DA, Bai JC, et al. The Oslo definitions for coeliac disease and related terms. *Gut*. 2013;62(1):43-52. doi:10.1136/gutjnl-2011-301346
3. Spagnoli C, Pisani F, Di Mario F, et al. Peripheral neuropathy and gastroenterologic disorders: an overview on an underrecognized association. *Acta Biomed*. 2018;89(9-S):22-32. Published 2018 Dec 17. doi:10.23750/abm.v89i9-S.7956
4. Ludvigsson JF, Bai JC, Biagi F, et al. Diagnosis and management of adult coeliac disease: guidelines from the British Society of Gastroenterology. *Gut*. 2014;63(8):1210-1228. doi:10.1136/gutjnl-2013-306578
5. Husby S, Murray JA, Katzka DA. AGA Clinical Practice Update on Diagnosis and Monitoring of Celiac Disease-Changing Utility of Serology and Histologic Measures: Expert Review. *Gastroenterology*. 2019;156(4):885-889. doi:10.1053/j.gastro.2018.12.010
6. Rubio-Tapia A, Hill ID, Kelly CP, Calderwood AH, Murray JA; American College of Gastroenterology. ACG clinical guidelines: diagnosis and management of celiac disease. *Am J Gastroenterol*. 2013;108(5):656-677. doi:10.1038/ajg.2013.79
7. Ludvigsson JF, Card TR, Kaukinen K, et al. Screening for celiac disease in the general population and in high-risk groups. *United European Gastroenterol J*. 2015;3(2):106-120. doi:10.1177/2050640614561668
8. Mooney PD, Kurien M, Evans KE, et al. Clinical and Immunologic Features of Ultra-Short Celiac Disease. *Gastroenterology*. 2016;150(5):1125-1134. doi:10.1053/j.gastro.2016.01.029
9. Romano C, van Wynckel M, Hulst J, et al. European Society for Paediatric Gastroenterology, Hepatology and Nutrition Guidelines for the Evaluation and Treatment of Gastrointestinal and Nutritional Complications in Children With Neurological Impairment. *J Pediatr Gastroenterol Nutr*. 2017;65(2):242-264. doi:10.1097/MPG.0000000000001646
10. Sanders DS, Hurlstone DP, McAlindon ME, et al. Antibody negative coeliac disease presenting in elderly people--an easily missed diagnosis. *BMJ*. 2005;330(7494):775-776. doi:10.1136/bmj.330.7494.775
11. Al-Toma A, Volta U, Auricchio R, et al. European Society for the Study of Coeliac Disease (ESsCD) guideline for coeliac disease and other gluten-related disorders. *United European Gastroenterol J*. 2019;7(5):583-613. doi:10.1177/2050640619844125
12. Oberhuber G. Histopathology of celiac disease. *Biomed Pharmacother*. 2000;54(7):368-372. doi:10.1016/S0753-3322(01)80003-2
13. Ilus T, Kaukinen K, Virta LJ, et al. Refractory coeliac disease in a country with a high prevalence of clinically-diagnosed coeliac disease. *Aliment Pharmacol Ther*. 2014;39(4):418-425. doi:10.1111/apt.12606
14. Leffler DA, Dennis M, Hyett B, Kelly E, Schuppan D, Kelly CP. Etiologies and predictors of diagnosis in nonresponsive celiac disease. *Clin Gastroenterol Hepatol*. 2007;5(4):445-450. doi:10.1016/j.cgh.2006.12.006
15. Al-toma A, Verbeek WH, Mulder CJ. The management of complicated celiac disease. *Dig Dis*. 2007;25(3):230-236. doi:10.1159/000103891
16. Daum S, Cellier C, Mulder CJ. Refractory coeliac disease. *Best Pract Res Clin Gastroenterol*. 2005;19(3):413-424. doi:10.1016/j.bpg.2005.02.001
17. Malamut G, Afchain P, Verkarre V, et al. Presentation and long-term follow-up of refractory celiac disease: comparison of type I with type II. *Gastroenterology*. 2009;136(1):81-90. doi:10.1053/j.gastro.2008.09.069
18. Verbeek WH, Goerres MS, von Blomberg BM, et al. Flow cytometric determination of aberrant intra-epithelial lymphocytes predicts T-cell lymphoma development more accurately than T-cell clonality analysis in Refractory Celiac Disease. *Clin Immunol*. 2008;126(1):48-56. doi:10.1016/j.clim.2007.09.002
19. Sergi C, Shen F, Bouma G. Intraepithelial lymphocytes, scores, mimickers and challenges in diagnosing gluten-sensitive enteropathy (celiac disease). *World J Gastroenterol*. 2017;23(4):573-589. doi:10.3748/wjg.v23.i4.573

20. Hussein S, Gindin T, Lagana SM, et al. Clonal T cell receptor gene rearrangements in coeliac disease: implications for diagnosing refractory coeliac disease. *J Clin Pathol*. 2018;71(9):825-831. doi:10.1136/jclinpath-2018-205023
21. Perfetti V, Brunetti L, Biagi F, Ciccocioppo R, Bianchi PI, Corazza GR. TCRβ clonality improves diagnostic yield of TCRγ clonality in refractory celiac disease. *J Clin Gastroenterol*. 2012;46(8):675-679. doi:10.1097/MCG.0b013e31823eff20
22. Jabri B, Sollid LM. T Cells in Celiac Disease. *J Immunol*. 2017;198(8):3005-3014. doi:10.4049/jimmunol.1601693
23. Ettersperger J, Montcuquet N, Malamut G, et al. Interleukin-15-Dependent T-Cell-like Innate Intraepithelial Lymphocytes Develop in the Intestine and Transform into Lymphomas in Celiac Disease. *Immunity*. 2016;45(3):610-625. doi:10.1016/j.immuni.2016.07.018
24. Malamut G, Cording S, Cerf-Bensussan N. Recent advances in celiac disease and refractory celiac disease. *F1000Res*. 2019;8:F1000 Faculty Rev-969. Published 2019 Jun 26. doi:10.12688/f1000research.18701.1
25. Cellier C, Delabesse E, Helmer C, et al. Refractory sprue, coeliac disease, and enteropathy-associated T-cell lymphoma. French Coeliac Disease Study Group. *Lancet*. 2000;356(9225):203-208. doi:10.1016/s0140-6736(00)02481-8
26. Comino I, Fernández-Bañares F, Esteve M, et al. Fecal Gluten Peptides Reveal Limitations of Serological Tests and Food Questionnaires for Monitoring Gluten-Free Diet in Celiac Disease Patients [published correction appears in Am J Gastroenterol. 2017 Jul;112(7):1208]. *Am J Gastroenterol*. 2016;111(10):1456-1465. doi:10.1038/ajg.2016.439
27. Ianiro G, Bibbò S, Montalto M, Ricci R, Gasbarrini A, Cammarota G. Systematic review: Sprue-like enteropathy associated with olmesartan. *Aliment Pharmacol Ther*. 2014;40(1):16-23. doi:10.1111/apt.12780
28. Al-Toma A, Verbeek WH, Mulder CJ. Update on the management of refractory coeliac disease. *J Gastrointestin Liver Dis*. 2007;16(1):57-63.
29. Sieniawski M, Angamuthu N, Boyd K, et al. Evaluation of enteropathy-associated T-cell lymphoma comparing standard therapies with a novel regimen including autologous stem cell transplantation [published correction appears in Blood. 2011 Feb 10;117(6):2077]. *Blood*. 2010;115(18):3664-3670. doi:10.1182/blood-2009-07-231324
30. Cellier C, Delabesse E, Helmer C, et al. Refractory sprue, coeliac disease, and enteropathy-associated T-cell lymphoma. French Coeliac Disease Study Group. *Lancet*. 2000;356(9225):203-208. doi:10.1016/s0140-6736(00)02481-8
31. Spencer J, Isaacson PG, MacDonald TT, Thomas AJ, Walker-Smith JA. Gamma/delta T cells and the diagnosis of coeliac disease. *Clin Exp Immunol*. 1991;85(1):109-113. doi:10.1111/j.1365-2249.1991.tb05690.x
32. Eiras P, Roldán E, Camarero C, Olivares F, Bootello A, Roy G. Flow cytometry description of a novel CD3-/CD7+ intraepithelial lymphocyte subset in human duodenal biopsies: potential diagnostic value in coeliac disease. *Cytometry*. 1998;34(2):95-102. doi:10.1002/(sici)1097-0320(19980415)34:2<95::aid-cyto6>3.0.co;2-b
33. Ferguson A. Intraepithelial lymphocytes of the small intestine. *Gut*. 1977;18(11):921-937. doi:10.1136/gut.18.11.921
34. 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(4):476-482. doi:10.1136/gut.35.4.476
35. Koskinen O, Collin P, Lindfors K, Laurila K, Mäki M, Kaukinen K. Usefulness of small-bowel mucosal transglutaminase-2 specific autoantibody deposits in the diagnosis and follow-up of celiac disease. *J Clin Gastroenterol*. 2010;44(7):483-488. doi:10.1097/MCG.0b013e3181b64557
36. García-Hoz C, Crespo L, Lopez N, et al. The Intracellular Intensity of CD3 on Aberrant Intraepithelial Lymphocytes Is a Prognostic Factor of the Progression to Overt Lymphoma in Refractory Celiac Disease Type II (Pre-Enteropathy-Associated T Cell Lymphoma). *Dig Dis*. 2020;38(6):490-499. doi:10.1159/000506305
37. van Wanrooij RL, Schreurs MW, Bouma G, et al. Accurate classification of RCD requires flow cytometry. *Gut*. 2010;59(12):1732. doi:10.1136/gut.2010.223438
38. van Wanrooij RL, Müller DM, Neefjes-Borst EA, et al. Optimal strategies to identify aberrant intra-epithelial lymphocytes in refractory coeliac disease. *J Clin Immunol*. 2014;34(7):828-835. doi:10.1007/s10875-014-0075-7

39. de Mascarel A, Belleannée G, Stanislas S, et al. Mucosal intraepithelial T-lymphocytes in refractory celiac disease: a neoplastic population with a variable CD8 phenotype. *Am J Surg Pathol*. 2008;32(5):744-751. doi:10.1097/PAS.0b013e318159b478
40. Ritter J, Zimmermann K, Jöhrens K, et al. T-cell repertoires in refractory coeliac disease. *Gut*. 2018;67(4):644-653. doi:10.1136/gutjnl-2016-311816
41. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. *Science*. 1999;286(5441):958-961. doi:10.1126/science.286.5441.958
42. Daum S, Weiss D, Hummel M, et al. Frequency of clonal intraepithelial T lymphocyte proliferations in enteropathy-type intestinal T cell lymphoma, coeliac disease, and refractory sprue. *Gut*. 2001;49(6):804-812. doi:10.1136/gut.49.6.804
43. Carbonnel F, Grollet-Bioul L, Brouet JC, et al. Are complicated forms of celiac disease cryptic T-cell lymphomas?. *Blood*. 1998;92(10):3879-3886.
44. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia*. 2003;17(12):2257-2317. doi:10.1038/sj.leu.2403202
45. Lonardi S, Villanacci V, Lorenzi L, Lanzini A, Lanzarotto F, Carabellese N, et al. Anti-TCR gamma antibody in celiac disease: the value of count on formalin-fixed paraffin-embedded biopsies. Virchows Arch [Internet]. 2013 Sep [cited 2021 Nov 25];463(3):409–13. Available from: https://pubmed.ncbi.nlm.nih.gov/23860877/
46. Tack GJ, van Wanrooij RL, Langerak AW, et al. Origin and immunophenotype of aberrant IEL in RCDII patients. *Mol Immunol*. 2012;50(4):262-270. doi:10.1016/j.molimm.2012.01.014
47. Patey-Mariaud De Serre N, Cellier C, Jabri B, et al. Distinction between coeliac disease and refractory sprue: a simple immunohistochemical method. *Histopathology*. 2000;37(1):70-77. doi:10.1046/j.1365-2559.2000.00926.x
48. Soderquist CR, Lewis SK, Gru AA, et al. Immunophenotypic Spectrum and Genomic Landscape of Refractory Celiac Disease Type II. *Am J Surg Pathol*. 2021;45(7):905-916. doi:10.1097/PAS.0000000000001658
49. Popp A, Taavela J, Graziano P, et al. A New Intraepithelial γδ T-Lymphocyte Marker for Celiac Disease Classification in Formalin-Fixed Paraffin-Embedded (FFPE) Duodenal Biopsies [published correction appears in Dig Dis Sci. 2020 Nov 25;:]. *Dig Dis Sci*. 2021;66(10):3352-3358. doi:10.1007/s10620-020-06680-x
50. Verkarre V, Romana SP, Cellier C, et al. Recurrent partial trisomy 1q22-q44 in clonal intraepithelial lymphocytes in refractory celiac sprue. *Gastroenterology*. 2003;125(1):40-46. doi:10.1016/s0016-5085(03)00692-9
51. Nicolae A, Xi L, Pham TH, et al. Mutations in the JAK/STAT and RAS signaling pathways are common in intestinal T-cell lymphomas. *Leukemia*. 2016;30(11):2245-2247. doi:10.1038/leu.2016.178
52. Sollid LM. The roles of MHC class II genes and post-translational modification in celiac disease. *Immunogenetics*. 2017;69(8-9):605-616. doi:10.1007/s00251-017-0985-7
53. Malamut G, Cellier C. Refractory Celiac Disease. *Gastroenterol Clin North Am*. 2019;48(1):137-144. doi:10.1016/j.gtc.2018.09.010
54. Cheminant M, Bruneau J, Malamut G, et al. NKp46 is a diagnostic biomarker and may be a therapeutic target in gastrointestinal T-cell lymphoproliferative diseases: a CELAC study. *Gut*. 2019;68(8):1396-1405. doi:10.1136/gutjnl-2018-317371