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The role of flow cytometry in complicated celiac disease

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Abstract

Background. Refractory celiac disease (RCD) is defined as persistence or recurrence of clinical symptoms of malabsorption and histologic signs of villous atrophy despite at least 1 year of strict adherence to a gluten-free diet. Two different entities of RCD have been described: RCD I shows a polyclonal pattern of intraepithelial lymphocytes (IEL), while in RCD II a monoclonal transformation of IEL can be identified (usually detected by means of TCR clonality analysis). Patients with RCD II have a poorer prognosis and a high risk of development of enteropathy-associated T-cell lymphoma (EATL), so that it has recently been proposed to consider RCD II as a form of low-grade intraepithelial lymphoma (pre-EATL). Recently, flow cytometric analysis of isolated intestinal lymphocytes has been introduced as new diagnostic modality for the detection of aberrant intestinal lymphocytes (ILs) and as a stronger predictor of EATL development than gene clonality analysis.

Aims. The aims of this study were (i) to evaluate the presence of aberrant ILs in a cohort of non-celiac, celiac and RCD patients by means of flow cytometry, (ii) to verify whether there is an association between clinical characteristics of RCD patients and presence of aberrant ILs, (iii) to compare different ILs detection strategies with the purpose of validating a simpler strategy than the ones currently proposed and (iv) to evaluate whether flow cytometric analysis of aberrant ILs could accurately identify RCD II/pre-EATL patients in our cohort.

Methods. Flow cytometry analysis of duodenal biopsy specimens from RCD, uncomplicated CD patients and controls was performed. Several lymphocyte markers (CD3, CD4, CD8, CD7, CD103, TCR$\gamma\delta$) were applied in order to identify aberrant ILs, defined by means of several gating strategies including cytCD3+surfCD3-CD7+ and surfCD3-CD7+CD107. Percentages of aberrant ILs as well as clinical characteristics in different patients groups were compared.

Results. A total of 130-flow cytometry assays were performed on 109 patients, including 42 controls, 21 active CD, 16 CD on GFD and 30 RCD. RCD patients were initially subgrouped according to the presence (TCRclon+, n=17) or absence (TCRclon-, n=13) of TCR clonality: the presence of elevated aberrant ILs was compared between the two subgroups, with elevated ILs detectable exclusively in the RCDclon+. Patients with elevated ILs also showed a significantly more severe malabsorption (assessed by a

composite score). A cut-off of 11% ILs for the most reliable strategy allowed to identify a subgroup of low risk (=21/30) RCD patients and a small group of high-risk RCD (n=5) that were classified as pre-EATL (two of them with a later EATL diagnosis). However, this technique was not able to correctly identify 1 patient with ulcerative jejunoileitis (who later developed a EATL) and proved negative in 2 cases of overt EATL. Alternative, simpler gating strategies for aberrant ILs showed similar accuracy to the principal strategy, however these results need further validation.

Conclusion: in routine clinical practice, flow cytometry for the assessment of aberrant ILs could prove a simple and accurate predictor for high-risk RCD. However, its use as a diagnostic strategy to classify patients into RCD I (low risk) and RCD II/pre-EATL could lead to missing cases of RCD patients with elevated risk. In order to prevent the consequences of false negative results, a multifaceted diagnostic approach taking TCR clonality and clinical manifestations (i.e. malabsorption) into account could maximize accuracy.

Introduction

Celiac disease: an overview

Celiac disease (CD) is a chronic autoimmune enteropathy caused by dietary exposure to gluten, a protein found in wheat, barley and rye [1,2]. It is considered the most common chronic enteropathy in Western countries, with an estimated prevalence of up to 1% and increasing incidence over the last few decades [1].

Epidemiology

The worldwide prevalence of CD is estimated between 0.5% and 1%, with a proportion of patients still undiagnosed [1]. In fact, serological screening studies have shown that only a small proportion of CD cases are clinically recognized [3-5].

In Western countries, the prevalence of CD reported in literature is close to 1%, with higher figures in Northern Europe [3,4,6]. The reported higher prevalence of CD in Western countries may be due to the high wheat consumption typical of the Western diet, but also to higher disease awareness: recent data suggest that the prevalence of CD in many developing countries may be higher than previously reported [7,8].

Despite its original identification and description as a pediatric disease [9], it is now clear that CD can develop at every age, with a first peak of incidence observed within 5 years of age (with a 1:1 female:male ratio) and a second peak between 30 and 40 years, in this case with a 2:1 female:male ratio [6].

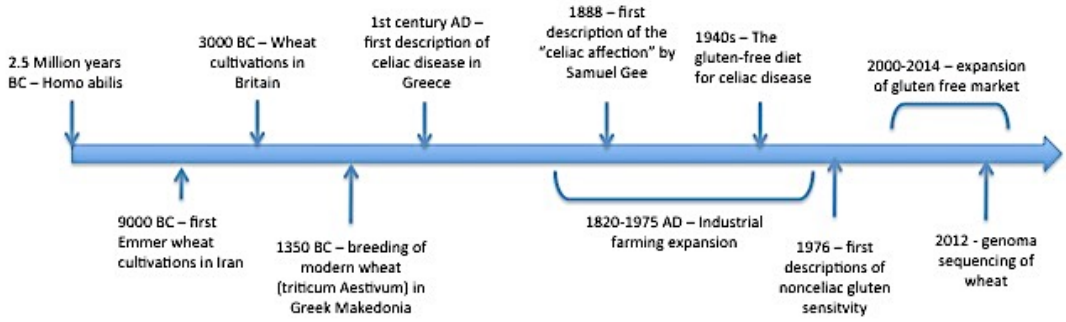
Pathogenesis

The pathogenesis of CD involves an altered T-cell mediated immune response triggered by dietary exposure to gluten.

The role of gluten. Gluten is a high molecular weight protein which can be found in the endosperm of grass-related grains, including wheat, barley and rye [10]. During the development and germination of plants, gluten storages in seeds ensure proper nourishing. Gluten is a composite of two classes of protein, a glutenin and a prolamin (gliadin in wheat, secalin in rye and hordein in barley). The latter can be further fractionated to produce alpha, beta and gamma peptides. Gluten-derived prolamins are responsible for the ability to process wheat to form dough by means of creating a viscoelastic network [11,12].

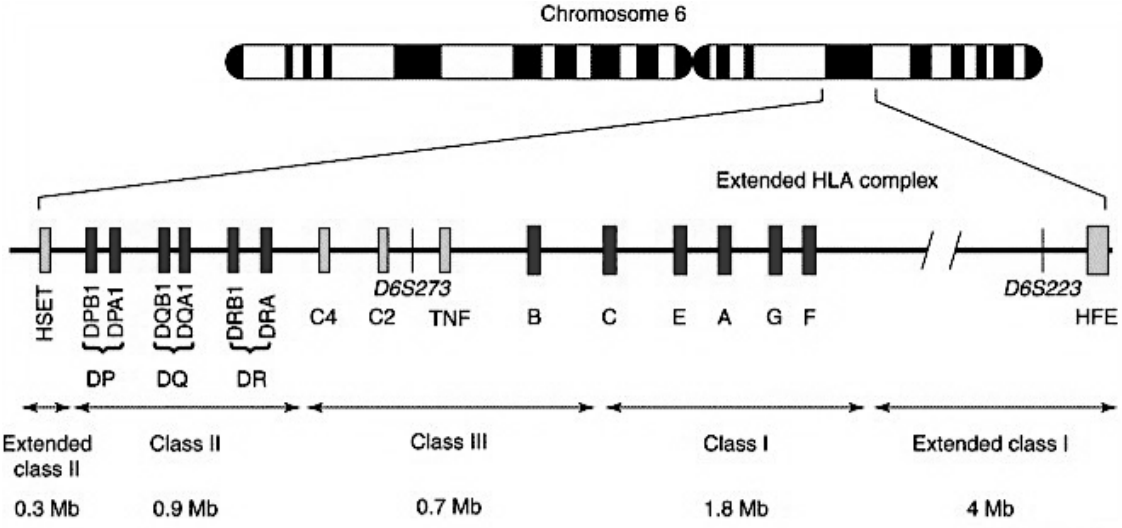
An interesting "evolutionary theory" about the pathogenesis of CD ventures that from the development of wheat cultivation to modern industrial production, the rate of increase in gluten exposure has been too high to give our immune system the time to develop optimal adaptive mechanisms [13]. In fact, cereal crops have been introduced as a component of the human diet around 10,000 years ago during the Neolithic Revolution in the Fertile Crescent in South West Asia (see Figure 1) [14,15]. Cereal harvesting and consumption has gradually increased since then, until its major outbreak in the twentieth century [16]. Over time, the need to provide an efficient agricultural production has led to the breeding and selection of wheat variants with better adaption to climate conditions, bread-making qualities and resistance to diseases [17]. As a consequence, the genetic variety and possibly immunogenic qualities of wheat have dramatically changed over time [17]. Furthermore, the awareness of the viscoelastic and stabilizing properties of gluten has led to its use in the baking industry as an additive [18].

Figure 1. Timeline of the history of wheat cultivation (an gluten consumption), from Aziz et al [14]



The role of genetics. As initially suggested by the presence of familiar clusters, genetic predisposition plays a role in the development of CD: in fact, the risk of developing CD in a first degree relative of a celiac patient is increased up to 10 times as compared to the general population [19,20]. A close association between CD and some genes codifying for class II molecules of the HLA system located in the short branch of chromosome 6 (6p21.31) has been identified (see Figure 2) [21,22]. The genes DQA1*05-DQB1*02 and DQA1*03-DQB1*0302 codify respectively for DQ2 and DQ8 molecules of the HLA system and can be found in more than 95% of celiac patients [21,22]. However, a predisposing HLA class II haplotype can be found in 20-30% of the general population, therefore this condition is not sufficient to develop the disease. Moreover, the concordance between monozygotic twins is around 75%, which confirms the relevant role of environmental factors in the pathogenesis of CD [20].

Figure 2. The HLA complex on chromosome 6 (from [23]).



The role of the epithelial barrier. In CD, gliadin peptides in the intestinal lumen cross the intestinal epithelial barrier and reach the extracellular matrix where they trigger an immune response [24]. Physiologically, the submucosal exposure to macromolecules is strictly regulated by the epithelial barrier by means of the tight junctions (TJ) between the enterocytes and by selected transcytosis [25]. In CD, an increased permeability due to epithelial barrier damage has been observed even at early stages of the disease and may enhance the exposure of immune cells in the extracellular matrix to gluten-derived peptides [26,27]. It is still unclear whether a defective epithelial barrier is a consequence of CD-related inflammation or if it actually contributes to the pathogenesis of CD [25,28]. In particular, defects in the TJ complex [29,30] and processes of altered transcytosis [31,32] have been described in this setting as potential ways through which gliadin peptides cross the epithelial barrier.

The role of the immune system. In order to trigger an immune response, gliadin peptides need to bind on the HLA molecules situated on the surface of antigen presenting cells and be presented to intestinal T lymphocytes. HLA DQ2/8 molecules preferably bind to negatively charged residues, but native gliadin peptides have very few negatively charged aminoacids and would therefore have low affinity for them [24]. However, gliadin can be

deamidated by tissue transglutaminase, an enzyme of the extracellular matrix: once its glutamine residues are converted into glutamate, gliadin shows an increased affinity for HLA molecules [33]. This process leads to a more effective antigen presentation and to T cell activation. The consequent Th1-dependent inflammatory reaction leads to cytokine production (IFN-gamma, IL21) and to various stages of epithelial and villous damage. At this stage, the increase in intestinal permeability causes further passage of gliadin peptides into the extracellular matrix and their exposure to tissue transglutaminase, thus perpetuating the immune reaction [24].

Clinical manifestations

The clinical manifestation of CD are heterogeneous, ranging from a clear malabsorption syndrome with diarrhea, weight loss and deficiency of liposoluble vitamins, which represents the classic manifestation of CD in the current nomenclature, to selective malabsorption of certain nutrients, leading to iron deficiency or osteopenia/osteoporosis [2]. In the past, most patients diagnosed with CD were children with severe organic manifestation, while in the last decades there has been an increase in diagnosis in adults with non-classic manifestations. Moreover, some patients are diagnosed asymptomatic (silent CD), often after screening in at-risk population [19]. A wide range of extra-intestinal manifestations, often but not necessarily consequent to malabsorption, have been described in association with CD [6,34,35], including:

– Disorders of hematopoiesis: iron deficiency anemia, megaloblastic anemia, vitamin K malabsorption; folic acid deficiency; hyposplenism and thrombocytosis
– Osteopenia and osteoporosis secondary to calcium and vitamin D deficiency
– Muscular hypotrophy, asthenia or tetany

– Neurological disorders: sensitive peripheral neuropathy; cerebellar and medullar ataxia; partial epileptic crisis with cerebral calcifications

– Disorders of the endocrine system: hyperparathyroidism (secondary to hypocalcemia); amenorrhea, infertility; repeated abortions

– Skin manifestations: glossitis, angular cheilitis, psoriasis

Association with other diseases

Down syndrome, inflammatory bowel diseases (including microscopic colitis), elevation in liver enzymes, congenital cardiopathy are among the wide range of conditions reported in association with CD. Most frequently, an association with other autoimmune disorders can be observed, such as connective tissue diseases (systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, myasthenia gravis, polymyositis) and skin diseases (psoriasis, vitiligo, and alopecia) [36]. Among patients with *type 1 diabetes mellitus* the prevalence of celiac disease is 4%, while the prevalence of type 1 diabetes among celiac patients ranges from 5.4 to 7.4% [1]. In patients with *autoimmune thyroiditis*, the prevalence of CD ranges from 3 to 8%, even though up to 43% patients may show activated T cells in the intestinal mucosa. On the other hand, the prevalence of autoimmune thyroiditis is estimated around 15% in celiac patients [36]. *Dermatitis herpetiformis (DH)* is considered the cutaneous equivalent of CD. Its clinical features include papulovesicular and itching lesions, mainly localized at the extensor surface of knees and elbows, buttocks and scalp. Among patients with DH, around 80% has villous atrophy. Skin lesions usually improve on a gluten-free diet (GFD) [2]. *Immunoglobulin A (IgA) deficiency* can be detected in around 2% of celiac patients, a prevalence 10 times higher than in the general population. This association is particularly

relevant because in case of IgA deficiency, the diagnosis of CD by can be more difficult (see below).

Diagnosis

The diagnosis of CD is based on a combination of clinical manifestations, serologic testing and gastroscopy with duodenal biopsies. Even in the absence of clinical symptoms, screening for CD has to be considered in first-degree relatives of celiac patients, patients with type I diabetes mellitus and patients with Down syndrome, given the high prevalence of CD in these groups [4,34].

Antibody testing

The optimal serologic test for the detection of CD in subjects older than 2 years is anti-transglutaminase IgA antibody (TTG), which shows a specificity and sensibility around 95% [37]. Anti-endomysium antibody (EMA) has a higher specificity (around 99%) and can be used as a confirmatory test in case of uncertain diagnosis in high-risk populations [38]. Deamidated gliadin peptide (DGP) IgA and IgG are used in combination with TTG IgA in children younger than 2 years of age [39].

Duodenal histology

Multiple biopsies of the duodenum are recommended as a critical component of the diagnostic evaluation in adult patients. The signs of gluten-related enteropathy at duodenal biopsies range from an increase in the intraepithelial lymphocytes to villous atrophy, as staged by Marsh-Oberhuber (details in Table 1) [40,41]. However, lymphocytic infiltration of the intestinal epithelium in the absence of villous atrophy is considered a non-specific finding, warranting further investigations [42].

Table 1: The Marsh-Oberhuber classification of CD related intestinal lesions (from [40,41])

Grade	Description
Type 0	IEL <25-40/100 enterocytes, normal villi and cryptae;
Type 1	IEL >40/100 enterocytes, normal villi and cryptae;
Type 2:	IEL >40/100 enterocytes, crypt hyperplasia, normal villi;
Type 3a:	IEL >40/100 enterocytes, crypt hyperplasia, slight villous atrophy;
Type 3b:	IEL >40/100 enterocytes, crypt hyperplasia, severe villous atrophy;
Type 3c:	IEL >40/100 enterocytes, crypt hyperplasia, absent villi

IEL, intra-epithelial lymphocytes

Genetic testing

In view of its high negative predictive value, genetic testing for HLA DQ2/DQ8 is suggested to rule out CD in selected clinical situations, as in case of "high risk" patients already on GFD (as mentioned above), or equivocal histologic findings in seronegative patients, or discrepancies between histology and serology [34]. Around 95% celiac patients carry the HLA-DQ2 heterodimer and the remaining 5% are mostly HLA DQ8 carriers, therefore a negative HLA genotyping can effectively exclude the presence of CD [20,43,44].

Differential Diagnosis

The diagnosis of CD is not always clear-cut: in particular cases, specific diagnostic strategies are needed to achieve a correct diagnosis. First of all, it is recommended to rule out IgA deficiency, that could lead to false negative results; in those cases, TTG IgG could prove a reliable test [45]. In case of evidence of villous atrophy with negative serology, other causes of villous atrophy such as common variable immunodeficiency,

autoimmune and chronic inflammatory disorders, drugs and neoplasia have to be excluded before hypothesizing a seronegative CD [46].

It is recommended to assess serology and duodenal histology while the patient is still on a gluten-containing diet [34]. Patients with suspected CD who are already on a GFD at the time of referral may not show histologic changes or antibody titers consistent with CD due to the improvement caused by the GFD itself [47]. In order to diagnose CD accurately, these individuals should be tested for the presence of HLA DQ2/DQ8 and, if positive, gluten should be reintroduced under medical supervision, in the so-called "gluten challenge", before planning serologic testing and duodenal biopsies [47]. It is still not clear which is the adequate daily intake of gluten and how long the gluten challenge should last in order to ensure a correct diagnosis. For long time, 10 grams of gluten per day for 6-8 weeks have been recommended [48]. However, recent data showed that the use of lower doses of gluten for shorter periods (3 grams per day for 2 weeks) are able to determine diagnostic changes in histology and/or serology in up to 90% subjects, with higher compliance and tolerability [47].

Treatment

To date, the only therapy for CD is a strict, long-life gluten free diet (GFD) [34]. Adherence to a restrictive GFD leads to a gradual healing of the mucosa of the small bowel (SB) and to the resolution of malabsorption symptoms, although there is a consistent proportion of patients who continue to show mucosal atrophy and/or persistence of symptoms even on GFD.[49,50]. A prolonged exposure to gluten over time, with subsequent immunological stimulation involving T-cell mediated reactions and cytokine production, is considered a focal player in the development of CD complications [51]. Therefore, the early adherence to a life-long, strict gluten free diet is viewed as crucial in the management of the disease for the prevention of its most

fearsome consequences. However, the evidence of the direct correlation between long-term gluten exposure and the development of associated autoimmune or neoplastic disorders is being questioned [52].

A future alternative treatment for CD?

There is strong interest among both patients and physicians regarding the possible future availability of a medical treatment for CD, which could represent an alternative to GFD [53]. In recent years, research has been actively focused on finding potential targets and developing drugs to treat CD. The improvement of gluten degradation, in order to reduce its immunotoxicity, could be achieved by means of oral administration of proteases [54-56]. Alternatives could be the development of non-immunogenic varieties of gluten [57] or of probiotics able to detoxify gluten [58]. Research has also been focused on the reduction of exposure to gluten derived toxic peptides: it is the case of zonulin-inhibitors, which have been specifically tested for their role in restoring intestinal permeability and reducing the paracellular transportation of toxic peptides in the subcellular matrix [27,59]. Oral-binding agents, designed to bind and neutralize gluten have also been tested [60]. Moreover, many potential options for the modulation of the immune response to gluten have been proposed, including a promising desensitization strategy based on the subcutaneous injection of increasing doses of toxic gluten-derived peptides [61].

Follow-up after CD diagnosis

Patients with CD should be monitored regularly to evaluate the presence of persistent or new symptoms, to verify a correct adherence to the GFD and to assess the risk of complications. Serological markers during follow-up may be used to monitor dietary adherence or to alert asymptomatic patients about the presence of contaminations in their diet [34]. In addition, general laboratory investigations are strongly recommended, including liver and thyroid function tests. Upper endoscopy with duodenal biopsy is only

indicated in case of lack of clinical response or relapse of symptoms despite treatment [62]. Furthermore, guidelines suggested to rule out osteoporosis with a DEXA scan. It should be performed at the diagnosis and every two years.

Complications of celiac disease

After CD diagnosis, adherence to a strict GFD leads to improvement of symptoms and of the signs of duodenal enteropathy in the wide majority of patients. However, in rare cases that account for up to 1% of all CD cases, the courses of the disease is complicated by a refractory celiac disease (RCD) or, even more rarely, by the onset of malignancy, including lymphoproliferative disorders and small bowel adenocarcinoma [63-65].

Refractory celiac disease

RCD is defined as the persistence of clinical and histological signs of enteropathy after at least 1 year of strict GFD [2]. It is a rare yet complex clinical entity, whose pathogenesis is still far from clearly understood.

Traditionally, two different variants of RCD have been described:

- Type I RCD (RCD I) shows a polyclonal pattern of intraepithelial lymphocytes (IEL) and a mild disease course, with most patients responding to cycles of corticosteroids (enteral delivering-budesonide or the proposed new strategy with open-capsule budesonide[66]) both in terms of symptoms and mucosal healing
- Type II RCD (RCD II) is characterized by a monoclonal transformation of IEL. Patients with RCD II have a poorer prognosis and a high risk of development of enteropathy-associated T-cell lymphoma (EATL) in the course of their disease. Moreover, up to 70% of RCD II patients may develop ulcerative lesions involving jejunum and ileum (ulcerative jejunoileitis) with clinical deterioration due to the

associated protein losing enteropathy [67]. In view of its poor prognosis, with 50% of cases developing enteropathy-associated T-cell lymphomas (EATL), RCD II is considered a pre-neoplastic condition [68,69] to the point that it has recently been proposed to consider it as a form of low-grade intraepithelial lymphoma (pre-EATL) [70].

Enteropathy-associated T-cell lymphoma

An association between CD and EATL has been demonstrated even in the absence of a known refractory CD [71]. In fact, cases of EATL can be diagnosed in undiagnosed CD patients, and it is believed that unrecognized CD cases with decade-long gluten exposure may be at risk. The pathogenesis of EATL is still far from clearly understood: an increased cellular turnover due to crypt hyperplasia or an alteration of mucosa-associated lymphoid tissue have both been proposed as possible triggers. Unfortunately, EATL is often diagnosed at a late stage, commonly during surgery for intestinal perforation, bleeding or occlusion. Consequently, the survival rate is very poor (11% at 5 years) [64].
RCD II, ulcerative jejunoilieitis and EATL are currently considered as part of the same clinical entity characterized by the presence of aberrant clones among intraepithelial lymphocytes (IEL). Diagnostic delay is the main issue affecting the prognosis of complicated CD and limiting the access to a curative therapy [72].

Management of RCD II/EATL

The introduction of high-dose chemotherapy, autologous stem cell transplantation and biologic drugs targeting the IL-15 pathway has represented a significant development in RCD II and EATL treatment [73]. To date, patients with a RCD II diagnosis without evidence or overt EATL are eligible for therapy with cladribin, a cytotoxic

chemotherapeutic agent. A recently published paper has shown satisfactory results in terms of symptoms and mucosal response with open-capsule budesonide in both RCD I and RCD II patients, though these data need further validation in the setting of RCD II [66]. In case of non-response or of EATL development, aggressive chemotherapeutic strategies (to which EATL has been shown not to respond optimally) or autologous/allogenic bone marrow may induce prolonged remission [74,75]. Early therapeutic intervention in this subgroup of RCD has been shown to significantly reduce morbidity and mortality[74]. However, poor prognosis and diagnostic difficulties make CD complications a challenging issue [76].

As a way of optimizing the early diagnosis of CD complications, several diagnostic strategies have been investigated in recent years. Capsule endoscopy and device-assisted endoscopy, have been proposed as a useful tool for the investigation of persistent or novel symptoms despite an ongoing GFD [77]. As opposed to cross-sectional imaging techniques, endoscopic techniques are able to directly visualize the mucosa of the small bowel and should be able to confirm or exclude the presence of early alterations. In RCD II patients, a more extensive involvement of the small bowel can usually be detected as compared to RCD I [77]. Moreover, RCD II patients often present small bowel erosive/ulcerative lesions, while EATL endoscopic features include nodules, plaques and ulcers [78,79]. A recent study suggested the sequential application of capsule endoscopy and device-assisted endoscopy as an accurate tool for the early diagnosis/exclusion of CD complications [80].

The role of flow cytometry in complicated celiac disease

Improving the management of refractory celiac disease

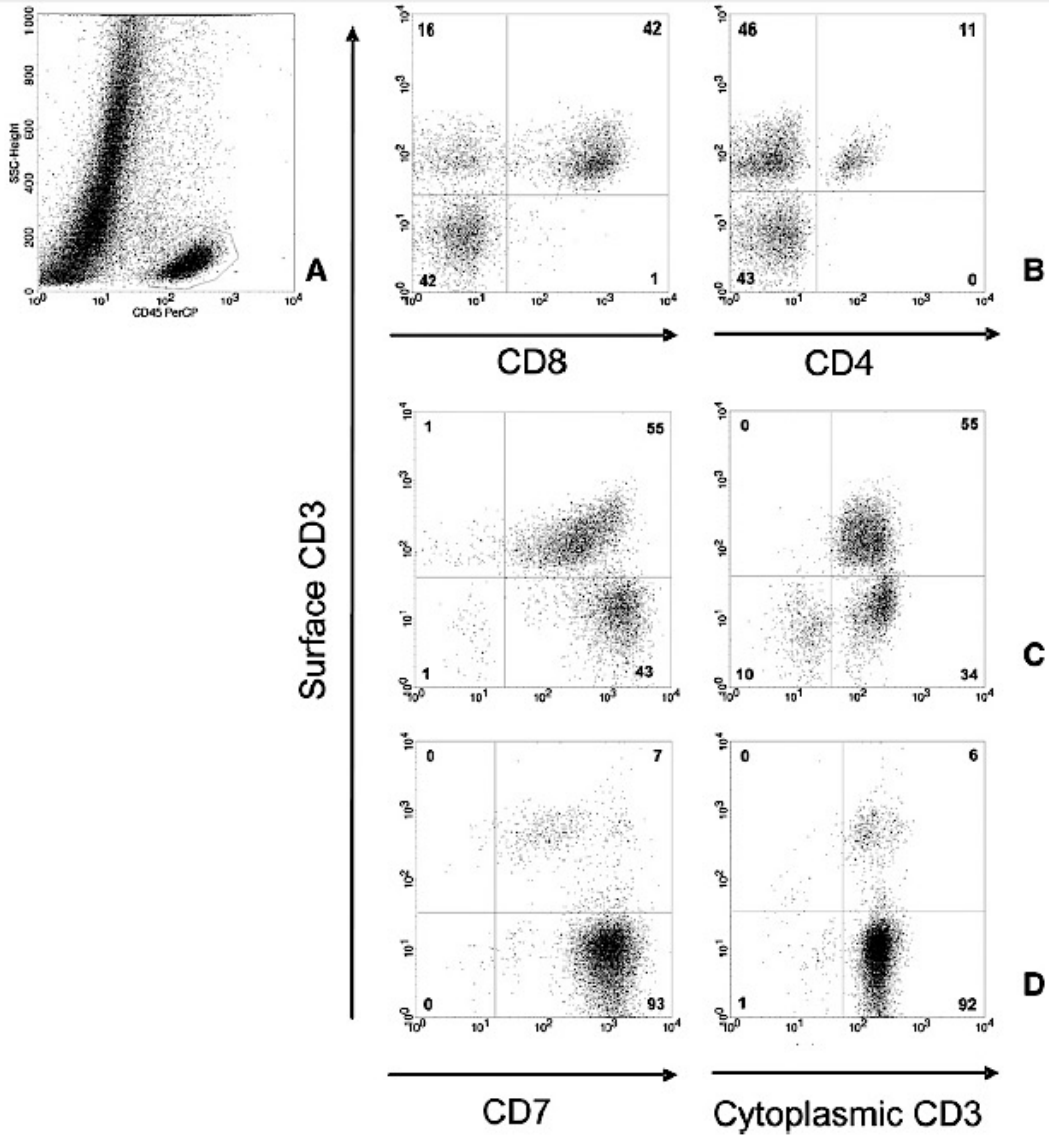
Considering the implications involved with a diagnosis of RCD II and the possible advantages of a prompt treatment start, it is crucial to recognize this entity of RCD reliably and in due time. The usual diagnostic tool for the identification of RCD II among RCD include CD8-immunohistochemistry - as a first step - and molecular histopathology analysis of duodenal biopsies. Molecular histopathology is a PCR-based investigation focused on the identification of a monoclonal pattern of the T-cell receptor (TCR) γ- and β- chains. This type of PCR-based investigation of the TCR has its limitations, however, as oligoclonal patterns in the amplified DNA are difficult to interpret. Furthermore, Liu et al. were able to show that an immunohistochemical CD8-antigen loss in combination with TCR monoclonality can also be transiently observed in active, non-refractory CD [81]. In this study, an acceptable specificity of clonality assessment was only reached when diagnostics were repeated several months after the initial endoscopy (including a repeat endoscopy, immunohistochemistry and molecular pathology) [81]. Patients with RCD, however, often need to immediately start therapy, therefore calling for an alternative or complementary method of diagnosis.

Evaluation of aberrant intestinal lymphocytes

In recent years, the group of Mulder in the Netherlands introduced an innovative technique that made it possible to identify intestinal lymphocytes (ILs) with an aberrant phenotype from single-cell isolates of duodenal mucosa [82,83]. Multiparameter flow cytometry is an accurate strategy for the analysis of cellular populations, which, among others, allows for discrimination between surface and cytoplasmic antigen expression (as opposite to immunohistochemistry)[82].

Aberrant ILs in RCD have been described as characterized by altered expression of common T-cell markers and by different expression of TCR rearrangements [84,85]. They originate by clonal expansion of deranged immature T-lymphocytes and show differentiation to a cytotoxic genotype [85]. In the study by Verbeek et al, the quantification of aberrant ILs was achieved by means of tracing different patterns in the expression of surface proteins in T- lymphocytes (CD, cluster of differentiation) and detecting the loss of surface CD3 in aberrant cells expressing cytoplasmic CD3 (see Figure 3) [82]. This finding is due to the aberrant cells' faulty assembly of the CD3/TCR complex resulting in a failed membrane shuttling of CD3 [86]. Another possible strategy to identify aberrant ILs consisted in testing for CD7 expression in the absence of surface CD3 expression, within lymphocytes expressing CD103 (a marker specific for intraepithelial localization).

Figure 3. Example of flow cytometric analysis of intestinal duodenal lymphocytes with illustration of the gating process (from Verbeek et al. [82]). In this study, lymphocytes were selected on the basis of CD45+ and low sideward scatter (Panel A). The determination of aberrant ILs was made through the gating of surfCD3-cytCD3+ within CD45+CD103+ lymphocytes (as shown in Panel C and panel D, the latter showing the results of flow cytometry of a RCD II patient)



The strategy for detection of aberrant ILs was validated in a cohort of patients with uncomplicated CD and RCD, and a 20% cut-off was proposed as a new parameter to discriminate RCD I from RCD II patients. Elevated aberrant IELs proved a better predictor of EATL development than assessment of TCR clonality [82] Another study from the same group confirmed a negative correlation between TCRγδ+ lymphocytes and aberrant IELs as well as EATL development, suggesting a possible protective role of these cells in the setting of RCD [83]. Further on, the utility of flow cytometric analysis of aberrant ILs was confirmed by other studies from the same group, in particular data from flow cytometry analysis of lamina propria lymphocytes suggested that aberrant ILs

in CD may not be confined in the intraepithelial compartment, thus questioning the need for CD103+ as a marker during the gating process [87].

Flow cytometry analysis for the detection of aberrant ILs has proven an accurate strategy for the stratification of RCD patients. Follow up data suggested a possible role of aberrant ILs assessment also in the setting of treatment monitoring, i.e. during cladribin treatment for RCD II [88]. However, as opposed to immunohistochemistry and TCR rearrangement studies, it is not a widely available technique and its application in routine clinical practice still needs further validation.

Aim of the Study

Among patients with RCD, those classified as RCD II show an elevated risk for developing EATL and a worse prognosis. Traditionally, RCD II patients have been identified based on TCR clonality. However, is has been proposed that determining the presence of a fraction of intestinal lymphocytes, denoted as "aberrant intestinal lymphocytes (ILs)" by flow cytometry is a better determinant for the identification of high-risk RCD. With this premise, the aims of this study were

1. To evaluate the presence of aberrant ILs in a cohort of non-celiac, celiac and RCD patients by means of flow cytometry of intestinal lymphocytes isolated from duodenal biopsies.
2. To evaluate whether there is an association between clinical characteristics of RCD patients and the presence of aberrant ILs.
3. To compare different IL detection strategies (based on various flow cytometric gating strategies) including the validation of a strategy that does not require cell permeabilisation prior to immunostaining. Such a technique would have the potential to use the identified/sorted cells in further experiments.
4. To evaluate whether flow cytometric analysis of aberrant ILs can accurately identify high-risk RCD patients (RCD II/pre-EATL) in our cohort.

Methods

Patients

The study was conducted at the Division of Gastroenterology, Infectious Diseases and Rheumatology of Campus Benjamin Franklin - Charité Universitätsmedizin, Berlin under the supervision of Dr. Michael Schumann.

Patients with and without CD, referred for gastroscopy as part of their clinical management, were consecutively enrolled in the study. The inclusion criteria were as follows and allowed to divide patients into four groups

1. Controls, in whom a gastroscopy was performed due to unspecific gastrointestinal symptoms, without evidence of pathologic findings both macroscopically and at histology;
2. Patients with an established diagnosis of active CD in line with international guidelines [34], with evidence of TTG IgA positivity, Marsh III duodenal mucosal alterations and an ongoing gluten containing diet;
3. Patients with an established CD diagnosis and adhering to a GFD for longer than a year, without evidence of villous atrophy at duodenal histology, thus considered GFD responders;
4. Patients with RCD, fulfilling the aforementioned criteria for the diagnosis of refractory disease, i.e. established CD diagnosis, persistence of Marsh duodenal lesions after >1 year GFD and after professional revision of dietary adherence and exclusion of differential diagnoses.

Exclusion of criteria were: age <18 years, active gastrointestinal bleeding at the time of endoscopy and - in case of controls - evidence of pathologic findings during endoscopy or at histology.

Flow cytometric analysis of duodenal biopsy specimens

Acquisition of specimens and ILs isolation

During the endoscopic examination, in addition to the formalin-fixed duodenal biopsies intended for histopathology investigation, another 4-8 biopsy specimens were obtained by forceps and placed in 10 ml Nalco 0.9% for ILs-isolation. The maximum delay between specimen acquisition and the beginning of ILs isolation was ca. 30 minutes. The buffer medium used for isolation was Phosphate-Buffered Saline (PBS; without Ca^{++} and Mg^{++}), to which 1 mM DTT and 1 mM EDTA were added. Biopsies were panned in 5 ml of this solution for 60 minutes at 37°C. Specimens were then transferred to 1.5 ml Eppendorf tubes and centrifuged (500 *g*, 5 minutes, ambient air temperature). The pellets containing the cells were resuspended in PBA (PBS containing 0,5% (w/v) bovine serum albumin and 0,02% (v/v) NaN_3 (both Sigma-Aldrich, Germany)) and further centrifuged (as above). This procedure was repeated twice. The cells were subsequently fixed in paraformaldehyde 4% (30 minutes at ambient air temperature), followed by another centrifugation and another PBA washing. Specimens were stored in PBA at 4°C until immunostaining. The protocol for ILs-isolation is simple and easy enough to be completed within 3 hours parallel to other laboratory work.

Immunostaining

Immunostaining of specimens was performed within a median of 3 days after PFA fixation of ILs isolates (range 0-24 days). Cells were stained in 50 µl of PBA, with the

addition of 2% Beriglobin and antibodies for 15 min at room temperature followed by washing cycles in PBA. Subsequently, after staining for extracellular markers, intracellular staining (CytCD3) was performed in the presence of 0.5% Saponin for permeabilisation (Sigma-Aldrich).

Florescein-isthiocyanate (FITC), allophycocyanin (APC), peridinin chlorophyll protein (PerCP), phycoerythirn (PE), Pacific Blue, and Amcyan conjugated monoclonal antibodies directed to CD3, CD4, CD8, TCR-γδ, CD7, CD103, CD11b, CD19, CD14, CD20, CD56, CD103 were used. Characteristics of the antibodies used for immunostaining are summarized in Table 2

Table 2 List of flourochrome-labeled antibodies used for immunostaining

mAb	Company	Dilution	Marker for cell type
CD3 Pacific Blue (UCHT1)	BD	1:20	T-lymphocyte
CD3 APC-H7 (SK7)		1:20	T-lymphocyte
CD4 Amcyan (SK3)		1:20	T- lymphocyte
CD8 PerCP (SK1)		1:10	T- lymphocyte
γδTCR PE (B1)		1:10	T- lymphocyte
CD7 APC (M-T701)		1:50	T/NK
CD11b FITC (D12)		1:20	Dendritic cell
CD19 FITC (HIB19)		1:20	B- lymphocyte
CD20 FITC (2H7)		1:20	B- lymphocyte
CD14 FITC (3G8)		1:20	Monocyte
CD56 FITC (ME188)	eBioscience	1:20	NK
CD103 PE-C7 (B-Ly7)		1:10	Intraepithelial T/NK

Flow cytometric analysis

Data were acquired on a FACSCalibur with the CellQuest software or a FACSCanto II with the FACSDiva software (all BD Biosciences) and were analyzed using the FlowJo software (Tree Star, Ashland, Oregon, USA). The minimum cell count analyzed via FACS was 10^4 lymphocytes defined by their granulation (using *Side Scatter*) and particle size (using *Forward Light Scatter*). The following gating strategies were used (see Table 3):

- For seven-color analysis lineage-negative cells were defined by exclusion of γδTCR+ cells and CD11b/CD14/CD19/CD20/CD56+ cells (Multitest). CD4 and CD8 expression was defined on lin- γδTCR- cells. Aberrant lymphocytes were determined either by gating on cytoplasmic (cyt) CD3+ surface (surf) CD3- lineage-negative cells followed by the analysis of CD7 and CD103 expression, or by gating on CD7+ lineage-negative cells followed by the analysis of cytCD3+surfCD3-. An alternative gating was performed on CD7+ surfCD3- lineage-negative (lin-) cells.
- For four-color analysis, CD4 and CD8 expression was analyzed in lineage-negative cells as defined by exclusion of CD11b/CD14/CD19/CD20/CD56+ cells. Analysis of CD7, surfCD3 and cytCD3 expression was performed after exclusion of γδTCR+ and CD56+ positive cells. Aberrant ILs were analyzed by means of staining CD7, CD103, surfCD3 and cytCD3 staining and defined after subsequent gating strategies.

Table 3 **Gating strategies used for the detection of aberrant ILs at flow cytometry**

Strategy	Definition of Aberrant ILs	
1	%cytCD3+surfCD3-within CD7+	
2	%CD7+ CD103+ within surfCD3-	
3a	%cytCD3+ surfCD3-within CD7+CD103+	
3b	%CD7+CD103+within cytCD3+surfCD3-	
4a	%CD4-CD8-within CD7+CD103+	*
4b	%CD7+CD103+ within CD4-CD8-	*
4c	%CD4-CD103+ within CD7+CD8-	*
5	%cytCD3+surfCD3- within CD4-CD8- within CD7+	*
6	%cytCD3+surfCD3- within CD7+CD103+within CD4-CD8-	*
7	%cytCD3+surfCD3- within CD4-CD103+within CD7+CD8-	*

All strategies are performed after gating for lin- cells. Lin- are also TCRγδ- with seven-color analysis

* tested with seven-color analysis only

Histopathology of duodenal biopsies

Conventional histology was performed on duodenal biopsies in order to ensure a correct management of patients. For this purpose, 4 to 6 formalin-fixed duodenal specimens were embedded in paraffin using conventional techniques, microtome-cut and stained using hematoxyline and eosine (H&E). In the pathology report, the enteropathy, if present, was graded according to the Marsh-Oberhuber-Classification [40,41]. The count of IEL defined as nr of IEL/100 enterocytes was reported, along with results of additional staining for CD8 and TCR in case of evidence of persistent villous atrophy. The presence of mucosal inflammatory/neoplastic infiltrate was reported and described.

T-cell clonality evaluation

As recommended by guidelines, patients with RCD were evaluated with molecular pathology in order to identify the presence of T-cell clonality. DNA was extracted from paraffin-embedded duodenal specimens and amplified by Multiplex PCR using Primers binding all TCR-γ V- and J- regions, as well as TCR-ß (based on previous data showing improvement in the diagnostic yield by means of TCR-ß clonality analysis along with TCR-γ analysis [89]). Analysis was performed using the GeneScan technology [90].

Ethics

The study was performed in accordance with the Helsinki Declaration. The examination with gastroscopy was performed only in participants who had given their informed consent and referred to gastroscopy for an appropriate clinical indication. The study was approved by the local Ethics Committee of Charité - Berlin (nr. EA4/016/14).

Statistical analysis

Descriptive statistics (medians and interquartile ranges as well as 10^{th}, 90^{th} and 95^{th} percentile) were calculated, Chi-Square or Fisher's exact test were used to compare proportions. All data sets were sampled for normality with the D'Agostino-Pearson omnibus normality test. Kruskall-Wallis test and Mann-Whitney's test were used to compare continuous variables unless specified otherwise. Correlation between continuous variables was evaluated by computing Spearman's r. Graphpad Prism software (version 6.0, GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis.

Results

Characteristics of patients

A total of 130 flow cytometry assays were performed on 109 patients, including 42 controls, 21 active CD, 16 CD responding to GFD and 30 RCD. In 13 cases of the latter group, more than one flow cytometry was performed over time. The demographic and clinical characteristics of the patients are summarized in Table 4.

Table 4. Characteristics of the subjects enrolled in the study

	Controls (N=42)	Active CD (N=21)	CD on GFD (N=16)	RCD (N=30)	p
Sex (F/M)	28/14	14/7	14/2	21/9	ns
Age at CD diagnosis	-	36 (8-80)	44 (26-70)	49 (2-76)	0.01*
Age at enrolment	43 (19-85)	41 (21-81)	53 (36-82)	64 (36-80)	<0.001^
Autoimmune comorbidities					
- Hashimoto	2 (5)	2 (10)	1 (6)	4 (13)	
- DM type I	0 (0)	0 (0)	1 (6)	0 (0)	
- Micr. colitis	0 (0)	2 (10)	1 (6)	2 (7)	
- other	0 (0)	2 (10)	3 (19)	4 (13)	
Neoplastic comorbidities					
- History of:					
Colon Ca	1 (2)	1 (5)	0 (0)	0 (0)	
- Breast Ca	0 (0)	1 (5)	0 (0)	0 (0)	
- SB Ca	0 (0)	0 (0)	0 (0)	2 (7)	
- other	2 (2)	0 (0)	1 (6)	1 (3)	
Symptoms/signs at enrolment					
- Anemia	3 (7)	9 (43)	0 (0)	10 (33)	
- Chr.diarrhea	13 (31)	13 (62)	4 (25)	14 (47)	
- Abd. pain	30 (71)	10 (48)	8 (50)	10 (33)	
- Weight loss	8 (19)	8 (38)	2 (13)	12 (40)	

CD, celiac disease; GFD, gluten free diet; RCD, refractory celiac disease; DM, diabetes mellitus; Ca, cancer
Data are expressed as median (range) or N (%)
*RCD vs. active CD, ^RCD vs. other groups

As expected, the median age at which the diagnosis of CD was made was significantly higher in RCD patients as compared to patients with active CD (p 0.01). The age at enrolment was also higher in RCD patients as compared to all other groups (p<0.0001), confirming that RCD represent a subgroup of CD patients characterized by comparatively older age and, as expected in this category of patients, longer periods of undiagnosed CD (i.e. prolonged gluten-containing diet).

In line with current routine clinical practice, all patients with a diagnosis of RCD were evaluated with molecular pathology and divided into two groups according to the absence or presence of a clonal population as assessed by GeneScan. Table 5 summarizes the characteristics of patients that belong to these two groups: However, as there have been various strategies to establish the diagnosis RCD type I and RCD type II published (TCR-GeneScan, CD8 staining in immunohistochemistry, flow cytometry of ILs), we renamed the groups to RCD without TCR clonality (RCDclon-) and RCD with TCR clonality (RCDclon+). This renaming contributed to clarity by denominating the crucial diagnostic parameter that caused assignment of the individual patient to the appropriate group. It can be observed that, within RCD patients, RCDclon+ patients are significantly older, including patients with a longer history of undiagnosed/untreated CD, while patients in the RCDclon- group show a similar age distribution compared to patients with active CD (median age at diagnosis 41 years, range 16-69 versus 36, range 8-80, p=0.1) and CD responsive to GFD (median age at diagnosis 44 years, range 26-70, p=0.9). With concern to clinical characteristics, a significant difference was observed in the type of presentation, with a higher proportion of RCDclon+ patients presenting with anemia (8/17 versus 1/13, p=0.04) and with alterations in several vitamins and micronutrients, including iron (iron deficiency defined as ferritin <10 ng/ml and/or transferrin saturation <16%), folate (<4.6 ng/ml), vitamin B12 (<200 pg/ml or holo-

transcobalamin <25 pmol/L) or zinc (<9 μmol/l), as well as with low serum albumin (<3.5 g/dl), low electrolyte levels (Ca, K, Na) and impaired coagulation (INR> 1.3).

Table 5. Characteristics of RCD patients stratified according to TCR clonality status

	RCDclon- (n=13)	RCDclon+ (n=17)	p
Sex (F/M)	9/4	12/5	ns
Age at CD diagnosis	41 (16-69)	54 (2-76)	0.04
Age at RCD diagnosis	54 (20-76)	66 (35-81)	0.01
Age at enrolment	62 (20-76)	73 (35-82)	0.01
Autoimmune comorbidities			
Hashimoto	3 (23)	1 (6)	ns
DM type I	0 (0)	0 (0)	ns
Microscopic colitis	2 (15)	0 (0)	ns
RA	2 (15)	0 (0)	ns
Sjögren	1 (8)	0 (0)	ns
Polyarthritis	1 (8)	1 (6)	ns
Symptoms/signs at presentation			
Anemia	1 (8)	8 (62)	0.04
Chr. Diarrhea	7 (54)	8 (62)	ns
Abdominal pain	3 (23)	7 (54)	ns
Weight loss	4 (31)	8 (62)	ns
Fever/Night sweats	0 (0)	4 (31)	ns
Malabsorption parameters			
Low levels of			
Iron	2/13	5/17	ns
Folate	1/10	4/15	ns
B12	1/13	2/15	ns
Zink	2/12	5/15	ns
Electrolytes	0/13	6/17	0.02
Albumin	0/13	6/17	0.02
High INR	0/13	6/17	0.02
Osteoporosis	0/10	8/17	0.01
Duodenal Histology^			
Marsh I	3	1	
Marsh II	4	1	
Marsh IIIa	2	5	ns
Marsh IIIb	3	3	
Marsh IIIc	1	7	

DM, diabetes mellitus, CD, celiac disease, RCD, refractory celiac disease,
Data are expressed as median (range) or N (%) unless specified otherwise. *low iron status defined as low ferritin or transferrin saturation; ^first histology since enrolment

With regard to the results of duodenal histology, most RCDclon+ patients presented with mild to severe villous atrophy (both patients without villous atrophy were on budesonide treatment at the time of the endoscopy), while a consistent proportion of patients in the RCDclon- group showed only increased IEL with or without crypt hyperplasia (7 patients, 4 of whom were on an ongoing treatment with budesonide, and one on azathioprine).

Flow cytometry results in celiac patients and controls

Assessment of aberrant ILs

The results of flow cytometry analysis are shown in Table 6. In line with previous work by Verbeek et al. [82], we initially defined aberrant ILs as CD3in+surfCD3-CD7+ among lin-TCRγδ- lymphocytes and expressed the results as % of total lymphocytes (Strategy 1). This strategy, however, slightly differs from the one initially proposed by Verbeek, since the initial gating for lin-TCRγδ- was performed before evaluating intracellular CD3 (CD3in), surface-bound CD3 (surfCD3) and CD7. Moreover, the initial selection for lymphocytes was not performed on the basis of CD45+ and low SSC, but in our case on low SSC and FSC (see Methods section). A second strategy for the identification of aberrant ILs proposed in previous publications [82], consisting in gating cells for surfCD3-CD7+CD103+ within lymphocytes (Strategy 2), was also evaluated. Moreover, alternative strategies have been tested in our cohort (see below).

The results of flow cytometry analysis with both strategies showed no significant differences between RCDclon- patients and uncomplicated CD. In fact, RCDclon- patients had low %aberrant ILs and moderately elevated %TCRγδ+, at a level comparable to other CD groups. No statistically significant difference was observed

between RCDclon+ and RCDclon- patients regarding aberrant ILs. However, the scatter in the RCDclon+ group was striking (Figures 4 and 5: RCD clon+ display a median of 2% and 1% of aberrant ILs according to the two different strategies, but a 90th percentile of 36 and 41 respectively!). This suggested that RCDclon+ was not a homogeneous group and that a subpopulation of patients with high aberrant IELs can be identified within the RCDclon+ group. Furthermore, these data confirm that the presence of TCR clonality in RCD patients does not predict malignant transformation as a single determinant of risk. Interestingly, the %aberrant ILs in control patients showed a wider variability than in active CD patients ($p<0.0001$) and RCDclon- ($p=0.009$). This observation may reflect the presence of an active T cell mediated response in active CD and RCDclon- (absent in controls and in GFD responders), with a subsequent "polarization" of T-cell profiles.

Table 6. Flow cytometry results

	Controls (n=42)	Active CD (n=21)	CD on GFD (n=16)	RCD clon- (n=13)	RCDclon+ (n=17)
N of assays	42	22	16	18	31
%Lymphocytes	13 (6-21)	24 (11-35)	15 (7-28)	16 (7-22)	20 (7-33)
%CD4+	14 (4-38)	9 (2-21)	11 (5-30)	10 (2-27)	8 (2-30)
%CD8+	33 (4-75)	44*(14-73)	40 (8-76)	37 (7-76)	31*(5-61)
%TCRγδ+	3 (0-8)	15 (2-29)	12 (3-35)	11 (2-36)	7 (1-47)
Aberrant ILs					
%CytCD3+surfCD3- CD7 within lin-TCRγδ-	3 (1-8)	1 (0-3)	1 (0-12)	1 (0-6)	2 (0-36)
CD7+CD103+ within surfCD3-	4 (0-11)	1 (0-2)	1 (0-12)	1 (0-5)	1 (0-41)

*p=0.048
Data are expressed as median, 10th and 90th percentile. ILs, intestinal lymphocytes.

Figure 4. %Aberrant ILs in controls and in CD groups as assessed by gating %CD3in+surfCD3-CD7 within lin-TCRγδ- lymphocytes (Strategy 1). The presence of subjects with high percentages of aberrant ILs in the RCDclon+, as opposed to RCDclon-, is not sufficient to determine statistical significance between the two groups (p=0.09). As pointed out in the figure, aberrant IL counts in the control group were more scattered (controls versus active CD p<0.0001, versus RCD clon- p=0.009). Also, patients with CD on GFD show a similar pattern as compared to active CD.

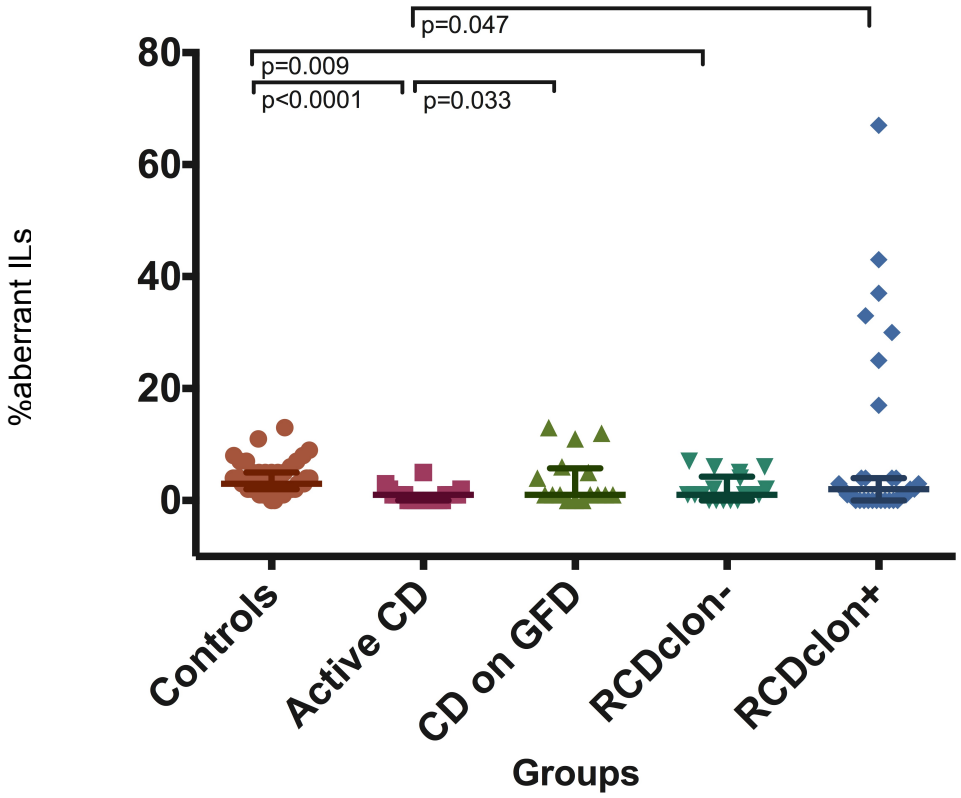
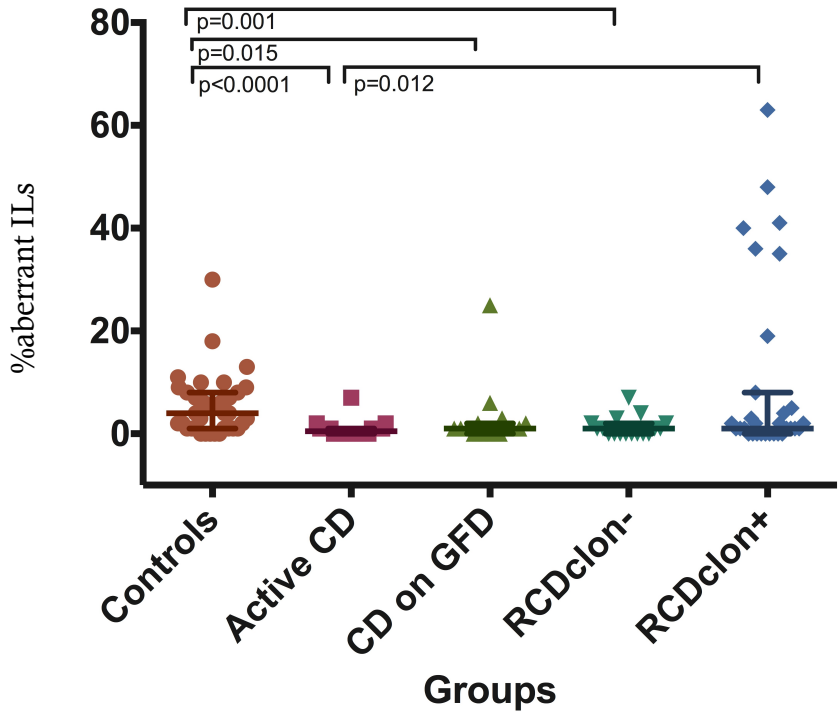


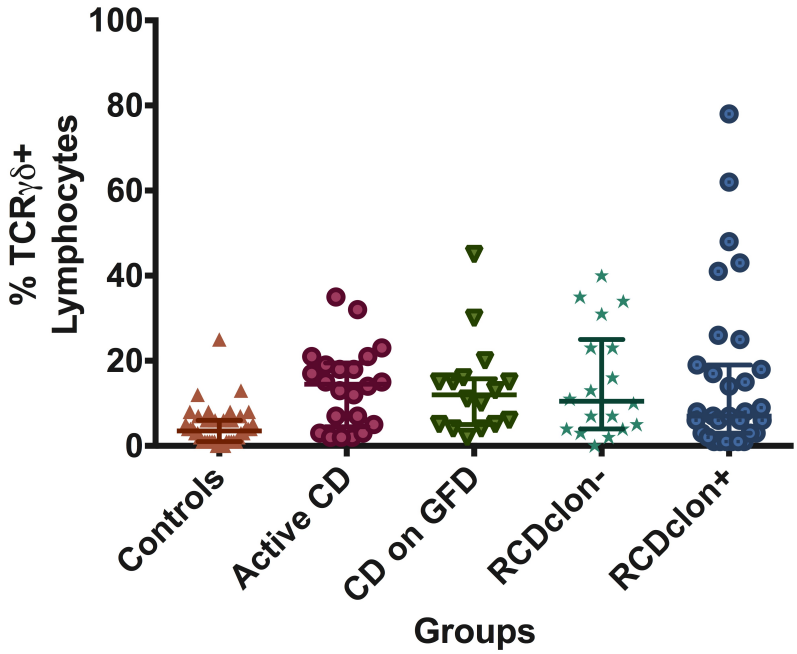
Figure 5. %Aberrant ILs in controls and in CD groups as assessed by gating CD7+CD103+within surfCD3- lymphocytes (Strategy 2). Data obtained from this strategy are similar to those shown in Figure 1. Also in this case, a high percentage of aberrant ILs is almost only observed in the RCDclon+ group, confirming previous observations that TCR clonality is not a sufficient parameter to identify high-risk RCD patients.



Assessment of TCRγδ+ lymphocytes

A higher percentage of TCRγδ+ at flow cytometry analysis has been associated with better prognosis in RCD [83], therefore we assessed TCRγδ+ and compared results in our groups. In this cohort, no significant differences were observed in TCRγδ+ between uncomplicated CD and RCD. However, an inhomogeneous distribution of results in the RCDclon+ groups suggests that a subgroup of RCDclon+ patients may reveal lower TCRγδ+ percentages, in line with previous observations showing a negative relation between TCRγδ+ and aberrant ILs (Figure 6).

Figure 6. Percentage of TCRγδ+ lymphocytes in controls and in different CD groups. All p-values between groups are nonsignificant. However, the RCDclon+ group shows a wider variability of %TCRγδ+.



Association between clinical characteristics of RCD patients and aberrant ILs

The initial results of the flow cytometric analysis in our patients showed (i) that high percentages of aberrant IELs are almost exclusively observed in RCD patients, specifically, in the RCDclon+ subgroup and (ii) that high aberrant IELs are only found in a subgroup of RCDclon+. In view of the results of key previous studies suggesting the close association between aberrant IELs and EATL development [82,87], the presence of elevated aberrant IELs may in fact be proposed as a prognostic determinant for the further course of RCD. With this in mind, we subsequently aimed at evaluating if the presence of aberrant IELs in a subgroup of RCDclon+ patients was associated with

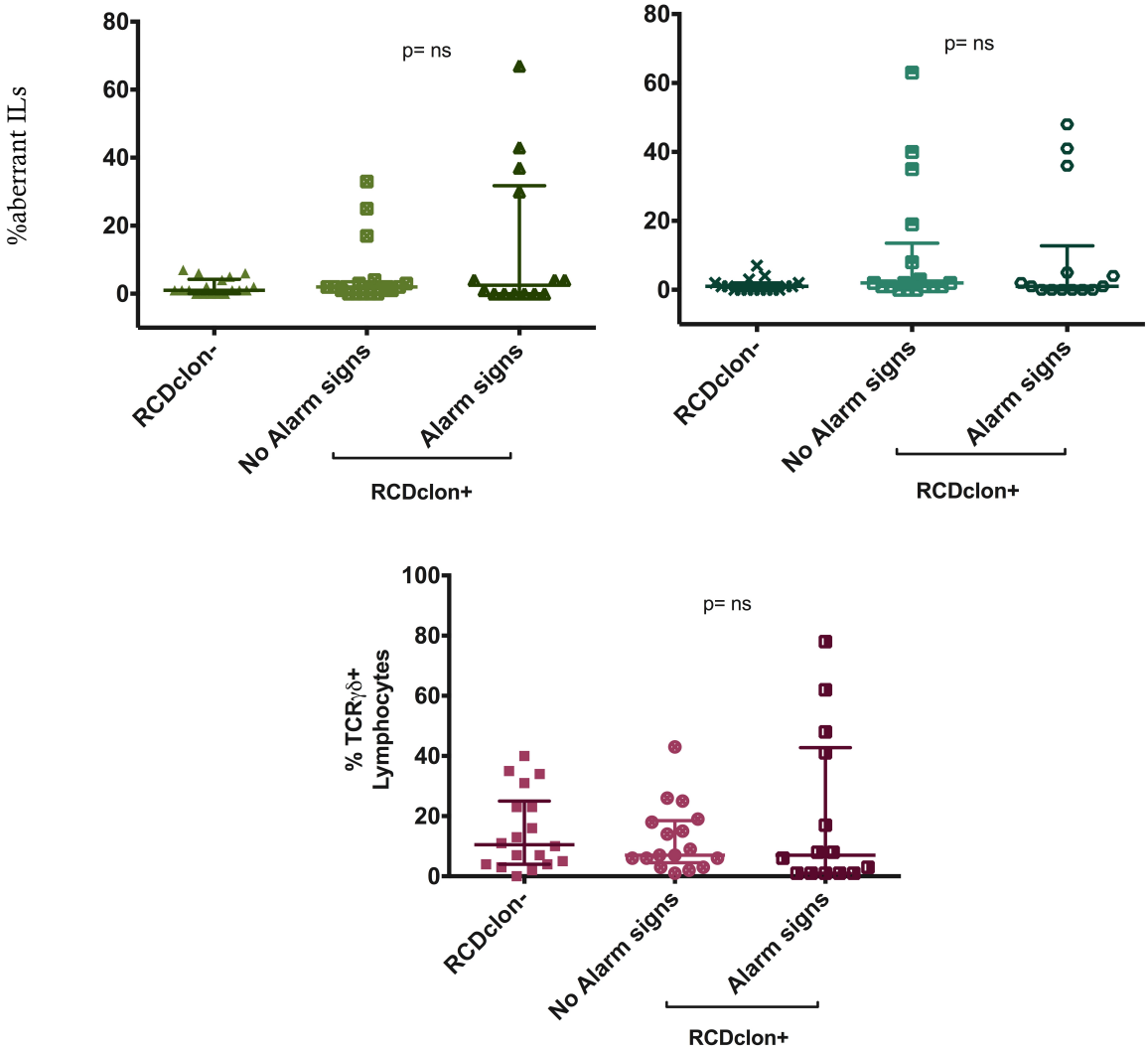
specific clinical characteristics, in order to identify a possible "clinical profile" of high-risk RCD patients. Particularly, in all RCD patients signs and symptoms at presentation (i.e. at the time of OGD) were assessed and data on "alarm signs" were collected. Moreover, available data on absorption parameters were collected (see Table 5 above)

Alarm signs and aberrant ILs

Anemia, chronic diarrhea, significant weight loss and fever/night sweats were defined as "alarm signs", considering the fact that they prompt urgent clinic evaluation when observed in a patient affected by CD on a GFD or in a "stable" RCD situation. When evaluated in RCDclon- versus RCDclon+, though, only anemia was significantly more frequent in RCDclon+ as compared to the other group (p=0.04). We hypothesized that the presence of more than one alarm sign at the same time could be considered a risk determinant in RCDclon+; therefore we grouped all four items in a simple score ranging from 0 (i.e. no alarm signs) to 4 (i.e. all four alarm signs present). Of note, abdominal pain was initially evaluated as an alarm sign, was then excluded because (i) initially reported abdominal pain was often described by patients as only abdominal discomfort (no specific questionnaire for the assessment of gastrointestinal symptoms was administered during this study) and (ii) it was reported by a high proportion of RCD on GFD and controls. Thus, it was considered to be too unspecific.

As shown in Figure 7, the stratification of patients according to the presence or absence of alarm signs, even when they had been grouped as an alarm score, was not able to discriminate between patients with and without elevated aberrant ILs. Median percentages of aberrant ILs were 2% (10th-90th percentile: 0-27) in patients with negative alarm score versus 3% (0-55) in patients with positive alarm score with the %CD3in+surfCD3-CD7 gating strategy (p=ns) and 2% (0-45) versus 1% (0-45) with the CD7+CD103+surfCD3- strategy (p=ns).

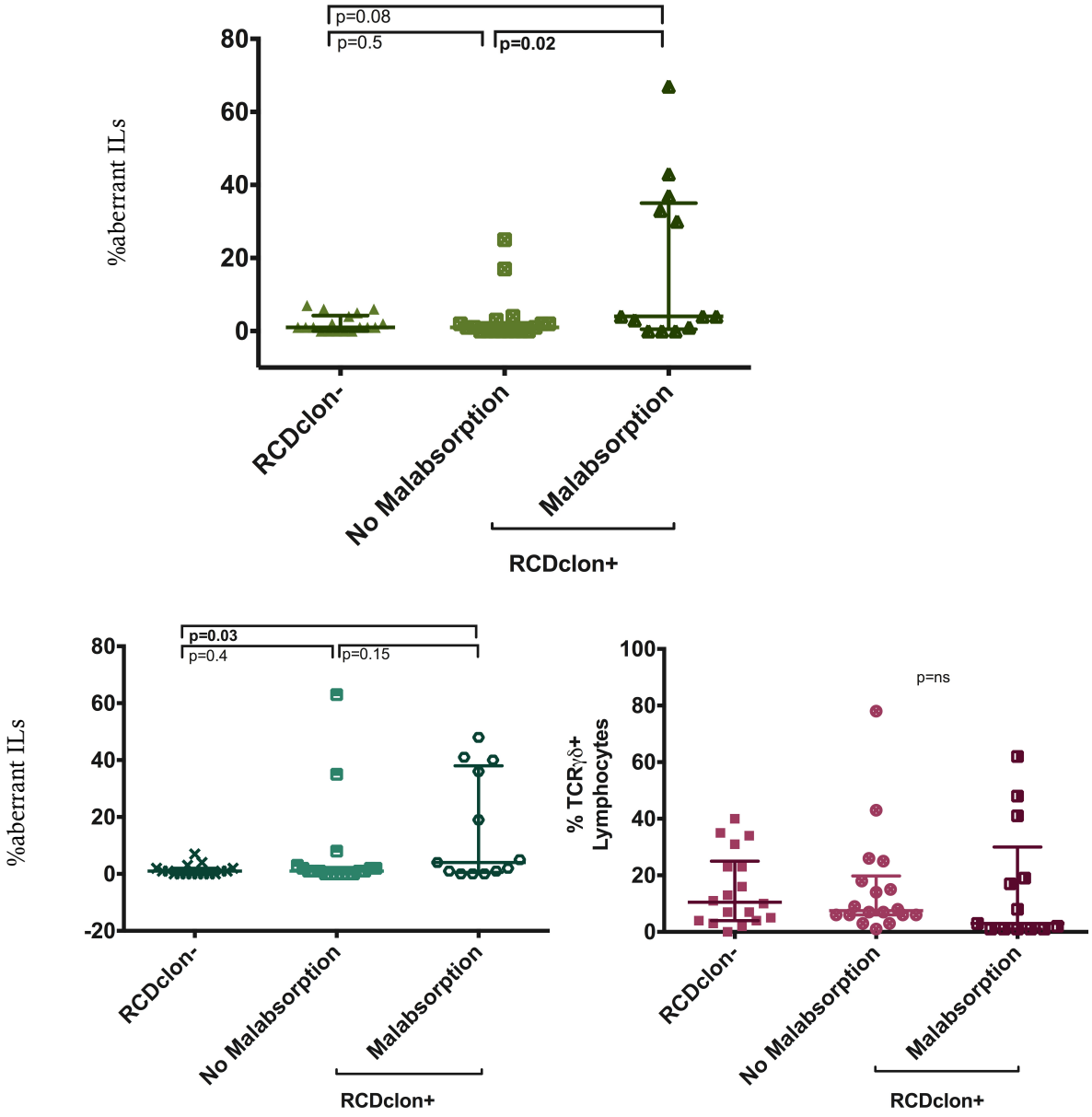
Figure 7. Stratification of flow cytometry results in RCDclon+ patients with regard to the presence or absence of alarm signs. The subgroups are stratified according to the aforementioned score, where results were dichotomized as "no alarm signs" (0-1) and "alarm signs" (2-4). As it can be observed, the presence of anemia, chronic diarrhea, weight loss and/or fever/night sweats, even grouped as a score, is not able to discriminate between RCDclon+ patients with or without elevated aberrant ILs (Panel a, %CD3in+surfCD3-CD7 gating, Panel b, CD7+CD103+ surfCD3- gating). A similar, non-significant result was observed when the number of TCRγδ+ lymphocytes was used as a read-out (Panel c). The RCDclon- group is shown as a reference control (p=ns between RCDclon- and both RCDclon+ subgroups in all Panels)



Malabsorption and aberrant ILs

On the other hand, malabsorption indexes routinely assessed by means of blood tests, including the iron status, folate, vitamin B12, zinc, as well as albumin, electrolytes, and vitamin K-dependent coagulation function (INR) already were observed more frequently (by trend, if not significantly) in RCDclon+ patients as in RCDclon- (see Table 5) and were therefore evaluated as potential determinants of higher risk or higher disease severity among RCDclon+ patients. As expected, no alteration in a single absorption parameter was associated with the presence of elevated aberrant ILs in the respective group. Therefore, we hypothesized (similar to our approach with the alarm signs) that the presence of more than one altered parameter could delineate a relevant malabsorption syndrome in RCD and show an association with aberrant IELs and/or poor prognosis. A score ranging from 0 to 8 was designed (1 point for deficiency in each of the seven aforementioned blood parameters plus 1 point for absence or presence of osteoporosis), with a score >2 considered positive. Patients in the RCDclonality+ group were stratified accordingly. As shown in Figure 8, a malabsorption score higher than 2 was able to divide RCDclon+ patients in two groups with significantly different median percentages of aberrant ILs ($p=0.02$). Although the presence of relevant malabsorption can certainly not be tested as single risk determinant in RCD, these results suggest the possibility that the malabsorption score in addition to determining %aberrant ILs may stratify RCD patients into a low risk and a high risk group regarding disease progression / development of EATL. This result was obtained only with the %CD3in+surfCD3-CD7, but not with the CD7+CD103+ surfCD3- gating strategy, which was previously shown to be less accurate compared to the former strategy [82]. A thorough evaluation of the performances of these strategies and of alternative strategies will be discussed further on.

Figure 8. Stratification of flow cytometry results in RCDclon+ patients with regard to the presence or absence of malabsorption. Grouping RCDclon+ patients along their malabsorption score (cut-off of 2) revealed a significantly higher %aberrant ILs in the RCDclon+ patients compared to the RCDclon- patients (p= 0.02, Panel A, %CD3in+surfCD3-CD7 within lin-TCRγδ- strategy). Similar results, but not reaching statistical significance, were observed using the CD7+CD103+surfCD3- strategy (Panel B). The behavior of TCRγδ+ lymphocytes did not show a significant tendency either (Panel C). The RCDclon- group is shown as a reference control.

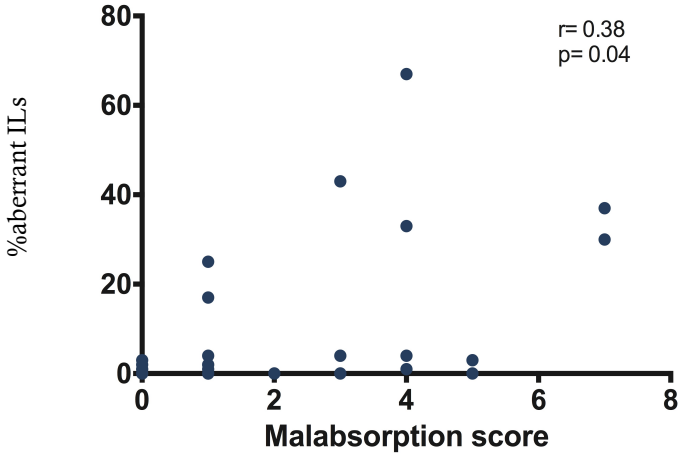


Correlation between clinical parameters and aberrant ILs

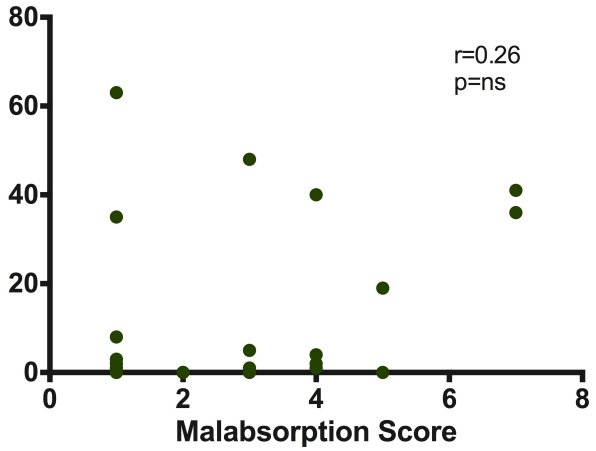
In order to further examine the association between clinical characteristics of RCD patients, a correlation analysis between the Malabsorption score and the %aberrant ILs was performed. As shown in Figure 9, Panel a, a weak to moderate positive correlation (r=0.38, 95% CI 0.02 to 0.65, p=0.04 for the CytCD3+surfCD3-CD7+ strategy and r=0.44, 95% CI 0.09 to 0.69, p=0.01 for the alternative gating strategy CytCD3+surfCD3-CD7+CD103+, further discussed below) could be observed between the Malabsorption score (i.e. number of deficient absorption parameters) and the %aberrant ILS at flow cytometry. Far from confirming a causal association, these results suggest that high-risk RCD patients could be characterized by both an elevation in aberrant ILs and severe malabsorption. In order to verify the persistence of this correlation in a wider group, the same analysis was performed on all RCD patients (clon+ and clon-), where a weaker but still significant correlation could be observed (r=0.35 and 0.39 with p=0.01 and p=0.005 with CytCD3+surfCD3-CD7+CD103+ gating strategies).

As expected, the Alarm score showed no significant correlation with aberrant ILs (r=0.27 and r=0.07 respectively for the CytCD3+surfCD3-CD7+ and for the CD7+CD103+surfCD3- strategy). All single parameters of the score were added to the malabsorption score in order to investigate whether they could add consistency to the results, but no parameter resulted strong enough to improve the performance of the malabsorption score. Another score, comprehending absorption parameters plus anemia and fever/night sweats (considered the two most specific alarm signs of the score already tested above), and thus ranging from 0 to 10, was then tested by means of correlation analysis, showing a moderate positive correlation with %aberrant ILs but still not adding much to the results obtained with the analysis of the malabsorption score alone, even when considered separately (Figure 10).

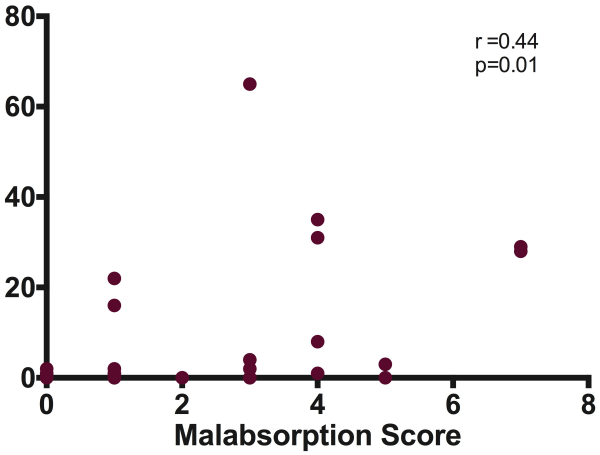
Figure 9. Correlation between Malabsorption score and aberrant ILs in RCDclon+ patients.



a) A weak yet significant positive correlation can be observed between malabsorption and %aberrant ILs assessed with the CytCD3+surfCD3-CD7 lin-TCRγδ- strategy (Strategy 1)

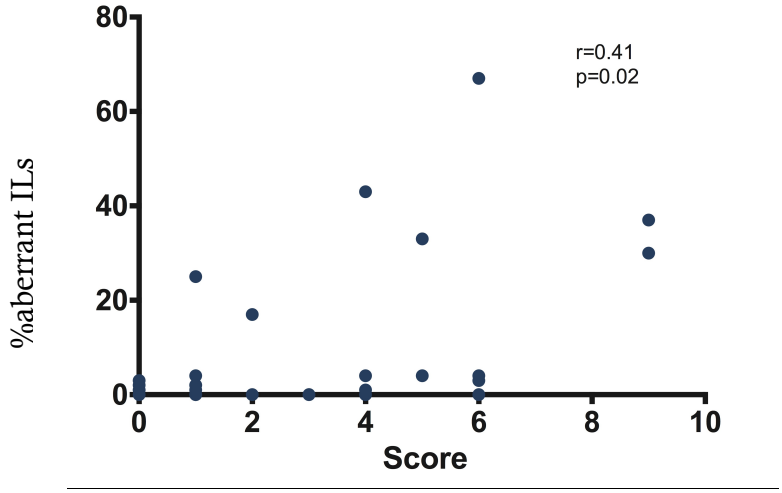


b) The correlation between malabsorption and aberrant ILs was not significant when the CD7+CD103+surfCD3- strategy (Strategy 2) was used. This strategy has already been described ass less specific than the former



c) Exemplary, the results of correlation analysis of an alternative gating strategy, defining ILs as CytCD3+surfCD3-CD7+CD103+ within lin-TCRγδ- (Strategy 6). This gating strategy may be too restrictive but is one of the most specific (see below)

Figure 10. Correlation between the composite score (combination of malabsorption and alarm signs) and aberrant ILs. Aberrant ILs are determined with the CytCD3+surfCD3-CD7 lin-TCRγδ- strategy. Also in this case, as for he malabsorption score, a positive correlation can be observed, with an r corresponding to moderate correlation (95% CI 0.05 to 0.67).

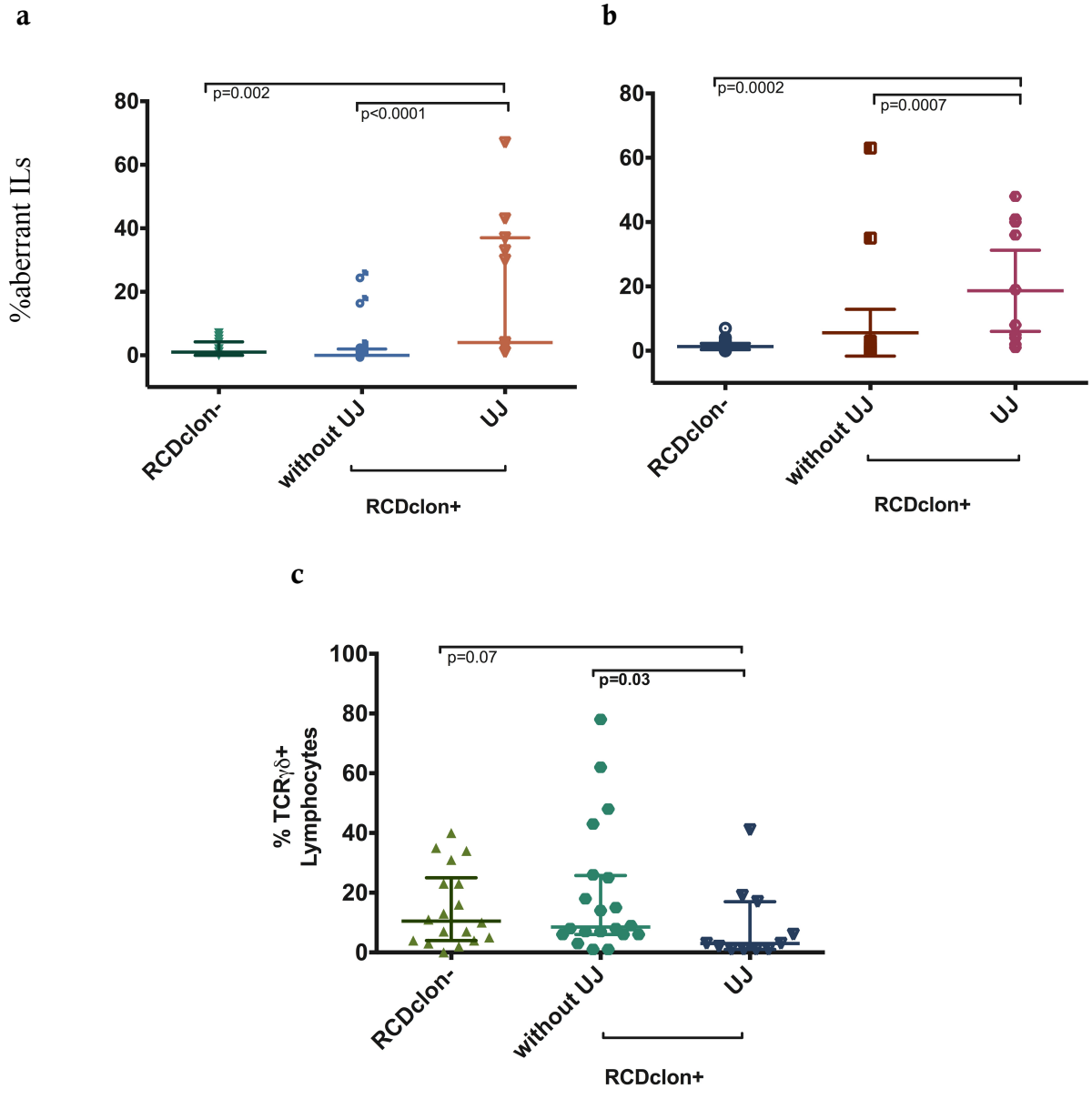


Identification of high-risk and low-risk subgroups within RCDclon+

As part of the diagnostic work up for RCD, patients underwent endoscopic evaluation of the small bowel (capsule endoscopy, push-enteroscopy and/or single-balloon enteroscopy) as well as cross-sectional imaging (abdominal CT or MRI) in order to assess the extent and severity of villous atrophy and to rule out the presence of malignancy. As regards the patients in our cohort, five RCDclon+ patients had an ulcerative jejunoileitis, of whom 2 developed EATL during follow up and 2 were diagnosed with EATL at the time of the first flow cytometry analysis. Moreover, one patient without ulcerative jejunoileitis developed EATL. As it is known, ulcerative jejunoileitis is per se considered an independent predictor of poor prognosis in RCD; therefore elevated aberrant ILs should be expected in this subgroup as well. As can be observed in Figure 11, in RCD patients with ulcerative jejunoileitis a significantly higher %aberrant ILs could be observed as compared to patients without ulcerative jejunoileitis

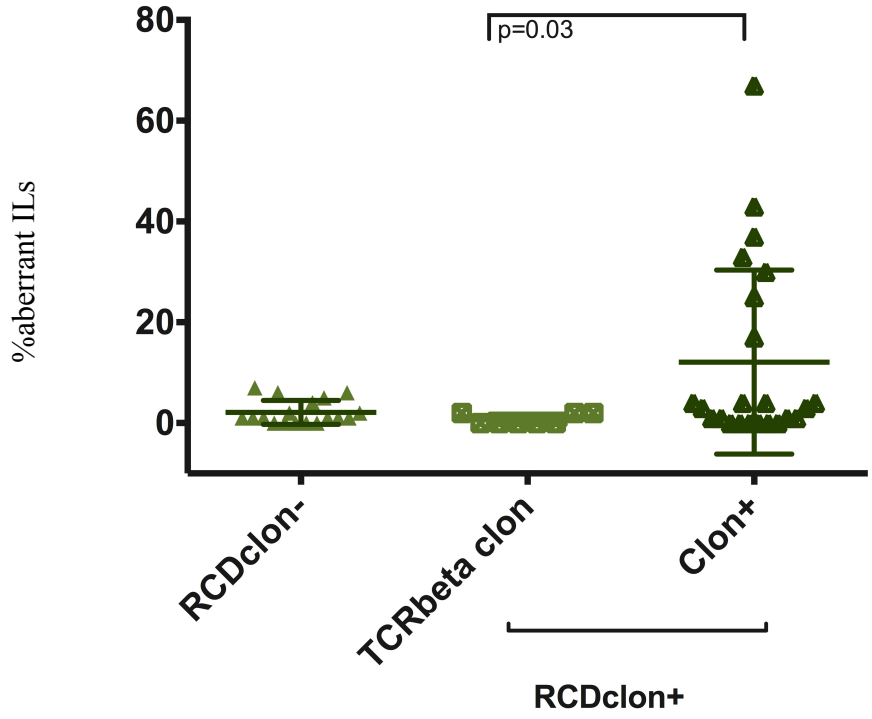
with a median of 4% (1-67) versus 0% (0-16), p<0.0001 with the CytCD3+surfCD3-CD7+ strategy and a median of 8% (1-47) versus 0% (0-32) with the CD7+CD103+surfCD3- strategy. Similarly, %TCRγδ+ were significantly lower in ulcerative jejunoileitis patients, with a median of 3% (1-36) versus 9% (1-61) in the other RCDclon+ subgroup.

Figure 11. Aberrant ILs in RCD patients with and without ulcerative jejunoileitis (UJ). Aberrant ILs are again tested with the CytCD3+surfCD3-CD7+ (Panel a) and the CD7+CD103+surfCD3- (Panel b). Panel c shows the differences in TCRγδ+ between UJ and non-UJ. TCRγδ+ are less in patients with UJ.



In an attempt to further stratify RCDclon+ patients according to their disease severity/risk of EATL development, we evaluated a subgroup of patients classified as RCDclon+ but presenting TCR clonality only at the analysis of the β-chain of TCR, while the molecular pathologic analysis of the γ-chain resulted in poli- or oligoclonality. The flow cytometry analysis of these patients with "atypical" clonality pattern revealed that their aberrant ILs profile (as well as their median Malabsorption score) was significantly different from the other RCDclon+ and similar to RCDclon- (Figure 12). These results suggest that RCD patients presenting with clonality for TCR-β only may be assimilated to RCDclon- and classified as low-risk CD patients.

Figure 12. Aberrant ILs in RCD patients with TCR-β clonality. As compared to the other RCDclon- patients, this subgroup of patients showed lower aberrant ILs (p=0.03) and similar characteristics to RCDclon-.



Re-classification of patients according to aberrant ILs

The results described demonstrated that among RCDclon+ a proportion of patients (but not the majority) had high aberrant ILs. Subsequent analysis allowed demonstrating that the %aberrant ILs was significantly higher in RCD patients with relevant malabsorption, as well as in patients with UJ. On the other hand, the presence of clonality for the TCR β-chain suggested a similar clinical profile as RCDclon- patients. In view of these preliminary analysis, we proceeded to analyze the clinical characteristics of patients according to their %aberrant ILs at flow cytometry.

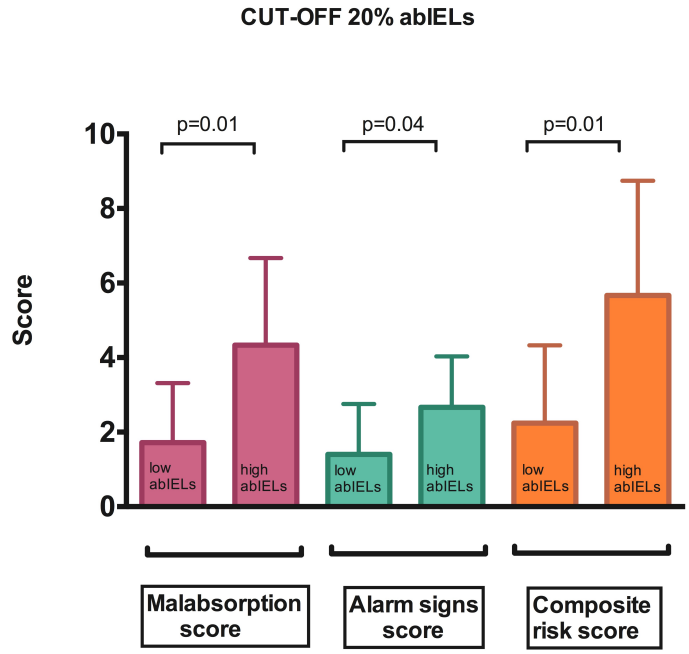
The initial works published on the role of aberrant ILs in RCD [82] have proposed a cut-off of 20% aberrant ILs to discriminate between RCD I and II. In this study, considering the methodological differences and the slightly different gating strategy as compared to the original papers (see Methods section), we proposed and tested a different cut-off for elevated aberrant ILs. The cut-off was developed as previously described, using the 95th percentile of aberrant ILs count in controls and non-refractory CD patients. Considering previous data suggesting that slightly elevated aberrant ILs in active and uncomplicated CD do not have clinical relevance [82], we used controls, active CD and CD on GFD as a whole as control group for the development of cut-offs. Table 7 (see below) summarizes the proposed cut-off for each strategy. For Strategy 1 the new cut-off was 11% while for Strategy 2 it was 7%.

In order to confirm data suggesting a close association between severe malabsorption and high-risk RCD, we compared malabsorption parameters and alarm signs in RCD patients with and without ILs elevation. As shown in Figure 13, for the *CytCD3+surfCD3-CD7+* gating strategy we compared subgroups using both the 20% cut-off proposed in literature of and the 11% cut off derived from our data set. The comparison of RCDclon+ with and without elevated aberrant ILs showed significantly higher values of Malabsorption score

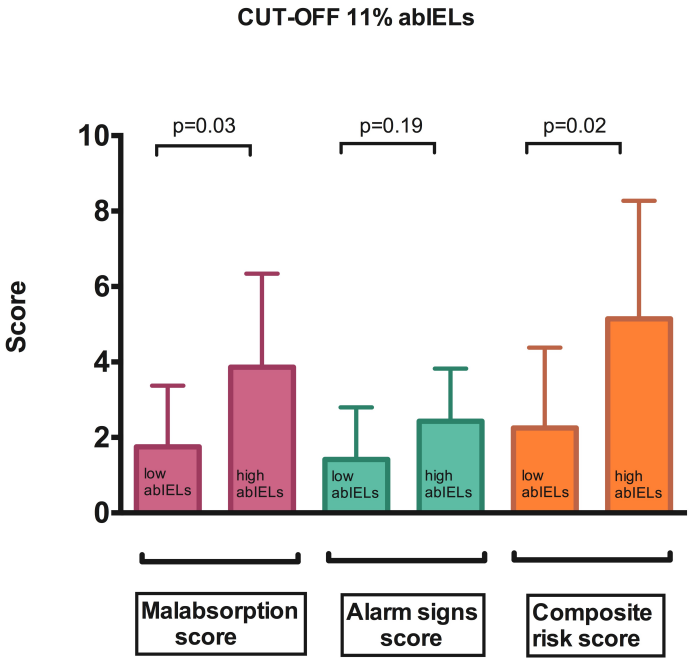
(and a tendency toward significance of the Alarm signs score), confirming the bilateral association between presence of aberrant ILs and signs of disease severity in RCD. As regards TCRγδ+ lymphocytes, higher percentages were observed, as expected, in patients with low aberrant ILs (with 11% cut-off: median 8%, (1-55) versus 2% (1-7), p=0.03).

Figure 13. Differences in malabsorption and alarm signs between patients with low (left side of each pair of data) and elevated aberrant ILs (right side). Strategy: CytCD3+surfCD3-CD7+ within lin-TCRγδ- lymphocytes. a) The cut-off is 20%, i.e. the cut-off proposed in literature for the discrimination of high- and low-risk RCD. b) Here with cut-off is 11%, i.e. the 95% percentile of controls and uncomplicated CD of our cohort.

a)



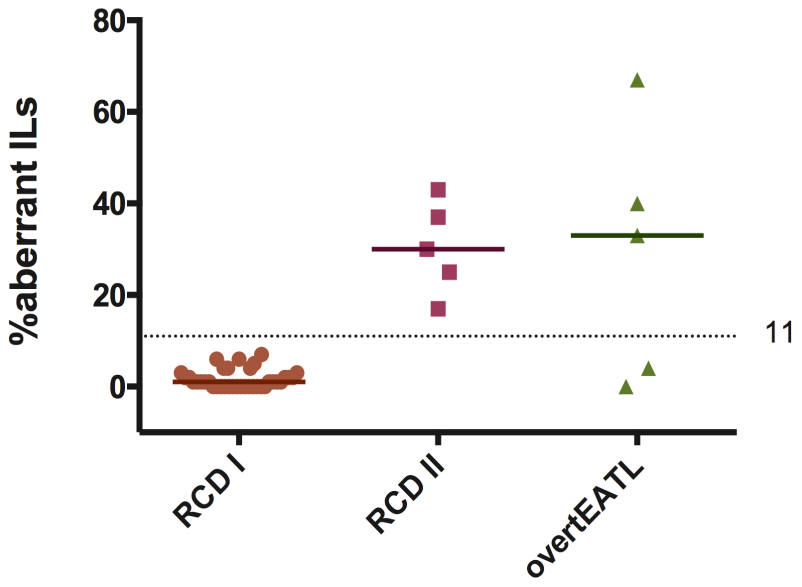
b)



As proposed by Verbeek et al [82], RCD patients from our cohort were re-classified into RCD I and RCD II on the basis of high or low aberrant ILs. According to this new classification strategy, the RCD I group includes patients with and without TCR clonality, considered at "low risk" for malignant development.

Clinical and prognostic data were used as reference standard in order to verify the accuracy of patient allocation in the RCD I – RCD II groups (i.e. low-risk versus high-risk groups) according to %aberrant ILs. Moreover, ILs measurements obtained in patients who received a diagnosis of overt EATL are shown separately. Figure 14 shows the distribution of patients in the groups according to the two strategies evaluated until now.

Figure 14. Classification of RCD patients according to presence or absence of elevated aberrant ILs. a) Strategy: CytCD3+surfCD3-CD7+ within lin-TCRγδ-, b) Strategy: CD7+CD103+surfCD3-. In the boxes to the right, details on incorrect classification of patients are shown

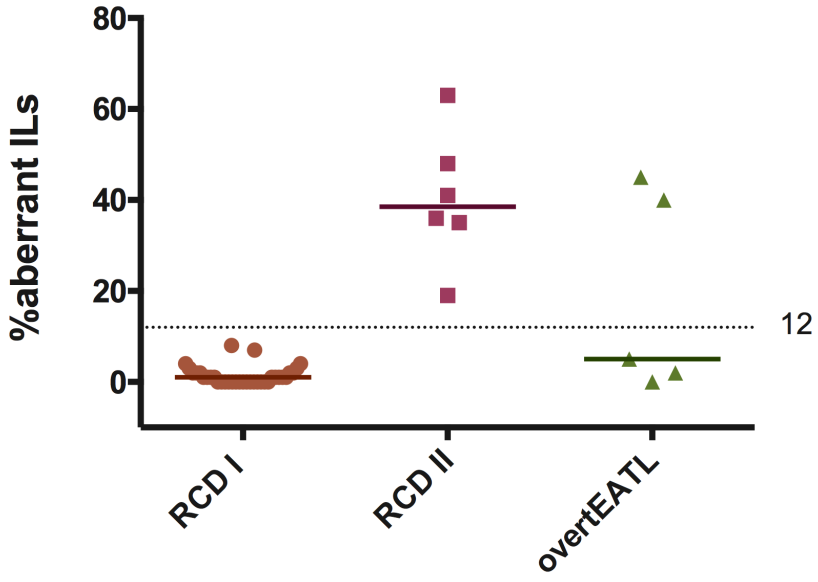


a) Strategy 1
CytCD3+surfCD3-CD7+ within lin-TCRγδ-

Incorrect classification: (False negatives)

3 High-Risk patients classified as RCD I:
1 patient who developed γδ-lymphoma
2 patients with UJ

2/5 Lymphoma patients with low aberrant ILs



b) Strategy 2
%CD7+CD103+surfCD3-

Incorrect classification: (False negatives)

3 High-Risk patients classified as RCD I:
1 patient who developed γδ-lymphoma
2 patients with UJ

3/5 Lymphoma patients with low aberrant ILs

A cut-off of 11% ILs for Strategy 1 and of 12% for Strategy 2 allowed to classify patients into a low-risk subgroup (RCD I =21/30) and to identify a small group of high risk RCD (n=5) that were classified as RCD II/pre-EATL (two of them with a later EATL diagnosis). However, both strategies classified as RCD I a patient with ulcerative jejunoileitis (who later developed EATL) and a patient with a later diagnosis of gamma-delta T-cell Lymphoma; moreover in 2 cases of overt EATL no aberrant ILs were detected. Despite the introduction of a lower cut-off for aberrant ILs detection, both strategies were unable to correctly identify a small number of high-risk patients.

A possible diagnostic approach in order not to miss high-risk RCD patients without aberrant ILs will be discussed further below. As regards Strategy 2, it was unable to correctly identify a third EATL patient, as opposed to Strategy 1, and will therefore be confirmed as less accurate.

Alternative strategies for the detection of aberrant ILs

As already mentioned in the Methods section, more criteria and gating strategies were applied on our cohort, in order to identify the most accurate one/s. Moreover, we aimed at evaluating whether a strategy without the need of cell permeabilization and staining for intraCD3 could be as accurate as Strategy 1 (CytCD3+surfCD3-CD7+ lin-TCR$\gamma\delta$-) and more accurate as Strategy 2 (CD7+CD103+surfCD3-) which is also without CytCD3 staining. In the setting of uncomplicated CD and controls, the presence of "false positive" results with our proposed cut-offs would not represent a clinically relevant problem (mildly elevated aberrant ILs can be observed in 5% controls without evidence or development of malignancy at follow-up). Therefore, the possible alternative strategies were principally evaluated for their accuracy in stratifying low- and high-risk RCD, with the aim of avoiding false negative results.

Of the 10 alternative gating strategies combining the available markers that were evaluated, 5 failed to reach satisfactory results, with too high proportions of false negatives (data not shown), probably due to the too restrictive gating strategy. The remaining 5 alternative strategies are detailed below (Table 7 and Figure 15).

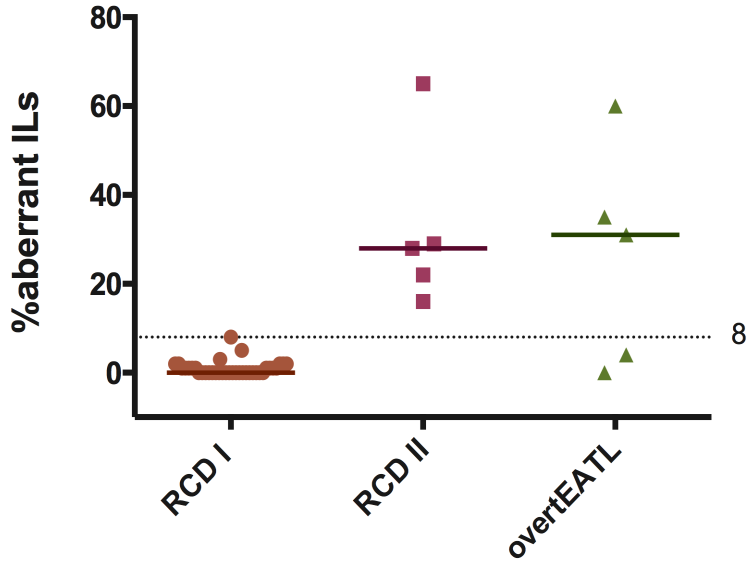
Of all the strategies evaluated, none appeared dramatically more accurate than strategy 1. However, strategies 3a and 3b are slightly more restrictive than Strategy 1, but strategy 3a was able to correctly identify one of the "false negative" high-risk RCD missed in the first place. As regards Strategies 4a-4c, the apparent better performance of strategies 4b and 4c is biased by the fact that all three strategies could be applied to a smaller population due to technical limitation of the four-color analysis. However, the gating for CD4-CD7-CD7+CD103+ within lin- TCR$\gamma\delta$- may prove a useful complementary – and maybe an alternative – to the classic CD7+surfCD3-CDin- and its accuracy should be validated in a specifically designed study.

Table 7. Alternative strategies for the assessment of aberrant ILs and their cut-offs

	Selection/gating strategy						
Cut-offs	**1**	**2**	**3a**	**3b**	**4a**	**4b**	**4c**
Controls	11%	14%	8%	7%	13%	10%	17%
Active CD	5%	6%	5%	5%	7%	15%	6%
CD on GFD	13%	25%	15%	11%	21%	16%	20%
Grouped	**11%**	**12%**	**8%**	**7%**	**15%**	**11%**	**12%**

For description of all strategies, see Table 3, Methods section

Figure 15. a-e) classification of RCD patients according to %aberrant ILs as defined by each alternative strategy.

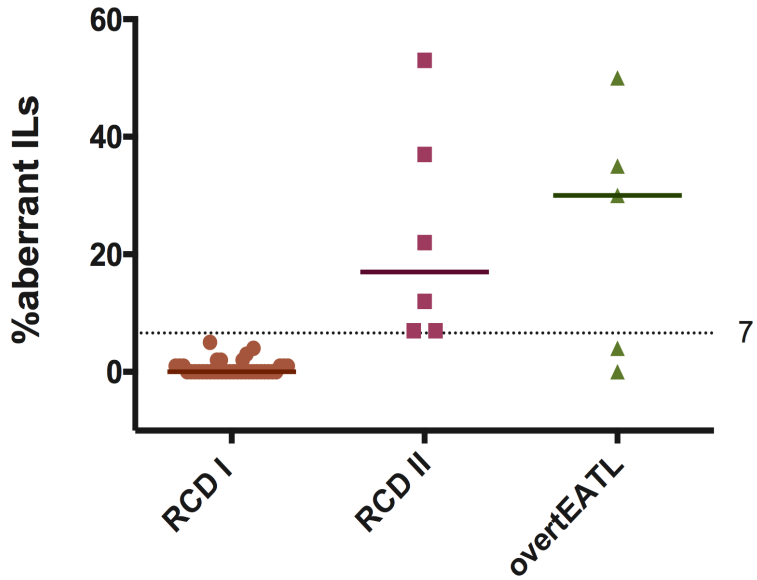


a) Strategy 3a
%CytCD3+SurfCD3 within CD7+CD103+ within lin-TCRγδ-

Incorrect classification: (False negatives)

3 High-Risk patients classified as RCD I:
1 patient who developed γδ-lymphoma
2 patients with UJ

2/5 Lymphoma patients with low aberrant ILs

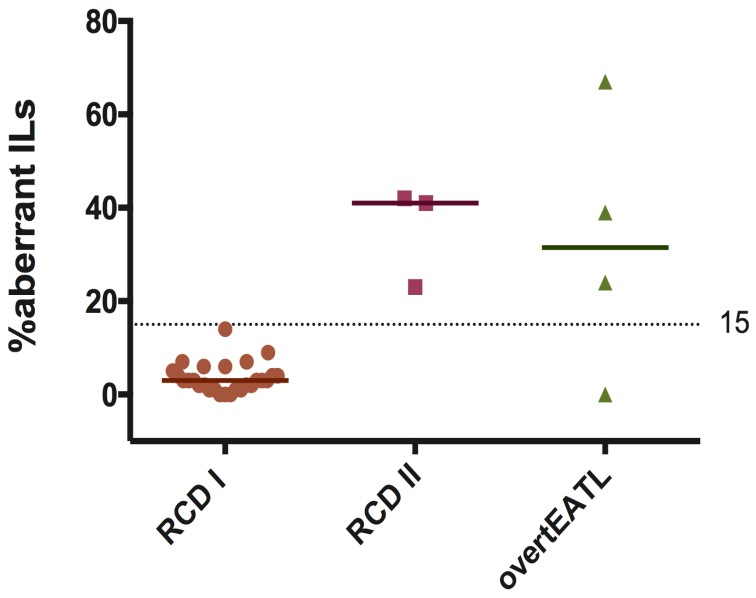


b) Strategy 3b
%CD7+CD103+ within CytCD3+SurfCD3 within lin-TCRγδ-

Incorrect classification: (False negatives)

2 High-Risk patients classified as RCD I:
1 patient who developed γδ-lymphoma
1 patient with UJ

2/5 Lymphoma patients with low aberrant ILs

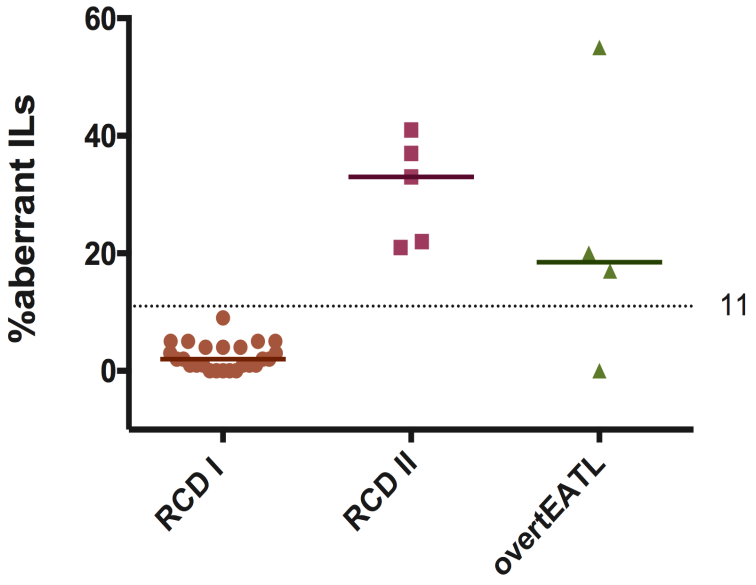


c) Strategy 4a
%CD4-CD8- within CD7+CD103+ within lin-TCRγδ-

Incorrect classification:
(False negatives)

3 High risk patients classified as RCD I
1 patient who developed γδ-lymphoma
1 patients with UJ
1 patient classified as RCD pre-EATL with other strategies

1/4 Lymphoma patient with low aberrant ILs

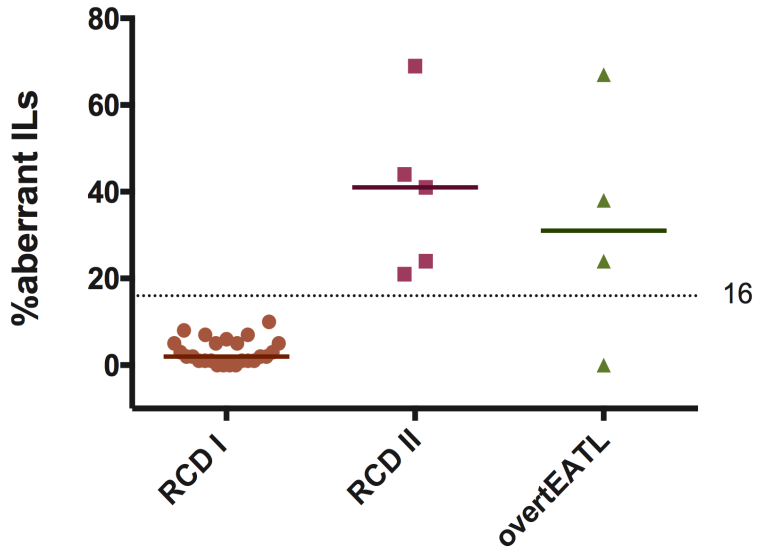


d) Strategy 4b
%CD7+CD103+ within CD4-CD8- within lin-TCRγδ-

Incorrect classification:
(False negatives)

1 High risk patient classified as RCD I:
1 patient who developed γδ-lymphoma

1/4 Lymphoma patient with low aberrant ILs



e) Strategy 4c
%CD4- CD103+ within CD7+CD8- within lin-TCRγδ-

Incorrect classification: (False negatives)

1 High risk patient classified as RCD I:
1 patient who developed γδ-lymphoma

1/4 Lymphoma patient with low aberrant ILs

High-risk RCD without aberrant ILs

Though small, a number of high-risk CD patients were incorrectly classified according to flow cytometry results. In particular, ILs level were found to be unexpectedly low in:

- One patient (M, 72 years) in the RCDclon+ group was diagnosed with Gamma-Delta T-cell Lymphoma and presented with extremely high levels of TCRγδ+ Lymphocytes (up to 70%) and, as a consequence, 0% aberrant ILs regardless of the strategy
- One patient (F, 75 years) in the RCDclon+ group had 4% aberrant ILs at enrolment despite severe malabsorption and evidence of erosions/ulcerations in the duodenum; at follow up, flow cytometry was repeated and this time revealed elevated aberrant ILs
- One patient (F, 80 years) in the RCDclon+ group, despite evidence of ulcerative jejunoileitis and development of Stage I EATL at follow-up, never showed elevated aberrant ILS

- One patient (M, 81 years) in the RCDclon+ group with delayed EATL diagnosis did not have aberrant ILs at flow cytometry.
- Of note, in one additional EATL patient (M, 75 years) a first flow cytometry analysis was negative for aberrant ILs but considered of poor quality given the small number of Lymphocytes analyzed ($<10^3$)

Interestingly, all the abovementioned patients, though not showing elevated aberrant ILs, presented a relevant malabsorption score and low %TCR$\gamma\delta$+ (<6%) at flow cytometry (apart from the patient with gamma-delta T-cell lymphoma). These data could represent useful additional information when evaluating RCD patients, as discussed below.

Discussion

Flow cytometry results in our cohort and their clinical significance

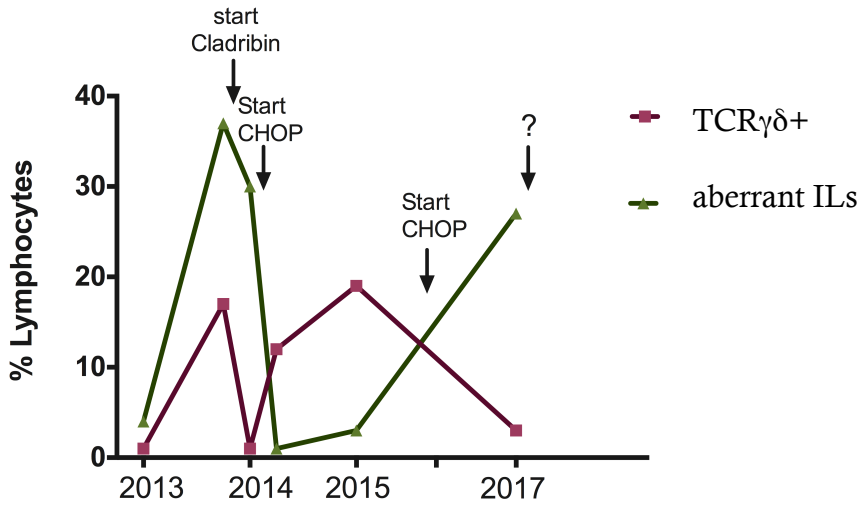
This study was aimed at evaluating flow cytometric analysis of aberrant in a cohort of patients affected by uncomplicated CD and RCD, in order to identify relevant differences among RCD patients able to identify high-risk RCD patients (pre-EATL) with better accuracy than TCR clonality assessment. In our cohort, the determination of aberrant ILs by means of flow cytometry proved a good yet imperfect diagnostic tool for the identification of high-risk RCD patients. To our knowledge, this study represents the first validation of flow cytometric analysis of ILs in a cohort of consecutive German patients affected by RCD.

First of all, the comparison of aberrant ILs counts between different CD groups and controls allowed to confirm that elevated aberrant ILs could be detected in a subgroup of RCDclon+ patients (traditionally defined RCD II as a whole), while seemed not to be present in patients in the RCDclon- group. This results corroborate our experience of the good diagnostic performance of GeneScan analysis for the detection of TCR clonality: in our cohort, in fact, the absence of TCR clonality was per se sufficient to classify RCD patients as "low risk" patients. RCDclon- patients also showed less severe malabsorption as compared to RCDclon+, thus further delineating as a low risk group. These observations differed from those published by other groups, who reported that a small proportion of RCDclon- patients may be at risk of EATL development [82] Interestingly, in a group of patients the presence of clonal rearrangement in the TCR-β chain only without clonal TCR-γ rearrangements corresponded to patients with low aberrant ILs and low malabsorption, thus suggesting that these patients could be assimilated as TCRclon-; however, these observations are in contrast with previous studies reporting

cases of EATL development in patients with isolated TCR-β clonality rearrangements and thus need careful consideration [89].

The importance of aberrant ILs assessment for their role as T-cell malignant precursors in RCD II can be better comprehended when examining their behavior over the course of time. Figure 16 shows the change in aberrant ILs and TCRγδ+ lymphocytes in a pre-EATL RCDclon+ patient over time. Interestingly, the first assessment of aberrant IELs proved negative (with low TCRγδ+). During follow up, the patient presented with worsening of symptoms and weight loss and was therefore re-evaluated: at this point, a treatment with cladribin was started in view of the increase in aberrant ILs observed (in absence of overt EATL), followed by a cycle of chemotherapy due to insufficient clinical response. The profile of both aberrant ILs and TCRγδ+ changed according with response to treatment and changed again when a relapse of symptoms was experienced.

Figure 16. Changes in % aberrant ILs and TCRγδ+ lymphocytes in a pre-EATL RCDclon+ patient over time



Aberrant IELs as specific diagnostic tool in RCD, but less sensitive as expected

The presence of elevated aberrant ILs in a group of RCD patients was used to identify a subset of high-risk RCD patients (RCD II/pre-EATL) who were also characterized by poorer clinical conditions, such as severe malabsorption and/or small bowel ulcerations. However, this diagnostic strategy missed two RCD patients that deserved classification as RCD II, the first being a RCDclon+ patients with ulcerative jejunoileitis and malabsorption symptoms, who later developed an EATL, the second (who could be considered a rare exception) a RCDclon+ patients in whom a diagnosis of gamma-delta T-cell lymphoma was made. Moreover, using clinically overt EATL (n=4) as a reference standard, negative aberrant ILs were observed (at repeated measurements) in two cases, both RCDclon+ patients. Those results were confirmed with both gating strategies (Strategy 1 and 2) initially developed on the basis of the work by Verbeek et al [82], using the cut-off of 20% proposed in that study as well as the cut-offs of 11% and 12% respectively developed on the basis of ILs counts in our internal control groups. As tested in the setting of RCD with clinical follow up as gold standard, aberrant ILs showed a Specificity of 100% but a Sensitivity of 67% for the detection of pre-EATL/EATL. Of note, the lower cut off identified by our strategy should be further validated but apparently did not result in higher percentages of false positive results.

Alternative strategies for the assessment of aberrant IELs

As already described in detail, several alternative gating strategies were tested, including alternative strategies not requiring the use of intracellular staining. An accurate detection technique without the need of cell permeabilization would have the potential to use the (viable) sorted T-cells in further experiments. At least two of the gating strategies for CD4-CD8-C7+CD103+ (Strategies 4a-c) showed promising results; however, they were tested in a smaller population due to technical reason (particularly, they were not tested

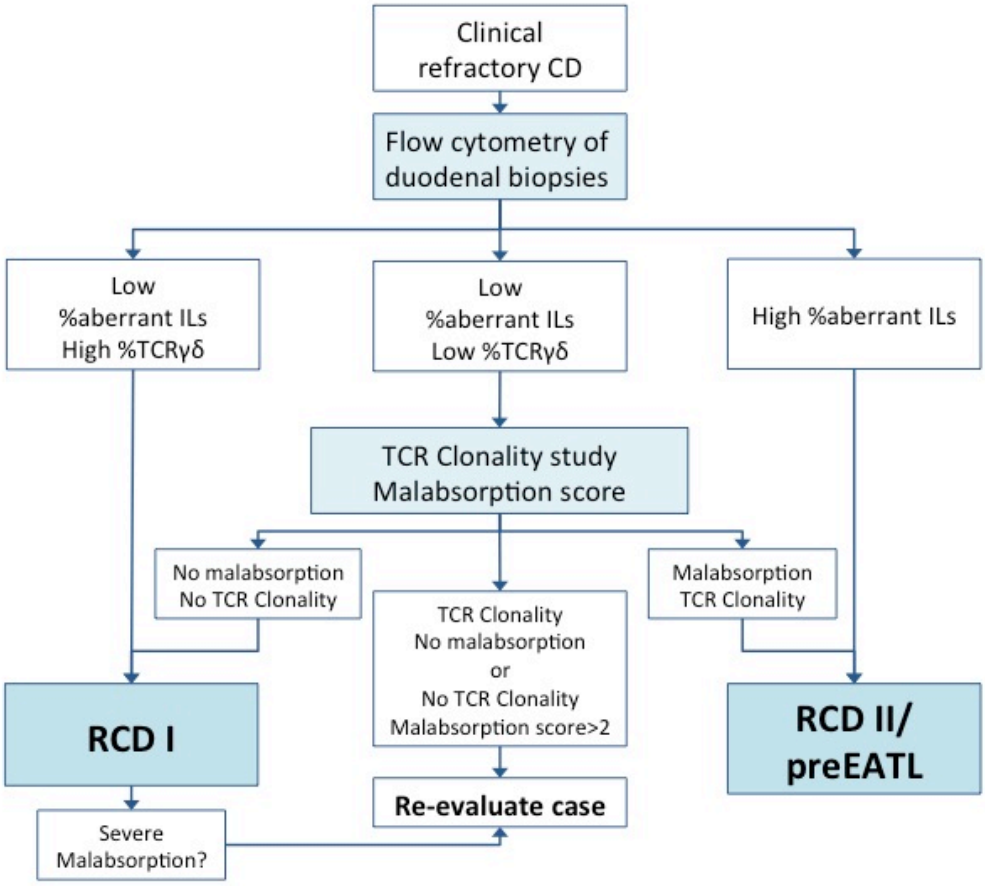
in 3 of the 4 controversial cases). Future perspective studies should be designed to validate these preliminary results.

A possible solution for an accurate classification of RCD patients

Results of flow cytometry analysis in our cohort prompted us to question whether the flow cytometric assessment of aberrant ILs is sufficiently accurate to discriminate between RCDI and RCDII/pre-EATL without the aid of TCR clonality studies.

In view of (i) the confirmatory data showing that high-risk RCD are found (almost) exclusively within the RCDclon+ subgroups, (ii) the fact that all of the patients incorrectly classified by means of aberrant ILs showed low %TCRγδ+ (<6%) at flow cytometry (apart from the gamma-delta T-cell lymphoma case), and (iii) the evidence (from our cohort and from a recent paper [91]) that a relevant malabsorption syndrome, defined as deficiency of 3 or more of relevant vitamins and/or micronutrients, is associated with a high-risk RCD, we developed a possible diagnostic strategy that could allow a correct identification of RCD patients at higher risk (RCD II or "pre-EATL") without missing those high-risk RCD patients in which aberrant ILs can not (yet) be detected (Figure 17).

Figure 17. Proposed diagnostic strategy for the identification of RCD II/pre-EATL patients



As described in the Figure, flow cytometry as a simple and accurate method can be easily performed as first step in the setting of follow-up gastroscopy. A TCR clonality study may be performed in all patients or – as future perspective - only in cases of unclear results of flow cytometry analysis and/or in patients with a relevant malabsorption syndrome.

In conclusion, considering the different prognosis and risk for malignant evolution associated with a diagnosis of RCD II as compared to RCD I, a correct classification of patients is of utmost importance. In fact, in order to prevent false negative results and verify for possible false positives, the classification of RCD patients into RCD I and RCDII/pre-EATL should be made by means of the combination of more diagnostic tools, among which flow cytometry will certainly play a key role.

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