Assessment of Duodenal Intraepithelial Lymphocyte Composition (Lymphogram) for Accurate and Prompt Diagnosis of Celiac Disease in Pediatric Patients

Cristina Camarero, MD, PhD[1,*], Ana De Andrés, MD[2,*], Carlota García-Hoz, PhD[2], Belén Roldán, MD, PhD[1], Alfonso Muriel, MD, PhD[3], Francisco León, MD, PhD[4] and Garbiñe Roy, MD, PhD[2]

INTRODUCTION: **Quantitative and phenotypic analyses of duodenal intraepithelial lymphocytes (IELs) by flow cytometry (IEL lymphogram) confer specificity and enable the diagnosis even in unconventional presentations of celiac disease (CD). To evaluate the validity of the IEL lymphograms in the pediatric population for new insights into their use as biomarkers in the natural history of CD.**

METHODS: **We retrospectively included 1,211 children (602 with active CD, 92 on a gluten-free diet, 47 with potential CD, and 470 nonceliac controls) who required duodenal biopsies in this study. The cutoff values for IEL subsets were established to calculate the probability of disease according to the lymphogram.**

RESULTS: **A celiac lymphogram (a ≥15% increase in gamma-delta T-cell receptor IELs and a simultaneous ≤6% decrease in CD3 surface-negative [sCD3$^-$]) IELs was strongly associated with the diagnosis of active CD, which was present in 89.7% of the confirmed patients. The remaining 10% of the celiac patients had a partial celiac lymphogram (≥15% increase gamma-delta T-cell receptor IELs or ≤6% decrease in sCD3$^-$ IELs), with lower diagnostic certainty. On a gluten-free diet, nearly 20% of the patients were indistinguishable from nonceliac subjects based on the lymphogram. In potential CD, a decrease in sCD3$^-$ IELs was a risk marker of progression to villous atrophy and a diagnosis of active CD.**

DISCUSSION: **If a biopsy is clinically indicated, the IEL lymphogram adds specificity to the histological findings, reducing diagnostic delays and misdiagnoses. The lymphogram is useful for monitoring the natural progression of the disease and predicting the transition from potential celiac to overt CD.**

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INTRODUCTION

Celiac disease (CD) is a highly prevalent but largely underdiagnosed autoimmune disease triggered by gluten consumption (1–5) in genetically susceptible HLA-DQ2/DQ8 individuals (6). The diagnosis relies on detecting highly specific serum immunoglobulin (Ig) A antitransglutaminase autoantibodies (7–9) and the presence of duodenal intraepithelial lymphocytosis, with variable degrees of duodenal villous atrophy, evaluated according to several classification schemes (10–13).

The European Society for Paediatric Gastroenterology, Hepatology and Nutrition 2012 and 2020 guidelines for the diagnosis of CD in children (14,15) do not require duodenal biopsies in certain clinical, serological, and genetic presentations. In the absence of these circumstances, and despite ongoing debate in the field, duodenal biopsy is indicated as a critical aspect of the diagnosis (16,17).

None of the histological changes that characterize CD are pathognomonic (17–20), and there are pitfalls in the pathological interpretation of intestinal biopsies (21–25).

The characteristic finding of the immune-mediated celiac enteropathy is an increase in the total intraepithelial lymphocyte (IEL) counts (26,27), which comprise the cytotoxic effectors responsible for the epithelial lesions (28) and an increase in gamma-delta T-cell receptor (TCRγδ) IEL subset. This increase is coupled with a decrease in the CD3 surface-negative (sCD3$^-$) IEL subset, which is highly characteristic of CD (29). Our group coined the term "IEL lymphogram" (30–32) to describe the duodenal flow cytometric IEL profile and coined the term "celiac lymphogram"

[1]Department of Pediatric Gastroenterology, University Hospital Ramón y Cajal, University of Alcal, Madrid, Spain; [2]Department of Immunology, University Hospital Ramón y Cajal, IRYCIS Madrid, Spain; [3]Clinical Biostatistic Unit, University Hospital Ramón y Cajal IRYCIS, CIBERESP Nursing and Physiotherapy Department, University of Alcalá, Madrid, Spain; [4]Provention Bio, New Jersey, USA. **Correspondence:** Cristina Camarero, MD, PhD. E-mail: cristina.camarero@salud.madrid.org.

*Cristina Camarero, MD, PhD, Ana De Andrés, MD contributed equally to this work.

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for the long-lasting imbalance in the ratio of TCRγδ vs sCD3$^-$ IEL subsets. This procedure has become a routine evaluation tool in numerous hospitals (33–38).

We present herein the diagnostic performance of the duodenal lymphogram in the largest pediatric series to our knowledge, adding new insights into the evolution of these TCRγδ and sCD3$^-$ IEL subsets in the natural history of patients with CD on a gluten-free diet (GFD) and patients with potential CD.

MATERIALS AND METHODS

Patients

This was a retrospective study conducted at the Pediatric Gastroenterological Unit and the Department of Immunology of University Hospital Ramón y Cajal (Madrid, Spain) between 2009 and 2019, as the result of routine clinical practice. A total of 1,211 patients undergoing upper digestive endoscopy during this period were included. Some of the patients analyzed in this study have been part of a previous study unrelated to the goals and methods of this report.

There were 4 patient subgroups included in the study: active CD group on a gluten-containing diet, patients with CD in remission on a GFD, patients with potential CD, and the control group.

Active CD group on a gluten-containing diet. This group was divided into 2 groups: (i) a new diagnosis of CD and (ii) gluten challenge in patients with previously diagnosed CD. The group with new diagnosis of CD (n = 602) included 197 boys, median age 3.4 years and a 25%–75% interquartile range (IQR) of 2.4–7.8. All the children in this group had elevated serum IgA anti-transglutaminase and antiendomysial antibody levels; 2 children with IgA deficiency had positive IgG-based tests. All patients had changes in their intestinal mucosa of stage 2 and 3 according to the Marsh-Oberhuber classification (10,12). All patients displayed HLA DQ2-or DQ8-positive antigens, except for 4 patients (1 DQ9/DQ5, 1 DQ9/DQ9, and 2 DQ7/DQ7). After a GFD was started, all patients became negative for antibodies in a maximum period of 14 months.

In the gluten challenge in patients with previously diagnosed CD (n = 15) group, the mean age was 7.7 years, range 4.5–18 years. Children who were previously diagnosed with CD required a provocation test to confirm the diagnosis. Median gluten intake time was 1.4 years (range 3 months–6.7 years).

Patients with CD in remission on a GFD. This GFD group (n = 92) had a median age 6.2 years, IQR 5.0–10.8. The mean period on a GFD was 46.2 months (range 2–204). The indication for biopsy was to demonstrate the recovery of the intestinal mucosa (n = 68) and comorbidities unrelated to CD (n = 21).

Patients with potential CD. The patients with potential CD (n = 47) had median age 5.6 years, IQR 3.3–8.6. These patients were asymptomatic, had positive serology, DQ2-or DQ8-positive antigens, and Marsh 0–1 stage in the biopsy histology.

Control group. The control group (n = 470) had a median age 6.8 years, IQR 2.1–12.7. Figure 2c,f detail the diagnoses of the included patients. All of the control patients consumed a gluten-containing diet. The intestinal mucosa was normal in all but 6 patients, with villous atrophy and normal IEL distribution, diagnosed with food allergy (n = 2), intestinal *Giardia lamblia* (n = 1), IgA deficiency and diarrhea (n = 1), microvillus inclusion disease (n = 1), and intractable diarrhea (n = 1).

METHODS

Small bowel biopsy

In each patient, upper gastrointestinal endoscopy was performed with 7 biopsies, 5 from the distal duodenum (4 for histology and 1 for cytometric analysis) and 2 from the duodenal bulb (1 for histology and 1 for cytometric analysis). The mucosal morphology was classified according to the Marsh criteria as modified by Oberhuber (10,12). Biopsies for flow cytometric analysis were collected in saline serum if they were to be processed immediately within 2 hours or in RPMI cellular complete medium (see below) if processing was to be postponed for up to 12 hours. Processing should not be delayed longer than 12 hours. Biopsies included in this study are from the distal duodenum.

Biopsy and flow cytometry

Single-cell suspensions were prepared from the epithelial layer of the duodenal biopsies by using a previously described protocol (39) with minor modifications. Briefly, IELs and epithelial cells were released from the mucosal specimens by a 1-hour incubation under gentle stirring with 1 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) in RPMI 1640 medium (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum and antibiotics. The suspension of released cells was collected by centrifugation, washed, and stained with fluorochrome-conjugated monoclonal antibodies (anti-CD45 APC, clone HI30, anti-CD3 PerCP, clone SK7, anti-TCRγδ PE, clone 11F2, anti-CD103 FITC, and clone Ber-ACT8; all from BD Pharmingen) to quantify the percentage of IELs relative to epithelial cells and the percentage of TCRγδ and sCD3$^-$ CD103$^+$ IEL subsets relative to total IELs. The expression of CD103 ensures an intraepithelial location of the analyzed CD45$^+$ IELs. The flow cytometry analysis was performed in a FACSCanto Flow Cytometry System (BD Biosciences, San Jose, CA), and the data were processed with DIVA software (BD Biosciences).

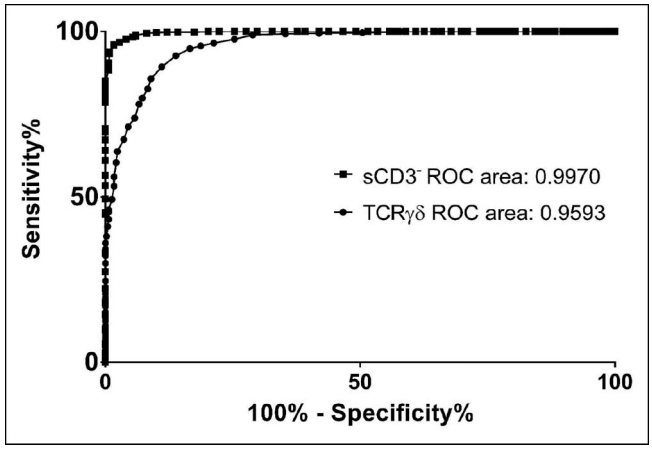

Figure 1. The receiver operating characteristics (ROC) curve analysis to define the cutoff values for the TCRγδ and sCD3$^-$ IEL subsets. CD, celiac disease; IEL, intraepithelial lymphocytes; TCRγδ, gamma-delta T-cell receptor.

All biopsy specimens were obtained for diagnostic purposes in accordance with the ethical guidelines of our institution after obtaining informed written consent.

Statistics

Absolute and relative frequencies were used to describe the categorical variables. Means, SDs or medians, and quartiles were used to describe the continuous variables. For the flow cytometric analysis, the following variables were considered: (i) the percent of TCRγδ IELs relative to total IELs and (ii) the percent of sCD3$^-$ IELs relative to total IELs. Intergroup differences in these variables were tested with the nonparametric Mann-Whitney test. Differences between matched-pairs data were tested with the Wilcoxon signed-rank test.

A receiver operating characteristic (ROC) curve analysis was performed to define the cutoffs of the variables, optimizing sensitivity and specificity to calculate the performance of these markers to diagnose CD (Figure 1). Sensitivity, specificity, and likelihood ratios were used.

To evaluate whether either 1 or both IEL subsets (sCD3$^-$ and TCRγδ) were needed to calculate the probability of having CD, we used binary logistic regression models and estimated the discrimination by means of the ROC curves. All measurements were performed using 95% confidence interval (CI) and using Stata 15.1 software (StataCorp LP, College Station, TX). A P-value < 0.05 was considered statistically significant. The figures in this article were created with GraphPad Prism Software (La Jolla, CA).

All biopsy specimens were obtained for diagnostic purposes after obtaining informed written consent and in accordance with the ethical guidelines of our institution. All the analyses presented in this study are included in the routine diagnostic protocol for CD.

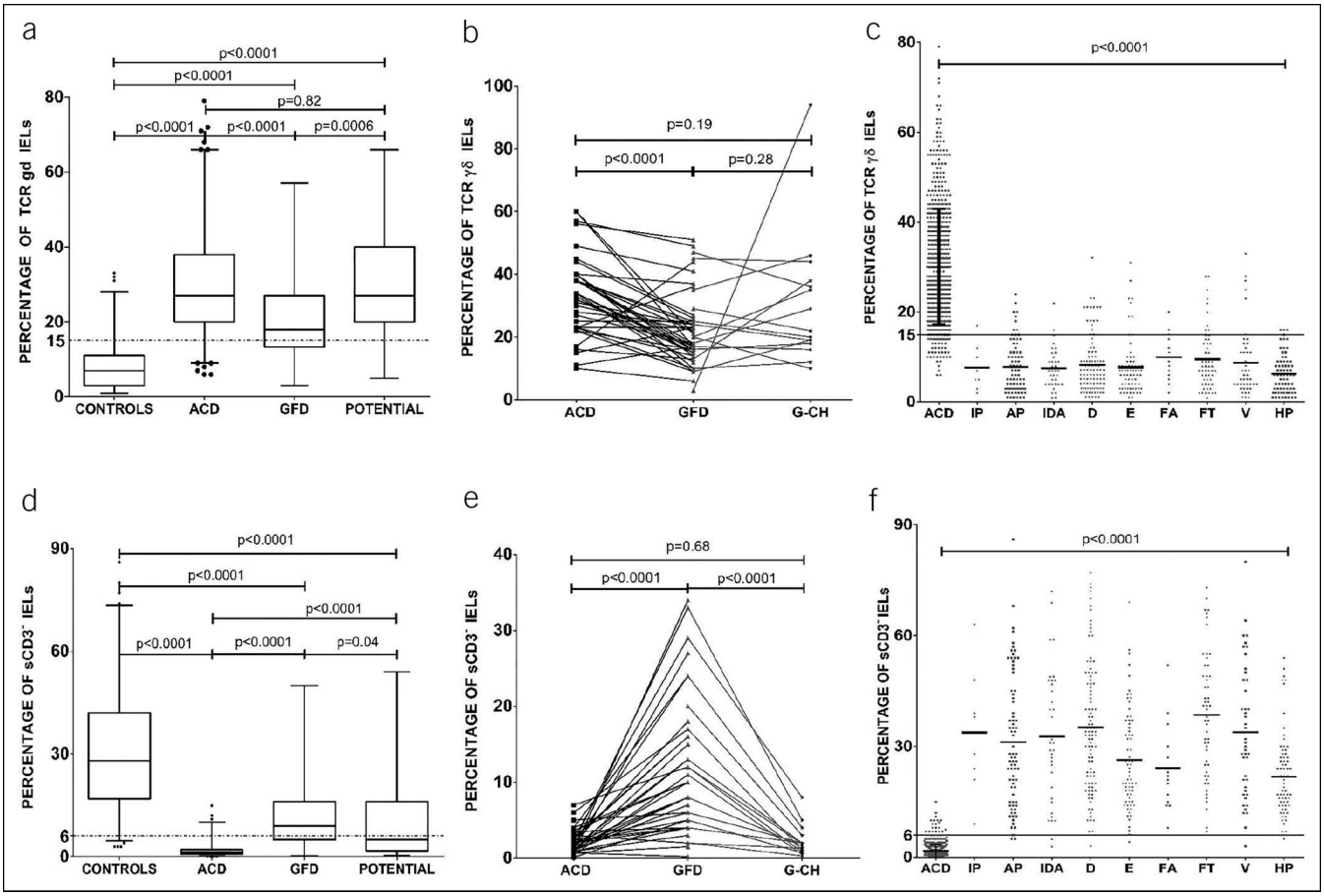

Figure 2. The distribution of TCRγδ and sCD3$^-$ IEL subsets in different forms of celiac disease and controls. (**a**) Medians, interquartiles, and ranges of TCRγδ in different patient groups. (**b**) The percentages of TCRγδ in active patients with CD at diagnosis compared with follow-up after introduction of a GFD (n = 38) and a posterior gluten challenge (n = 15). Patients included in the ACD → GFD series do not overlap with the GFD → G-CH set. (**c**) The distribution of TCRγδ IEL densities in ACD vs the different clinical conditions included in the control group. TCRγδ IEL densities are expressed as a percentage of total intraepithelial lymphocytes. A cutoff at 15% TCRγδ is indicated, and the mean value markers are shown for each group. (**d**) Medians, interquartiles, and ranges of sCD3$^-$ IELs in different patient groups. (**e**) The percentages of sCD3$^-$ in active patients with CD at diagnosis compared with follow-up after introduction of GFD (n = 38) and a posterior gluten challenge (n = 15). Patients included in the ACD → GFD series do not overlap with the GFD → G-CH set. (**f**) The distribution of sCD3$^-$ IEL densities in ACD vs the different clinical conditions included in the control group. CD3$^-$ IEL densities are expressed as a percentage of total intraepithelial lymphocytes. A cutoff at 6% sCD3$^-$ is indicated, and the mean value markers are included for each group. ACD, active celiac disease at diagnosis; AP, abdominal pain; CD, celiac disease; D, unspecific chronic diarrhea and postenteritis syndrome; E, gastroesophageal reflux; FA, food allergy; FT, failure to thrive; G-CH, gluten challenge; GFD, gluten-free diet; IDA, iron deficiency anemia; IEL, intraepithelial lymphocytes; IP, intestinal parasites; HP, *Helicobacter pylori*; V, various; TCRγδ, gamma-delta T-cell receptor.

Table 1. Distribution of TCRγδ and sCD3⁻ IEL subsets in CD groups

	% TCRγδ IEL mean ± SD (95% CI)	% sCD3⁻ IEL mean ± SD (95% CI)
Active CD n = 602	30.1 ± 12.9 29.1–31.1	1.8 ± 1.7 1.6–1.9
GFD n = 92	22.2 ± 12.2 19.6–24.7	12.1 ± 10.6 9.9–14.3
Potential CD n = 47	30.4 ± 15.2 25.9–34.9	11.4 ± 13.5 7.4–15.3
Controls n = 470	7.9 ± 6.0 7.4–8.5	30.9 ± 16.8 29.4–32.5

The table shows the mean percentages of TCRγδ and sCD3⁻ IEL subsets relative to the total CD45⁺ IELs.
CD, celiac disease; CI, confidence interval; GFD, gluten-free diet group; IEL, intraepithelial lymphocytes; TCRγδ, gamma-delta T-cell receptor.

RESULTS

TCRγδ IELs and sCD3⁻ IELs are the main components of the duodenal lymphogram

Figure 2a confirms the significantly higher percentage of TCRγδ IELs in any of the various forms of CD compared with the controls ($P < 0.0001$) (Table 1).

Although always elevated, there was a significant decreasing trend in TCRγδ IEL percentages on the initiation of a GFD (Figure 2b), as illustrated by the small group of patients who underwent a follow-up after the GFD was initiated (n = 38), with a mean of 33.8% before the GFD (95% CI, 29.3–37.2) vs a mean of 22.1% after starting the GFD (95% CI, 19.0–25.4). No significant increasing tendency in TCRγδ IEL percentages was observed in those who required a gluten challenge (n = 15) ($P = 0.281$).

In parallel, Figure 2d illustrates how the sCD3⁻ IEL density values observed in either CD group were significantly lower than in the control group ($P < 0.0001$) (Table 1). Note the significant increasing trend in sCD3⁻ IEL densities after initiating a GFD (Figure 2e), from a mean of 2.2% before the GFD (95% CI, 1.7–2.7) to a mean of 12.3% after initiating the GFD (95% CI, 9.3–14.0; $P < 0.0001$). This trend was also illustrated in the patient group who underwent a follow-up after initiating a GFD (n = 38) and in the drastic drop in the small number of patients who required a gluten challenge (n = 15), back to a mean of 2.3% (95% CI, 1.1–3.4).

Wide individual variations in TCRγδ IEL and in sCD3⁻ IEL densities were demonstrated in each patient group, with some overlap among groups and between the different clinical conditions included in the control group and the active CD group (Figure 2c,f).

Permanent imbalance of TCRγδ IEL and sCD3⁻ IEL subsets: the duodenal lymphogram as a powerful predictor of CD

To calculate the optimal diagnostic performance of these cytometric parameters, the cutoff values for TCRγδ IEL and sCD3⁻ IEL were established from the ROC curves. A cutoff of ≥15% for TCRγδ IEL and a cutoff of ≤6% for sCD3⁻ IEL optimized the sensitivity and specificity to calculate the performance of these parameters to predict CD (Table 2).

The combination of the 2 variables, according to the compliance of the above established cutoffs, gave rise to 4 distinct lymphogram profiles: a celiac lymphogram (TCRγδ ≥15 and sCD3⁻ ≤6), 2 partial lymphograms (1 with a ≥15% increase in TCRγδ density, with sCD3⁻ >6% and the other with a ≤6% decrease in sCD3⁻ density, with TCRγδ <15%), and a nonceliac lymphogram (TCRγδ <15 and sCD3⁻ >6) (Table 3). In the active CD patient group, a celiac lymphogram profile had a disease probability of 100% in 89.7% of these patients, whereas the remaining 10% of the patients in this group presented with a partial lymphogram, with lower predictive values. When patients were on a GFD, only 30% presented a celiac lymphogram profile, whereas most of the patients showed a partial lymphogram, with 20.6% of the patients presenting a nonceliac lymphogram and the consequent loss of diagnostic accuracy.

The sensitivity and specificity of the combination of the 2 variables were calculated and associated with convincing diagnostic evidence of CD (Table 4). In the case of a celiac lymphogram, the combination of the 2 variables yielded a specificity of 100% and a sensitivity of 89.7%. The decrease in sCD3⁻ IEL density provided the best contribution for the CD diagnosis, with a positive likelihood ratio of 34.9 vs 6.7 when the partial lymphogram was due primarily to an increase in TCRγδ IELs.

Duodenal lymphogram: a useful prognostic marker in the natural history of potential CD

Figure 2a,d and Table 1 show the distributions of IEL subsets in the patients with potential CD (n = 47), revealing interesting and statistically significant differences. The median follow-up time for this cohort was 117 months (range 3–240).

When comparing the potential CD group with the active CD group, there were no significant differences in TCRγδ density ($P = 0.82$), whereas the sCD3⁻ IEL subset was significantly increased in the potential CD group ($P < 0.0001$), as also occurs in the GFD group, both characterized by a preserved mucosal architecture. However, the increase in the TCRγδ IEL subset was significantly higher in the patients with potential CD than in those on a GFD, which could suggest the direct involvement of

Table 2. Statistical performance of IEL lymphography TCRγδ and sCD3⁻ IEL parameters in CD prediction

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	+LR (95% CI)	−LR (95% CI)	OR (95% CI)	AUC (95% CI)
TCRγδ ≥ 15%	92.7% (90.6–94.7%)	86.2% (83.1–89.3%)	89.6% (87.2–91.9%)	90.2% (87.4–92.9%)	6.7 (5.3–8.4)	0.08 (0.06–0.11)	79 (52.8–118)	0.9593 (0.9487–0.9698)
sCD3⁻ ≤ 6%	96.7% (96.2–98.1%)	97.2% (95.7–98.7%)	97.8% (96.6–99.9%)	95.8% (94.0–97.6%)	34.9 (20.4–59.7)	0.03 (0.02–0.05)	1,023 (506–2068)	0.9970 (0.9954–0.9980)

The table shows the diagnostic performance of TCRγδ and sCD3⁻ IELs as independent variables, according to the established cutoffs from ROC curves (≥15% for TCRγδ and ≤6% for sCD3⁻ IELs).
AUC, area under the curve; CD, celiac disease; CI, confidence interval; IEL, intraepithelial lymphocytes; LR, likelihood ratio; NPV, negative predictive value: OR, odds ratio; PPV, positive predictive value; TCRγδ, gamma-delta T-cell receptor.

Table 3. Disease probability of lymphogram analysis combining both dichotomized variables TcRγδ and sCD3⁻ IELs

	Active CD		Gluten-free diet	
Lymphogram	Disease probability (95% CI)	Individuals % n = 602	Disease probability (95% CI)	Individuals % n = 92
TcRγδ < 15 and sCD3⁻ > 6 nonceliac lymphogram	0.5 (0.06–1.8)	0.33 (2/602)	4.6 (2.8–7.4)	20.6 (19/92)
TcRγδ ≥ 15 and sCD3⁻ > 6 partial lymphogram	21.7 (13.4–32.1)	2.9 (18/602)	38.1 (28.8–47.1)	43.0 (40/92)
TcRγδ < 15 and sCD3⁻ ≤ 6 partial lymphogram	76.3 (63.0–86.8)	6.8 (42/602)	31.6 (12.6–56.6)	6.5 (6/92)
TcRγδ ≥15 and sCD3⁻ ≤ 6 celiac lymphogram	100 (63.0–86.8)	89.7 (540/602)	100 (87.0–100)	29.3 (27/92)

Four different lymphogram profiles that combines the TCRγδ and sCD3⁻ IELs dichotomized variables according the cutoff values. Disease probabilities are calculated for the active CD group and for the group on gluten-free diet. Individual % (the number of patients in a group that fit the corresponding lymphogram profile/total number of patients in the group).
CD, celiac disease; CI, confidence interval; IEL, intraepithelial lymphocytes; TCRγδ, gamma-delta T-cell receptor.

TCRγδ in the ongoing active immunological process elicited by gluten.

Approximately 53% (25/47) of the IEL analyses in their first diagnostic biopsy of patients with potential CD fit a celiac lymphogram pattern, whereas 43% (20/47) adopted a partial lymphogram due mainly to a ≥15 increase in TCRγδ % with sCD3⁻ % ≥6. When comparing the 2 patterns, the probability of developing villous atrophy was 1.5-fold higher for the patients presenting a celiac lymphogram pattern (56% vs 35%), which reinforces the pivotal role of the decline in sCD3⁻ IELs in achieving an active disease state. Only 2 patients of the total potential CD cohort had a nonceliac lymphogram: 1 was a first-degree family relative of a patient with CD with fluctuating serology, whereas the other became seronegative and was lost to follow-up.

Figure 3 describes the potential CD cohort progression. All patients in the potential CD cohort who developed villous atrophy (n = 17) presented a celiac lymphogram at the time of the second follow-up biopsy, with a drastic drop in the sCD3⁻ IEL subset, whereas the patients in the potential CD cohort who were clinically considered to still have potential CD after a second biopsy tended to preserve higher sCD3⁻ IEL subset densities in these lymphograms. The mean time between the first diagnostic biopsy and the second follow-up biopsy was 45 months (range 12–216 months).

DISCUSSION

The small bowel IEL compartment experiences profound changes in the pathogenesis of CD (40,41). The introduction of flow cytometry in clinical analyses has enabled further phenotypic and functional characterization of IEL subsets (30,32,42). Our group coined the term "celiac lymphogram" to describe the near-pathognomonic imbalance in the ratio of TCRγδ IELs vs sCD3⁻ IELs in pediatric patients with CD (31,43).

In this study, we were able to confirm the value of the lymphogram as a diagnostic tool not only for an ongoing active celiac process but also for remission stages in patients on a GFD and the diagnosis of potential celiac stages along the disease's natural history.

Approximately 90% of the active CD group had a celiac lymphogram, with a 100% probability of disease, whereas none of the individuals in the nonceliac group fulfilled this phenotypic profile. The remaining 10% of patients in the active CD group had a partial lymphogram, associated with a lower probability of disease. In this scenario, isolated low CD3⁻ IEL counts (<6%) yielded a higher sensitivity and specificity for CD detection than the isolated TCRγδ density evaluation, which explains the higher probability of active CD when a partial lymphogram was found to be mainly because of decreased sCD3⁻ IEL counts (≤6%) than when it was mainly because of a ≥15% increase in TCRγδ density (76.3% vs 21.7%). Therefore, in the nonceliac pediatric group used as a control, an isolated increase in TCRγδ density was a more frequent finding than a decreased density of sCD3⁻ IELs, as has occasionally been found in other situations, such as cow's milk intolerance, food allergy, giardiasis, cryptosporidiosis, Sjögren syndrome, and IgA deficiency (44–46). However, defining diagnostic cutoffs in the adult population will require further analysis (33), given that environmental inflammatory factors and therapeutic interventions frequently interfere in IEL compartment homeostasis (47).

In the GFD group, only 29% of the patients displayed a celiac lymphogram, whereas approximately 50% presented a partial lymphogram, mainly because of an isolated ≥15% increase in TCRγδ density, as an unequivocal marker of a celiac condition,

Table 4. Statistical performance of duodenal lymphogram in CD prediction

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Celiac lymphogram[a]	89.7 (87.3–92.1)	100	100	88.3 (85.6–91.1)
Celiac + partial lymphograms[b]	99.7 (99.2–100)	83.4 (80.0–86.8)	88.5 (86.1–90.9)	99.5 (98.8–100)

CD, celiac disease; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.
Diagnostic performance of the complete celiac lymphogram when
[a]Both variables, TCRγδ and sCD3, fit into the cutoff values.
[b]When partial lymphograms are also considered.

accompanied by a moderate rise in sCD3⁻ IEL density above 6%, as a useful marker to monitor GFD compliance. However, 20% of our patients on a GFD had a nonceliac lymphogram pattern, which indicates a natural tendency to resolve the TCRγδ/CD3⁻ imbalance after gluten withdrawal. This finding also emphasizes the relevance of the presence of gluten in the diet when interpreting the initial diagnosis of CD in a patient. An increase in TCRγδ IELs is a well-documented finding in celiac duodenal mucosa (48–50), and their numbers remain increased long after initiating a GFD (51).

In our potential CD cohort, we found evidence of the dynamic changes in TCRγδ and sCD3⁻ IELs along the natural progression of this celiac condition. An expansion of TCRγδ IELs has been a constant hallmark in the lymphograms of patients with potential CD at any time of their progression (52,53). A recent report by Auricchio et al. (54) considered high TCRγδ levels to be a risk factor for developing atrophy. All patients with potential CD who progressed to active CD showed a drastic drop in sCD3⁻ IELs in the second confirmatory biopsy, whereas a tendency to preserve higher levels of sCD3⁻ IELs was observed in the group that remained as having potential CD. High sCD3⁻ IEL densities seem to protect against progression to villous atrophy. In addition, all patients who became symptomatic and who started a GFD without histological confirmation had a celiac lymphogram profile at the initial diagnostic biopsy, highlighting this profile as a risk marker to progress to an overt celiac process.

This study was not prospective but rather the result of the routine follow-ups of patients with CD in our clinical practice. It

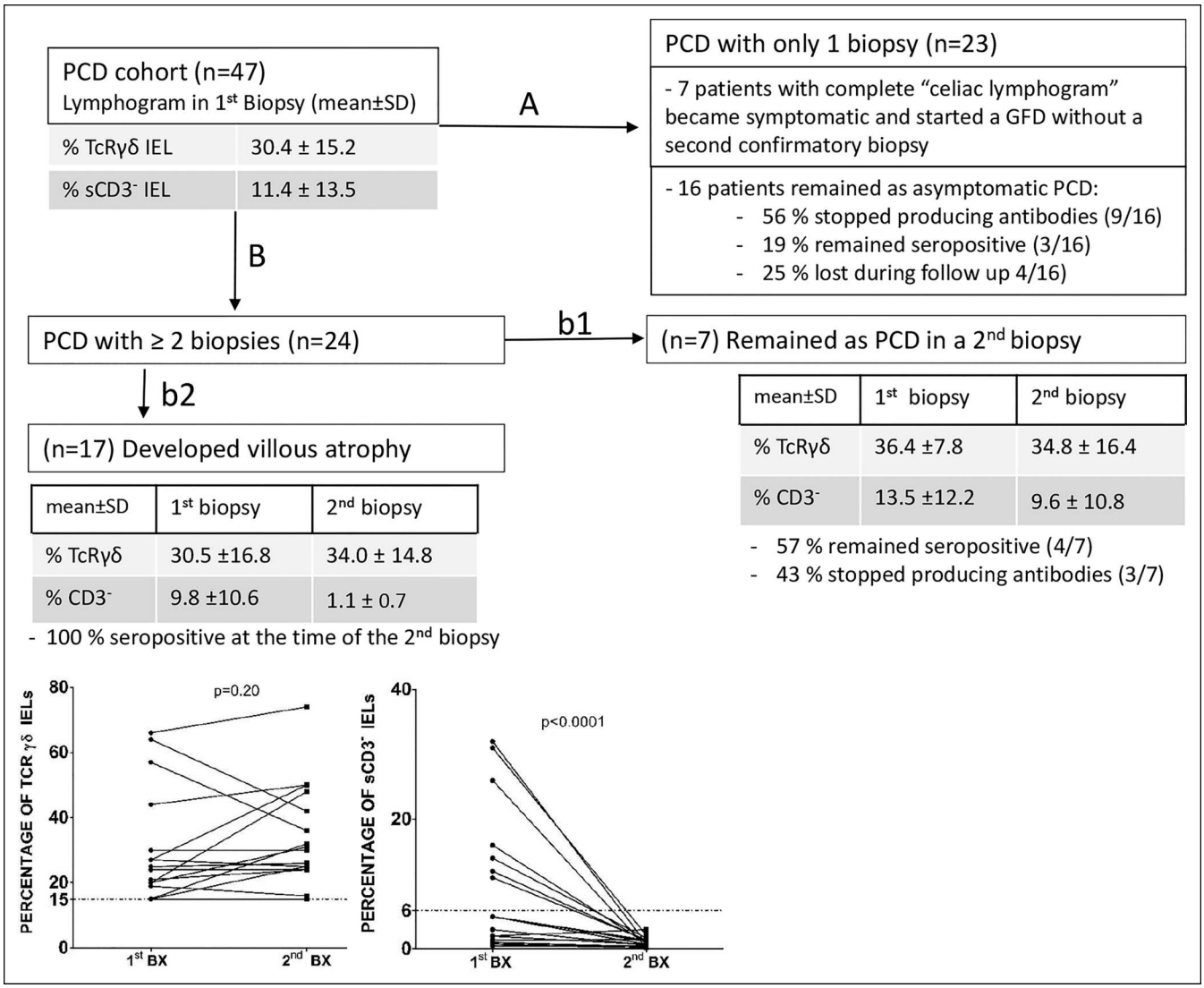

Figure 3. Flow chart of the potential celiac disease (PCD) cohort progression. The table in the upper left corner shows the mean densities of the TCRγδ and sCD3⁻ IELs at the first diagnostic biopsies of the patients included in this cohort. (**a**) The progression of patients with PCD with only a first diagnostic biopsy and on a gluten-containing diet. (**b**) The progression of patients with PCD for whom a second follow-up biopsy was clinically indicated: (**b1**) patients who remained as having PCD after the second follow-up biopsy. The table contains the comparative lymphograms between the first and second biopsies and the IgA antitransglutaminase progression and (**b2**) patients who progress to overt celiac disease. The table shows the comparative lymphograms between the first and the second biopsies and these patients' final serological status. The figure shows a matched-pairs distribution of TCRγδ and CD3⁻ IEL subsets corresponding to the first diagnostic and second follow-up biopsies from these patients with PCD who developed villous atrophy. First biopsy (BX): initial biopsy at diagnosis; second BX: a second follow-up biopsy.

was therefore difficult to estimate time intervals, such as the time required to develop epithelial lesions once sCD3$^-$ IEL counts have dropped and to estimate the cumulative incidence of villous atrophy. In our potential CD cohort, 17 of the 47 children progressed to proven villous atrophy (36%), in line with reported ratios (54–56).

Each celiac patient develops a unique immunological response elicited by gluten, presumably determined by genetic, microbiome, dietary, and other environmental factors (51,57), which result in sequential and dynamic changes in IEL subsets with functional repercussions and different disease outcomes. CD3$^+$ TCRγδ IELs expand in all celiac conditions and remain increased long after gluten withdrawal; however, their pathogenic role in CD remains elusive. Recent reports have described changes in the receptor repertoire of the resident TCRγδ IELs induced by celiac inflammation (58,59), with functional reprograming toward regulatory and inflammatory pathways (60–63). Our results support that a relative increase of TCRγδ IELs is a hallmark finding in all presentations of CD (Figure 2a), even if their absolute values decrease from the initial peak when celiac patients maintain a GFD. Gluten intake might therefore be a pivotal modulator of TCRγδ functionality and/or numbers. The sCD3$^-$ IELs represent a heterogeneous compartment that contains a diversity of innate lymphoid cell subtypes and lymphoid precursor cells, with still poorly defined functions (42,64,65). This sCD3$^-$ IEL subset is highly represented in healthy mucosa (47) and drops drastically in active CD. Our study showed the decreasing tendency of this subset coinciding with mucosal lesions in patients with potential CD and its increasing tendency in healing mucosa in patients after a GFD. The sCD3$^-$ IEL subset level is a sensor of the inflammatory cascade responsible for villous atrophy and could be considered a biomarker in the clinical management of potential CD.

In conclusion, once a diagnostic or follow-up biopsy is clinically indicated, the lymphogram analysis by flow cytometry performed in a single duodenal biopsy sample is a rapid, simple, and inexpensive method that allows a higher yield from the whole diagnostic procedure.

1. The lymphogram confirms active CD, conferring specificity to the histological and serological findings, not only in typical presentations but also in asymptomatic patients and in the clinically atypical forms. This confirmation is extremely useful in cases of ambiguous histology and can assist in the diagnosis in cases of seronegative CD.
2. The lymphogram identifies potential CD in suspected patients without histological lesions. A celiac or partial lymphogram confirms the increase in TCRγδ IEL density as a celiac marker, whereas the density of sCD3$^-$ IELs acts as a risk marker for progression to an active form.
3. The lymphogram can be highly useful in the differential diagnosis of CD vs other enteropathies.

CONFLICTS OF INTEREST

Guarantor of the article: Cristina Camarero, MD, PhD.

Specific author contributions: C.C.: responsible for patient recruitment, clinical data input, and critical reading and discussion of the manuscript. A.D.A.: provided analytical data, statistical and cytometric data analysis, and graphic work. C.C.-H.: contributed to database interpretation. A.M.: conducted statistical analysis. B.R.: provided clinical data and critical reading of the manuscript. F.L.: helped with the edition and critical reading and discussion of the manuscript. G.R.: designed, conducted the study, and drafted the manuscript. All the authors approved the final version of the article.

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Potential competing interests: None to report.

Study Highlights

WHAT IS KNOWN

- ✓ Intestinal histological lesions in celiac disease (CD) are characteristic but not pathognomonic.
- ✓ A seemingly normal or mildly abnormal intestinal mucosa does not rule out a diagnosis of CD.
- ✓ Flow cytometry of duodenal intraepithelial lymphocytes (IELs) represents a powerful diagnostic tool for CD, given the characteristic IEL imbalance (celiac lymphogram).

WHAT IS NEW HERE

- ✓ By analyzing a large pediatric cohort, this report provides the first pediatric reference values for IEL subsets, which enables the interpretation of the lymphogram in the differential diagnosis of pediatric CD.
- ✓ The celiac lymphogram is a highly specific signature of the ongoing immunopathogenic process in children.
- ✓ An increase in gamma-delta T-cell receptor IEL density remains the pivotal finding in all forms of CD.
- ✓ The density of CD3 surface-negative IELs is a biomarker of mucosal integrity, useful in monitoring the natural history of CD.

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