International Research Journal of Pharmacy and Medical Sciences
ISSN (Online): 2581-3277

Quantifying Intraepithelial Lymphocytes with CD3 Marker: Insights into Marsh Classification in Celiac Patient

Rose Razzaq Fadhil[1], Hadi Muhammad Ali Almosawi[2]
[1]Pathology dept. Al-Hilla Teaching Hospital, Al-Hilla, Iraq
[2]Department of Pathology, Hammurabi medical college, University of Babylon, Babylon, Iraq
Email address: rosefadhil75@gmail.com

Abstract—Background*: Early celiac alterations may be difficult to diagnose via mucosal biopsy due to the overlapping nuclei of enterocytes and intraepithelial lymphocytes (IELs). CD3 was suggested as an intraepithelial lymphocytosis marker.* ***Aim****: To evaluate CD3 immunohistochemical marker for IELs to confirm Marsh categorization of celiac disease.* ***Methods****: A cross-sectional observational comprised 52 routine diagnostic duodenal biopsies from Al-Hilla Teaching Hospital, Al-Imam Al Sadiq Hospital, and private laboratories that were "consistent with" or "suggestive of" celiac disease. Clinical and demographic data from patient records were obtained for all instances. All sections were CD3-stained immunohistochemically. IELs were examined for 100 enterocytes in hematoxylin and eosin (H&E) slides and CD3.* ***Results****: Patients aged 18-48 years, with a mean of 25.2 (±12.53) years, and a male-to-female ratio of 1:1.6. Marsh I was 17 (32.7%), Marsh II and IIIc were 10 (19.2%), and Marsh IIIa and b were 15.4% and 13.5%. In H&E sections, the IELs count was 38.8 (± 7.4) cells, whereas CD 3 immunostaining showed 47.10 (±7.0) cells (P<0.001). CD3 IELs counts correlated well with H&E (r=0.842). However, CD3 counts were consistently higher, with a mean difference of -8.29 cells and significant dispersion (P<0.001). CD3 detected more lymphocytes than H&E regardless of age, sex, or Marsh group. Higher significance was seen in Marsh I (P<0.001) and II (P=0.005).* ***Conclusions****: CD3 immunostaining improved IELs counting. Marsh I shows a greater CD3 IELs count than H&E. Thus, CD3 immunostaining may diagnose early celiac disease in individuals with positive serology and Marsh 0 or borderline lymphocytosis.*

Keywords*— Celiac disease, intraepithelial lymphocyte count, CD3.*

I. INTRODUCTION

Celiac disease is a devastating inherited autoimmune illness with no cure or medication. Celiac disease is increasing globally with a pooled frequency of 1.4% [1], although prevalence may vary greatly across nations despite close proximity. High incidence in the Middle East affects high-risk and general populations. This is due to diets high in barley and wheat and DR3-DQ2 haplotypes. [2].

Celiac disease (CD) is an immune-mediated illness that is induced by gluten. The mucosal pathology seen in celiac disease encompasses a range of abnormalities, such as the presence of intraepithelial lymphocytosis (IELs), the degeneration of the intestinal villi, the excessive proliferation of crypts, the increased presence of mononuclear inflammatory cells in the lamina propria [3, 4]. However, these changes are not exclusive to celiac disease, since they may also be caused by several other illnesses such as immunodeficiency, infection-related disorders, inflammatory bowel disease, and autoimmune conditions [5]. A patient is diagnosed with CD when they have a genetic predisposition, show compatible clinical symptoms, test positive for very specific celiac serologic results, have duodenal biopsies that confirm enteropathy, and experience improvement in following a gluten-free diet [6].

Diagnosing CD in the presence of mild histological lesions (Marsh 1) may present challenges owing to the limited specificity and sensitivity of lymphocytic enteritis and serology [7]. However, it is crucial to identify Marsh 1 patients with CD due to the similarity of their clinical manifestations to those of patients with atrophy, which can be reversed through the implementation of a gluten-free diet [8]. When there is uncertainty, especially when Marsh-1 or -2 classifications are used, other methodologies can be applied besides morphology [9]. The utility of special immunohistochemistry markers in identifying and characterizing lymphocytes has been investigated in several studies.

The purpose of this study was to evaluate tiny endoscopic biopsies in the diagnosis of celiac disease according to Marsh classification and to assess the value of CD3 immunohistochemical marker in the detection of intraepithelial lymphocytosis to confirm the diagnosis of celiac disease.

II. METHODS

This cross-sectional study included 52 Hematoxylin and eosin slides of routine diagnostic duodenal biopsies reported as "consistent with" or "suggestive of" celiac disease were retrieved from Al-Hilla Teaching Hospital, Al_Imam Al Sadiq Hospital, and private laboratories during the period between January- 1st and November -30th 2023. The study was approved by the Scientific Committee of Al-Hilla Teaching Hospital under the registration number (EAC-6332).

All the cases were reviewed for histological features specifically IEL, crypt hyperplasia, and villus atrophy, and classified according to Marsh criteria by a consultant pathologist. Clinical and demographic data were collected for

International Research Journal of Pharmacy and Medical Sciences
ISSN (Online): 2581-3277

all cases from associated patients' notes. Samples with inadequate material and those with no clinical correlation were excluded from the study.

Lymphocyte counting: Under a bright field and at x400 magnification, H&E slides were assessed for the IEL in 100 enterocytes. Then, an image was taken for the field and IELs were recounted using Microsoft photo software. For each case, the average of IELs in the upper third of three villi was recorded. In cases with complete villus atrophy, IELs of the surface epithelium were counted. Thirty lymphocytes per 100 epithelial cells or more were considered as intra-epithelial lymphocytosis [10]. Recorded counts were subsequently transferred to an Excel sheet.

CD3 immunohistochemistry: A 4-µm slice was prepared for immunohistochemistry (IHC) using Polyclonal Rabbit Anti-Human anti-CD3 (Code A0452, Dako, Glostrup, Denmark) according to the instructions provided by the manufacturer.

Sections were initially dewaxed through graded alcohol. Epitope retrieval was conducted by subjecting them to a heating process (water bath) using a target recovery solution at a pH of 9 (Code S2368, Dako) for 20 minutes. Primary antihuman CD3 was applied to each section at a dilution of 1:50 and incubated for 30 minutes at room temperature. Subsequently, the sections were washed with a washing buffer. Dako system was used for detection, and DAB was utilized in accordance with the manufacturer's instructions for visualization. Sections were counterstained with Hematoxylin, dehydrated, and mounted in synthetic resin. As a negative control, an isotope-matching secondary antibody (0.5 mg ml) was substituted for the primary antibody, and each staining run included an external positive control (reactive cervical lymph node).

Interpretation: Slides were examined and reassessed for Marsh classification without knowledge of the clinical and H&E details. The field for CD3 labeled cell counting was selected with the help of the prior image taken for the H&E slides. Using the same method, CD3 labeled lymphocytes were counted under a light microscope, then imaged and counted with Microsoft Photo. Data were then transported to the Excel sheet. Then the two methods were compared using statistical analysis.

Statistical analyses: All statistical analyses were carried out using Statistical Package for Social Sciences (IBM SPSS) software version 25. Continuous variables were expressed as mean ±SD. Categoric data was presented as frequency and percentage.

As the data were not normally distributed, the Mann-Whitney and Kruskal Wallis tests were used to compare groups. The Wilcoxon signed-rank test was utilized to compare the matched H&E and CD3 scores. Using Pearson's test for bivariate correlation, the association between various examination methods was examined. The mean of IEL count and the mean difference for both methodologies were calculated and Bland Oltman blot was constructed. T-test was used to determine whether or not there were differences between the two methods. P values less than 0.05 were statistically significant.

III. RESULTS

Study group characteristics

A total of 52 cases were collected. Patients' age ranged between 18 and 48 years with a mean of 25.2 (±12.53) years, 17 (32.7%) of them were younger than 20 years, and only one (1.9%) was older than 60 years. Females constituted more than two-thirds of the cases (61.5%) with a male-to-female ratio of 1:1.6. Patients had different combinations of symptoms and signs, however, the most frequent was malabsorption signs (weight loss, pallor, and iron deficiency anemia) reported in 22 (42.3%) followed by abdominal pain 19 (36.5%) and diarrhea 18 (34.6%), further details are illustrated in Table 1.

TABLE 1. Demographic and clinical features.

Characteristics		Frequency	Percent
Age	≤19	17	32.7
	20-39	28	53.8
	40-60	6	11.5
	>60	1	1.9
Sex	Female	32	61.5
	Male	20	38.5
Symptoms	Abdominal pain	19	36.5
	Diarrhea	18	34.6
	Constipation	2	3.8
	Malabsorption signs	22	42.3
	Vomiting	3	5.8
	Screening\ known case	3	5.8
	Bloating\ abdominal distention	2	3.8
	Diabetes	2	3.8
	Fatigue	4	7.7

Marsh Classification

Approximately a third of the collected cases were Marsh one 17 (32.7%), Marsh II and IIIc constituted 10 (19.2%) of the cases each. Less frequent, Marsh IIIa and b accounted to 8 (15.4%) and 7 (13.5%) respectively as further shown in Figure 1.

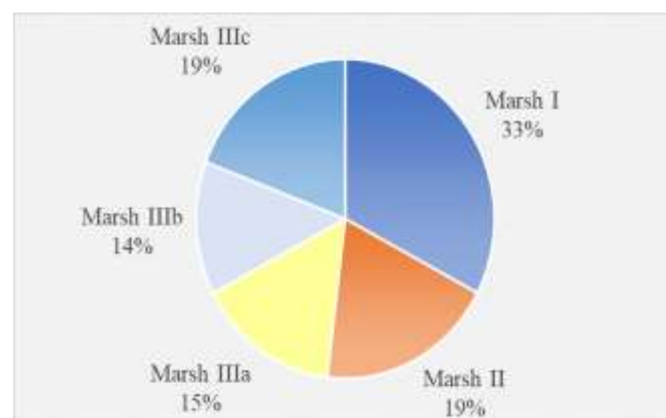


Figure 1. Marsh classification of the study group.

Intraepithelial lymphocytes assessment

The overall mean IEL count in H&E sections was 38.8 (± 7.4) cells /100 enterocytes compared to 47.10 (±7.0) cells using CD 3 immunostaining which was statistically different (P<0.001). As Table 2 shows, there was one case (1.9%) with IEL count of less than 25 cells and 4 (7.7%) with borderline

IEL when assessed in H&E sections. By contrast, all cases illustrated ≥30 IEL using CD3.

TABLE 2. Intraepithelial lymphocytes evaluation by H&E vs CD3

	Normal <25 IEL/100 EC		Borderline 25-29 ILC/100 EC		Lymphocytosis ≥30 ILC/100 EC	
	No	Frequency	No	Frequency	No	Frequency
H&E	1	1.9%	4	7.7%	47	90.4%
CD3	0	0%	0	0%	52	100%

There was a strong correlation between IEL count using H&E and CD3 (r=0.842), as shown in Figure 2.

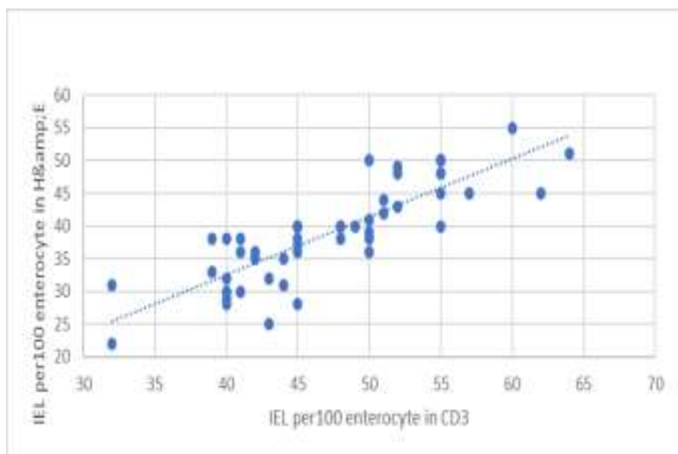
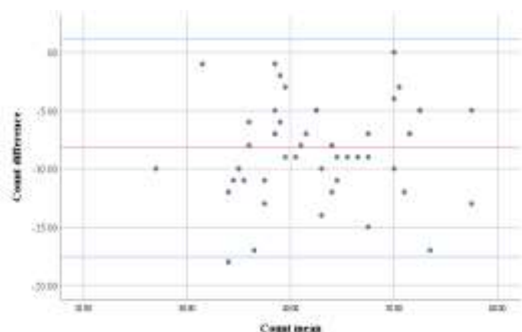


Figure 2. The correlation between H&E and CD3 in the assessment of lymphocyte r=0.842, P<0.0001.

However, counts with CD3 were consistently higher with a mean difference of -8.29 cells, and the dispersion around the mean was significant (P<0.001) as illustrated in Bland Altmann Figure 3.



	Mean	95%Confidence Interval		P value
		Lower	Upper	
Difference between two methods	-8.29	-9.426	-7.150	<0.001

Figure 3. Bland Oltmans plot showing the variation between H&E and CD3 in the assessment of intraepithelial lymphocytes.

The paired comparison showed a significantly higher number of lymphocytes detected with CD3 compared to H&E regardless of the age group, sex, or Marsh category. However, higher significance was identified in Marsh I (P<0.001) and II (P=0.005) as shown in in Table 3.

When comparing IEL count in H&E across different variables, no significant difference was observed between age groups (P=0.945) and sex (P=0.888). However, it was significantly higher in Marsh III compared to II and I. Similarly, IEL count using CD3 was not significantly different in age groups and sex but showed higher significance in Marsh categories (P=0.001) as further detailed in Table 3.

TABLE 3. The difference in Intraepithelial lymphocytosis assessment by the two methods relative to demographic and Marsh classification.

Variables		Mean IEL H&E	Mean IEL CD3	Mean Difference	P value [b]
Age	≤19 years	39.0 (7.03)	47.0 (7.27)	-8.0 (4.2)	<0.001
	> 19 years	38.71 (7.72)	47.14 (7.02)	-8.42 (4.08)	<0.001
	P value [a]	0.945	0.984	0.977	
Sex	Female	39.16 (7.58)	47.59 (6.35)	-8.43 (4.62)	<0.001
	Male	38.25 (7.34)	46.3 (8.11)	-8.05 (3.13)	<0.001
	P value [a]	0.888	0.637	0.977	
Marsh classification	I	35.29 (7.29)	44.71 (6.18)	- 9.4 (3.42)	<0.001
	II	35.5 (3.13)	41.8 (2.25)	-6.3 (2.86)	0.005
	IIIa	41.50 (9.24)	50.25 (7.14)	-8.75 (5.03)	0.012
	IIIb	39.86 (5.11)	49 (9.69)	-9.14 (5.11)	0.018
	IIIc	45.20 (6.07)	52.6 (4.195)	-7.4 (4.03)	0.007
	P value [c]	0.009	0.001	0.302	

[A] comparison between age groups and sex groups using the Mann Whitney test, [b] comparison between the H&E and CD3 method using the Wilcoxon Signed Rank test; [C] comparison between Marsh grades using the Kruskal Walls test

IV. Discussion

During the initial phases of celiac disease, the histological assessment and ultimate diagnosis are predicated on the presence of mucosal intraepithelial lymphocytosis of the small intestine [11]. This procedure is relatively challenging, and the contrast between the blue color of the lymphocyte nucleus and that of the epithelial cell nucleus can be inconclusive at times [9]; therefore, CD 3 has been proposed as an efficient method for assessing intraepithelial lymphocytosis in early stages of celiac disease.

In the current study, diagnostic Marsh II and III accounted for 19% and 49% respectively based on H&E, while suspicious for celiac disease Marsh I constituted 33%. A similar distribution of the Marsh grouping was reported by other local studies. Hammo *et al.* study included 52% Marsh III, 20% Marsh II, and 30% Marsh I [12]. A lower Marsh II rate (8%) was reported by Hamdi and colleagues on the advantage of the class III rate (56%) and a similar proportion of Marsh I (30%) [13]. IEL was confirmed in 90.4% of the cases while the count was borderline (25-29 cells/100EC) in 7.7% and in one case (1.9%) the count was < 25 cells/100EC. In the presence of villus atrophy and crypt hyperplasia, intraepithelial lymphocytosis confirms the presence of celiac disease even in the absence of serological study, however,

International Research Journal of Pharmacy and Medical Sciences
ISSN (Online): 2581-3277

missing intraepithelial lymphocytosis in the early stage of the disease when it is the only histopathological change may lead to misdiagnosis and may prohibit the further serological investigation. Therefore, CD3 immunolabeling highlights this early change and improves the early diagnosis, particularly in those with persistent signs and symptoms [9]. In keeping with this, we found that using CD3 immunostaining was associated with persistent higher counts of IEL and a mean difference of 8.29 cells, compared to H&E, nevertheless, a strong correlation between the two methods was observed (r=0.842). The involvement of CD3 in assessing IEL in duodenal biopsy samples, which are suspected to have celiac disease, has been examined in various regions of Iraq. Shihab *et al*. described the expression of CD3 and CD20 in the lymphocytic population in a sample of 60 patients in Al-Qadisiyah governorate, however, they did not show a correlation with Marshal groups. Further, the authors did not use intra-epithelial lymphocyte count, instead, they estimated the level of expression in the lining epithelium and lamina propria as (<50%, 50%, and >50%) for CD3 expression and reported that 16% had low CD3 expression and 56.7% had >50% expression. They further highlighted that the CD20 expression (B lymphocytes) pattern was different and mostly involved the crypts [14]. A larger retrospective study was conducted in Ninawa governorate including 100 duodenal biopsies from archives of 2019 -2020. The study has correlated the significance of CD3 expression in intraepithelial lymphocytic population to histopathological changes in Celiac disease. Authors observed differences in intraepithelial lymphocyte count using H&E and CD3 expression only in the Marsh I group, where 2/30 (6%) cases diagnosed as Marsh I by H&E showed CD3 count less than 30 cells /100 EC and 11/30 (36%) where <25 cells /100 EC. By contrast, all other Marsh groups had consistent H&E and CD3 counts over 30 cells /100 EC [12]. The exact difference between the two methods, however, was not addressed since cutoffs were used rather than actual counts. A comparable Indian study confirmed CD 3 positivity in all Marsh groups with a distribution concentrated on both the lateral aspect as well as the tip of the villous [15]. In a Western study, Mubarak and colleagues found that when comparing H&E stains alone to CD3 stains, there were significant variations between the two methods in 12.6% (20/159) of patients. Out of 93 Marsh III and 3 Marsh II cases evaluated by CD3, 9 (9.4%) were underdiagnosed by H&E (8 were considered as Marsh 0 and one was diagnosed as Marsh I). The probability of these individuals being over-diagnosed was quite low, considering that all of them tested positive for celiac disease serology [9].

We did not see a significant difference between IEL counting in either method in terms of patients age (H&E, P=0.945, CD3, P=0.984) and sex (H&E, P=0.888, CD3, P=0.637), although, counts using CD3 was significantly higher than that using H&E (P<0.001) when each variable compared individually. In terms of Marsh groups, there was a significant increase in IEL counts in Marsh IIIc compared to Marsh I when using H&E (P=0.009) and CD3 (P= 0.001). The difference between IEL counts using the two methods was significantly different in each Marsh group but the highest significance was observed in Marsh I. Although Marsh I changes do not occur exclusively in celiac disease and positive serology is mandatory to confirm the presence of celiac disease, early diagnosis can initiate early management and reduce the need for gluten challenge which may lead to the mucosal deterioration [16].

V. CONCLUSION

A substantial association between intraepithelial lymphocyte count and CD3 was observed, with the maximum significance in Marsh I, and CD3 immunostaining was shown to be more efficient than H&E in assessing IEL count.

REFERENCES

1. Singh, P., et al., *Global prevalence of celiac disease: systematic review and meta-analysis.* Clinical gastroenterology and hepatology, 2018. 16(6): p. 823-836.
2. El-Metwally, A., et al., *The Epidemiology of Celiac Disease in the General Population and High-Risk Groups in Arab Countries: A Systematic Review.* BioMed Research International, 2020. 2020: p. 6865917.
3. Ludvigsson, J.F., et al., *The Oslo definitions for coeliac disease and related terms.* Gut, 2012. 26(1): p. 43-52.
4. Lindfors, K., et al., *Coeliac disease.* Nat Rev Dis Primers, 2019. 5(1): p. 3.
5. Aziz, I., et al., *The clinical and phenotypical assessment of seronegative villous atrophy; a prospective UK centre experience evaluating 200 adult cases over a 15-year period (2000–2015).* Gut, 2017. 66(9): p. 1563-52.
6. Oxentenko, A.S. and A. Rubio-Tapia, *Celiac disease.* Mayo Clinic Proceedings, 2019. 94(12): p. 2556-2571.
7. Vivas, S., et al., *Age-related clinical, serological, and histopathological features of celiac disease.* Official journal of the American College of Gastroenterology| ACG, 2008. 103(9): p. 2360-2365.
8. Ruiz-Ramírez, P., et al., *Intraepithelial Lymphocyte Cytometric Pattern Is a Useful Diagnostic Tool for Coeliac Disease Diagnosis Irrespective of Degree of Mucosal Damage and Age-A Validation Cohort.* Nutrients, 2021. 13(5): p. 1684.
9. Mubarak, A., et al., *Immunohistochemical CD3 staining detects additional patients with celiac disease.* World J Gastroenterol, 2015. 21(24): p. 7553-7557.
10. Sergi, C., F. Shen, and G. Bouma, *Intraepithelial lymphocytes, scores, mimickers and challenges in diagnosing gluten-sensitive enteropathy (celiac disease).* World J Gastroenterol, 2017. 23(4): p. 573-589.
11. Kondrashova, A., et al., *Lower economic status and inferior hygienic environment may protect against celiac disease.* Annals of medicine, 2008. 40(3): p. 223-231.
12. Hammo, S.H. and M.A. Hayawi, *The Significance of CD3 Marker in the Diagnosis of Celiac Disease.* Annals of the College of Medicine, Mosul, 2021. 42(2): p. 99-108.
13. Hamdi, E.A., K.T. Tawfeeq, and K.W.A. Khattab, *Immunohistochemical Evaluation of CD20 in Patients with Celiac Disease in Nineveh Province.* International Journal of Current Medical And Applied Sciences, 2021. 34(2): p. 35-38.
14. Shihab, M.A. and H.M. Enaya, *Immunohistochemical evaluation of CD3 T-cell lymphocyte and CD20 B-cell markers in Iraqi patients with celiac disease.* EurAsian Journal of BioSciences, 2020. 14(1): p. 2023-2028.
15. Iftikhar, R., et al., *Histopathological and immunohistochemical analysis of small intestinal biopsies in adults suspected of celiac disease.* Journal of the college of physicians and surgeons-Pakistan, 2016. 26(10): p. 827-830.
16. Wahab, P.J., et al., *Gluten challenge in borderline gluten-sensitive enteropathy.* Official journal of the American College of Gastroenterology| ACG, 2001. 96(5): p. 1464-1469.