



DIAGNOSTIC STUDY OF THE PATIENTS WITH CELIAC DISEASE VIA USING DUODENAL BIOPSY DEPENDING ON INTRAEPITHELIAL LYMPHOCYTES ALONE

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Abstract: Objective: The aim of the present study was reported to evaluate the dispersal pattern of T-bet stained intraepithelial lymphocytes (IELs) in the patients with the celiac diseases (CD).

Methods: 250 patients had participated in this study. These patients were diagnosed with the CD by relying on the assimilation of clinical pathology features. A routine process of duodenal biopsied tissues were conducted onto the formalin-fixed paraffin-embedded (FFPE) blocks. Hematoxylin and eosin (H and E), and T-bet were used to prepare and stain the sections. The distribution of intraepithelial lymphocytes was evaluated. IBM Statistical Package for the Social Sciences (SPSS) analytic software was used to analyze the results.

Results: T-bet stained cells counts and the rise of intraepithelial lymphocytes P-value = 0.001 are significantly correlated. The IELs distribution was regular distribution in the majority of cases and only one case had IELs distribution in the body of the villi. The IELs distribution pattern in CD cases might be an early indicator of the disease.

Conclusions: This can be used in the Diagnosing and follow up of the patients with celiac disease.

Keywords: Celiac Disease, Intraepithelial Lymphocytes, Hematoxylin, Eosin, T-bet, Coeliac disease, Diagnosing, Gluten sensitive.

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INTRODUCTION

Celiac disease (CD) also known as gluten sensitive enteropathy, celiac sprue or non-tropical sprue^{1,2}, is a chronic, immune-mediated disease occurring in genetically predisposed individuals due to an intolerance to gluten-containing foods, in particular, to some of its proteins, called gliadins found in wheat, rye, barley, and some varieties of oats³. This intolerance leads to abnormal immune response, which is followed by a chronic inflammation of the small intestinal mucosa with progressive disappearance of intestinal villi⁴.

CD is increasing in prevalence, which is estimated at 1:100 of the Western countries⁵. Its prevalence is rising in the Middle Eastern populations to 1.5:100⁶. Celiac disease may occur in adults and children with a female/male ratio of 3:1⁷.

The intestinal epithelial cells and IELs constitute the intestinal mucosal barrier, which is considered as the first line of defense in the host immune system. IELs possess hallmark features of tissue-resident T cells: they are long-lived non-migratory cells capable of rapidly responding to antigen challenges independent of T cell recruitment from the periphery. Gut-resident T cells have been implicated in the relapsing and remitting course and persisting low-grade inflammation of chronic gastrointestinal diseases, including celiac disease (Lutter et al., 2018). On routinely H and E stained sections, IELs have a basophilic nuclear chromatin pattern, irregular nuclear outline and clear peri-nuclear halo. Phenotypically, in normal individuals IELs are T lymphocytes, of which the majority 70% are CD8+ T cells and less than 20% are CD4+ T-cells. B cells are not present Of the

immunological functions of IELs are cytotoxic activity and secretion of cytokines^{8,9}.

The diagnosis of CD requires a joint clinicopathological approach; the recommended first line test is serology with immunoglobulin A (IgA) tissue transglutaminase, deaminated gliadin and/or endomysial antibodies. These serological tests show high levels of sensitivity and specificity, but biopsy is the gold standard to confirm the diagnosis¹⁰. It is important that these tests are performed before the introduction of a gluten-free diet¹¹. Although the classical histopathology changes of celiac disease with partial or total villous atrophy are well recognized, the pathology classification of celiac disease is changing, with recognition that celiac disease may show minimal pathology (normal architecture and an intraepithelial lymphocyte count / 100 enterocytes \geq 25). This entity is also described as lymphocytic duodenosis, and recommendation of follow-up serology testing is paramount in this condition¹².

Treatment of CD is strict and lifelong removal of the offending antigen, a gluten-free diet (GFD). Gluten describes the storage proteins (prolamins) from wheat, barley and rye. In some countries such as Australia and New Zealand, oats are also excluded from a GFD as they contain gluten-like proteins that are immunogenic in some CD patients¹³. A strict GFD resolves symptoms and normalizes CD-specific antibodies and enteropathy. However, maintaining satisfactory GFD compliance is challenging and many patients fail to achieve full disease remission¹⁴. Therefore, the development of more effective treatment and diagnostic approaches are major research goals.

An acquired chronic immune-mediated enteropathy is referred to as CD, which happens in a susceptible individual that shows an immune reaction towards gluten. The symptoms range from life-threatening to non-symptomatic. The classical features of the disease include a rise in the count of intraepithelial lymphocytes, crypt hyperplasia and villus atrophy. Not all celiac disease patients exhibit architectural changes in their intestinal villi. It is normal for celiac disease to be associated with a normal small bowel biopsy. It is crucial to find patients with celiac disease. To avoid complications of the disease, chronic iron deficiency anaemia, malignant neoplasms and osteoporosis.

IELs are non-recirculating, resident T-cells (1). IELs consist of many lymphocyte populations that exist at a close distance to the intestinal lumen located in the middle of enterocytes in the intestinal epithelium (2-5). IELs employ cytolytic functions for the elimination of cells that are infected, while damaged and regulatory functions are imparted to the remedial and restoration of the epithelium (5-9). IELs are adaptive and innate immune cells that give them the capability to survey the tissues by recognizing certain antigens and stress signals. In contrast, they also add to the inflammatory and destructive reaction to the tissue as seen in CD.

Many studies investigated the possibility that celiac disease is of Th1 mediated autoimmune reaction (10-14). The predominant cytokine made by T-cell isolated from patients with celiac disease mucosa functions significantly in the formation of lesion known as IFN gamma¹⁵. Other studies also supported the view regarding the intestinal lamina propria isolated T cells, underlined with the rise of cytokines IFN gamma and IL-18 (15-19). In T-box transcription, a T-bet is a definite factor. T-box transcription associates with the

expression of IFN gamma in Th1 and natural killer cells¹⁶. Several reports recommended that the factor of T-bet transcription is regulated in active celiac disease, then reduce to regular levels in treated (on GFD) celiac disease patients. The transcription factor T-bet protein functions mainly in regulating the cytokine for the Th1 production¹⁷.

According to our more than 20 years' experience of reading duodenal biopsies of healthy individuals across ages, we can state that only very few lymphocytes can be usually seen among the epithelial cells. However, the IELs may vary during life and possibly in a circadian cycle. The IELs usually do not go over 5-10 per 100 epithelial cells in healthy individuals. The cut-off between pathological and normal has been decreased in the last three decades from 40 to either 20 or 25 lymphocytes per 100 epithelial cells. Between 5-10 and the pathological threshold (20 or 25), there is a gap that has probably been inadequately investigated. The unveiled and/or underlying causes of the "near normal" cases (5-20 IELs/100 epithelial cells) may be intriguing. The presence of scattered normal lymphocytes in the surface epithelium of the duodenum is not well understood, although the prominent role of the duodenum in assessing the epitopes present in the food should be considered^{18,19}.

Based on these findings and a finding in (24), CD is a response of Th1 mediated immune. We used the T-bet monoclonal antibody to study 40 cases with patients diagnosed with the disease and 10 patients where celiac disease was considered, but not yet confirmed. The study aims to examine the distribution pattern of the IELs in the duodenal villi of celiac disease patients. A 'regular distribution' is referred to as an equal distribution of the IELs alongside the villi and the tip of the villi. While IELs aggregation along the surface of the villi was documented as "aggregated in the base". And an aggregation of the IELs in the tip of the villi was considered that.

The villous character of the small bowel is intrinsically linked to the aim of an organism to increase its absorptive surface area. In early embryogenesis, development of the duodenal epithelium takes place from simple endodermal tubules between the 9th and 10th wk of gestation, when the epithelium converts to simple columnar epithelium. The epithelium ends its differentiation just 4-5 d before birth. The usual configuration of the duodenal mucosa contains slender structures protruding from the surface, with 3-5 times the height of the crypts. The patchiness of the lymphoid nodules or mucosa-associated lymphatic tissue (MALT) needs to be considered in assessing the duodenal histology and can constitute one of the first pitfalls in interpreting a small intestinal biopsy.

MATERIALS AND METHODS

T-bet, GATA3 and CD3 monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Dallas, Texas, and United States. Anti-tissue transglutaminase IgG - IgA and anti-deaminated gliadin IgG - IgA, was purchased from Generic Assay, EUROIMMUN AG, Germany.

250 samples have been taken from patients with CD. The duration time for the collected samples were 6 months (from August 2019 until February 2020). The samples have been collected from Karbala city (Al-hussainy hospital, medical laboratory of Al Sajjad and Al-Kafeel hospital). The samples

were formalin-fixed paraffin-embedded tissue (FFPE), which then treated and stained with H and E. All steps were performed at room temperature, and the manufacturer's instructions were followed in both the immunohistochemical and the ELISA procedures. All kits and chemicals were stored at 2-4 C° along the duration of the study.

RESULTS AND DISCUSSION

Based on Table 1, 190 out of 250 samples were females (76%) and the remaining were males (24%). This brings the ratio of the female to the male was 3.2:1. The patients' age ranges between 3 to 65 years old.

Table 1. Characteristics of the Patients.

Characteristics		Frequency	Percent%
Gender	Female	190	76.0
	Male	60	24.0
Age Groups	0-10	1	2.3
	11-20	12	27.3
	21-30	14	31.8
	31-40	9	20.5
	41-50	7	15.9
	>51	1	2.3
Marsh grade	0*	17	34.0
	1	10	20.0
	2	6	12.0
	3*	17	34.0

* Grade III in this table is referred to all grades of Marsh (IIIa, IIIb, IIIc).

* Grade 0 is referred positive serology patients and a borderline increase of IELs, which counted as (20-29/100EC) in this study.

Under the microscope, CD3 stained IELs showed a brown color, of the DAB, in contrast to the blue shown in the H&E

staining. The CD3 staining is membranous while the H&E staining is nuclear and cytoplasmic.

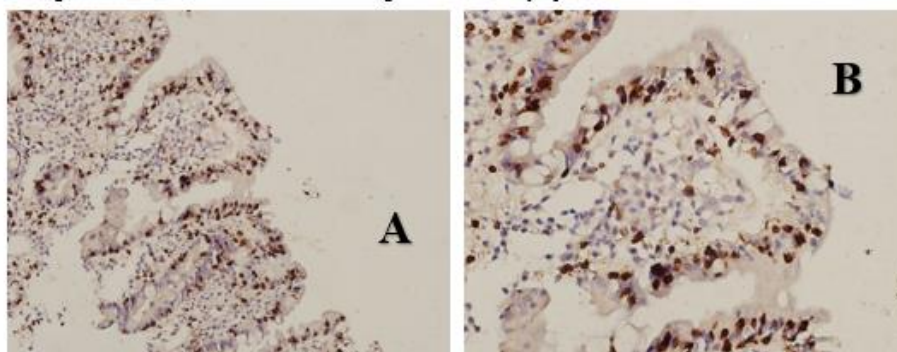


Figure 1. View of CD diagnosed patient Marsh IIIb, Expression of CD3 monoclonal antibody in IEL of duodenal villi in a cytoplasmic staining pattern. A: X200, B: X400.

In the lamina propria of the 250 selected cases, after the IHC technique was implemented the observed response of the immune cells to the CD3 was apparent, but in no more than 60% of all laminal cells. The distribution patterns of IELs were counted by the T-bet monoclonal antibody. **Table 2**

presents that 68% of the cases exhibited normal segregation of T-bet stained IELs all over the villi body, while one case (2%) exhibited an accumulation of IELs in the base of villi. This shows that there is a significant connection between grades of Marsh and the T-bet stained IELs distribution pattern.

Table 2. The Distribution pattern of T- bet stained IELs in correspondence with the grades of Marsh in the duodenal villi of celiac disease suspected patients.

Marsh grade	Distribution pattern of T-bet stained IELs in villi			
	Flat villi	Aggregated in the tip	Aggregated in the base	Regularly distributed
0	0	0	0	17
1	0	2	0	8
2	0	0	1	3
3*	3	10	0	6
Total	3	12	1	34

P<0.001

This study presented that the early stages of celiac disease can be distinguished. We examined the distribution pattern of the IELs. The regular segregation of IELs along the villi exhibits the feature of "luminopetal" distribution (17). This indicates that the IELs are of "crescendo" pattern with the rise of IELs

in the base of villus and loss of IELs at the upper part of the villus and tip (25-27). In this study, we classified the distribution pattern of the IELs into: regularly distributed (along the tip and sides of villus), aggregated in the tip and aggregated in the base of the side of the villi.

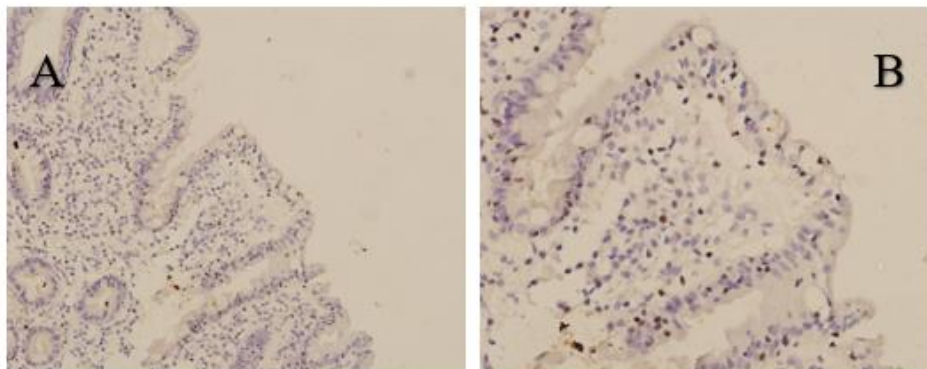


Figure 2. View of CD diagnosed patient Marsh IIIb. T-bet staining of the Th1 type IELs (the responsible in CD pathogenesis), P-value < 0.001. A: X200, B: X400.

IELs distribution pattern in villi

Regarding the distribution patterns of the intraepithelial lymphocytes (T-bet marked) in the villi of the duodenum mucosa. Table 3. Shows the majority 68% of the cases showed regular distribution of the T-bet marked IELs throughout the villi body, while only 2% of the cases showed an aggregation of the IELs in the base of villi. It is important to note that the 6% of the cases were flat (Marsh 3C) meaning there was no villi present so the pattern of distribution was not apparent.

We found the majority of the samples of celiac diagnosed patients to have a regular distribution of the IELs along the

whole villus. This could be a result of the aggregation of the IELs in the villus tip, leading to the loss of the normal "decrecendo" pattern. These results which were examined using the T-bet mononuclear antibody showed a high statistical significance ($P < 0.001$). Based on our reviews, other studies found in the literature have studied the IELs distribution with either the H&E stain or the CD3 monoclonal antibody. These results could be very useful as a predictive of early-stage, potential and/or latent CD, especially with the use of the T-bet mononuclear antibody, which is a useful tool to count the specific celiac disease immune cells (Th1).

Table 3. The Connection between the IELs and their distribution pattern in the patients of celiac disease.

Distribution pattern of T-bet marked IELs in villi	Number of IELs in the duodenal villi			Total
	1-19	20-29	≥30	
flat villi	0	0	3	3
aggregated in the tip	0	0	12	12
aggregated in the base	0	0	1	1
regular distribution	12	6	16	34

$P=0.039$

A study discovered that a pattern with normal distribution along the villi was the most sensitive among the celiac Marsh I lesion (28). The rise of IEL in the villus tip was reported to be closely related to celiac disease (28). When Mino et al. observed the staining of CD3 IHC, they discovered that the accumulation of IELs in the tip of villi is reminiscent of celiac disease (29). Another study with more than 400 biopsies reported that celiac disease IELs were not distributed in a normal pattern (30). Dickson et al. proposed that the absence of the normal "decrecendo" form of IEL normal distribution of villi in a patient is reminiscent of celiac disease (31). Likewise, they recommended that the normal distribution of IELs should be ≥ 25 in normal villus to indicate a sign of CD. They also highlighted that when assessing the biopsy, the Gestalt approached should be applied by the pathologists.

DISCUSSION AND CONCLUSION

In this study the highest distribution of age groups between (20-30) years, other ages including children under 11 years and elderly showed in less frequency. The frequency of celiac disease among this study sample group was low in the children, the most probable cause is that the parental consent was not giving to perform a biopsy for children under this age group. The ESPGHAN guidelines stipulate avoiding the biopsy in children for the diagnosis of celiac disease²⁰. Controversy has arisen regarding these guidelines²¹. In the elderly, the clinical diagnosis of the disease in this age group can be quiet challenging to the primary care physician. It was thought that celiac disease was a disease of children and thought that it rarely occurred in adulthood or elderly people²². But the evidence is showing the diagnosis of celiac disease

among adults is increasing. Studies of the disease, suggests an increase rate of the disease among adults especially the elderly^{23,24}. The reason why the diagnosis is tricky in the elderly might be partially as to the subtle clinical symptoms, the low index suspicion of the disease in elderly, and distraction to more threatening conditions like malignancies^{25,26}. For instance, the subtle changes in the bowel habits could be attributed to diseases like irritable bowel syndrome, mood disorders or even missed as a part of the normal aging process²⁷.

In this study the female to male ratio was 3:1. These results were found to be similar to what was found in the literature. Celiac disease is of a female predominance with 2.8:1 or 3:1 ratio^{28,29}. The celiac disease diagnosis of female is more frequent than the diagnosis of males³⁰. A possible explanation for this is the difference in the clinical presentation and its severity between females and males. The menstrual cycle, pregnancy and fertility can all be affected adversely. Other possibilities is the lack of the physician's awareness of the gender related differences in the clinical presentation of the disease³¹. Some authors suggest that since during pregnancy some aspects of immunity are suppressed, and celiac is an immune mediated it is possible that celiac disease may be exaggerated^{32,56,57}. This gender difference is less apparent in diagnosed children with 1:1 female to male ratio. The gender distribution varies depending upon different parts of the world, as some geographical areas shows a female to male equality of distribution^{33,34}.

In this study, most of the samples were seropositive. Few of the samples had false negatives. The false positive or false negatives are not uncommon in celiac disease cases. Anti-tTG IgA may be negative in (5-16%) of patients with biopsy confirmed celiac disease on a gluten containing diet³⁵. The seronegative celiac disease has a prevalence of (6-22%) of the diagnosed cases as mentioned in the 2014 guidelines from British society of gastroenterology³⁶. Not all patient have a positive celiac disease serology at presentation. There has been discussions in the literature regarding the fact that the appearance of celiac disease related antibodies in the patients serum correlates with the degree or level of atrophy³⁷. Meaning patients with lesser degree of villus atrophy are less likely to be of a positive serology³⁸⁻⁴⁴.

A positive serology is supportive for celiac disease diagnosis but no single test is a (100%) specific, the serologic accuracy varies dramatically between different laboratories. The serological tests in a clinical settings may perform less well than in research settings^{39,58}.

The presence of increased IELs with normal villus architecture can be an indicative of celiac disease but only in correlation with other indications like positive tTG-IgA or clinical presentation. This especially true in relatives of celiac disease patients, patients with anemia and patients with DH⁴⁰.

In an attempt of an early diagnosing the mild enteropathy celiac disease to avoid the complication of a delayed diagnosis, over the years the cutoff or the upper normal limit of the IELs was subject to many variations. A significant reduction in the highest normal value of IELs has occurred. The past proposed increase number of IELs in the duodenum was 30 per 100 epithelial cells, then the number 25 IELs per 100 enterocytes was proposed as borderline increase in IELs. Some studies later proposed that the upper limit of normal IELs is 22 per 100 enterocyte, while other studies suggested a

mean of upper limit of 20.9 IELs/100 enterocyte⁴¹. Pellegrino *et al* (43) suggest an even lower threshold of 20, 20.5 IELs as indicative of celiac disease.

In this study all of the samples when stained with H&E showed a significant increase of IELs. In the clinical settings, the count of IELs is performed using the H&E stains. The H&E is helpful in the examination of cases with changes to the villus architecture, Marsh III and II. But in cases with Marsh stage I, some difficulties may arise. These stains could complicate the accuracy of quantifying the IELs count. Nuclear overlap and heterogeneity of nuclear shapes could make it difficult to distinguish epithelial cells from enterocytes and granulocytes.

The guidelines of British society of gastroenterology stipulate that use of IHC staining in borderline cases is vital⁴⁵⁻⁴⁸. Many other studies used the CD3 monoclonal antibody to improve the count of IELs in an attempt to either detect the early stages of celiac disease or improve contrast to count IELs in seropositive cases or simply to study the celiac related IELs⁴⁴. Since the CD3 in of cytoplasmic expression pattern, the issues of overlapping of the nucleus faced with H&E has become less prominent.

When stained with CD3 monoclonal antibody, most of the sample were of increased IELs, while the remainder of the samples showed a borderline increase (20-29) count of IELs. Some cases that showed a borderline increase of 25 on CD3 were sero-positive. A highly significant correlation was found between the increase of IELs stained with H&E and those stained with CD3 ($P < 0.001$).

The use of CD3 may have improved the diagnosis of few celiac disease cases. CD3 eliminated the issue of miscounting the IELs that was faced with the H&E. However, CD3 is not specific for CD. Recent study proposed that the CD3 was of no more use in the diagnosis of the normal histology cases of celiac disease than is the staining with H&E⁴⁵. Therefore, the search of a more specific marker for celiac disease would serve as a more reliable method for diagnosing CD, especially in early, latent and potential cases.

Many studies investigated the possibility that celiac disease is of Th1 mediated autoimmune reaction⁴⁶. The predominant cytokine produced by T-cell isolated from celiac disease patients mucosa which have a significant role in the lesion formation is IFN gamma. This view is supported by other studies on the intestinal lamina propria isolated T cells, underlined by the increase of cytokines IFN gamma and IL-18. T-bet is a specific T box transcription factor which correlates with IFN gamma expression in Th1 and natural killer cells. Reports suggest that the T-bet transcription factor is up regulated in active celiac disease then down regulated to normal levels in treated (on GFD) celiac disease patients. The transcription factor T-bet protein plays a key role in regulating the cytokine for the production of Th1⁵⁰⁻⁵⁵.

Another criteria was examined in this study to distinguish early stages of celiac disease is the distribution pattern of the IELs. The normal distribution of IELs along the villi assumes a characteristic "luminopetal" distribution. Meaning that the IELs are of "crescendo" pattern with increased IELs in the basal part of the villus with loss of IELs along the upper part of the villus and tip. In this study a classification of the distribution pattern of the IELs into: regularly distributed (along the tip and sides of villus), aggregated in tip and aggregated in the base of the side of the villi.

Here it was found the majority of the samples of celiac diagnosed patients to have a regular distribution of the IELs along the whole villus. This could be a result of the aggregation of the IELs in the villus tip, leading to the loss of the normal "decrecendo" pattern. These results which were examined using the T-bet monoclonal antibody showed a high statistical significance ($P < 0.001$). As to the best of our knowledge all of the studies found in the literature have studied the IELs distribution with either the H&E stain or the CD3 monoclonal antibody. These results could be very useful as a predictive of early stage, potential and / or latent CD, especially with the use of T-bet monoclonal antibody, which is a useful tool to count the celiac disease specific immune cells (Th1).

Intraepithelial lymphocytosis in an otherwise normal small bowel biopsy is somewhat nonspecific, The pattern of IELs distribution in cases of CD can be an early symptom of the disease. Therefore all patients with this finding should be investigated for GS. Owing to the specificity of T-bet to Th1 and being Th1 response is a major immunological response in the pathophysiology of CD, this study conclude that counting of IELs using T-bet immunostaining would be usefully in the diagnosis and follow up of the disease. Studying of T-bet immunostaining in other conditions that are associated with increased IELs and changes in the architectural villi. In situ, studying of the cytokines that are regulated by T-bet positive cells to examine their utility in the pathophysiology and diagnosis of CD. Studying the usefulness of modifying marsh staging to include the count of T-bet cells.

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