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***Prospective Study***

**Immunohistochemical** **CD3 staining detects additional patients with celiac disease**

Mubarak A *et al.* CD3 staining in diagnosing celiac disease

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**Abstract**

**AIM:** To investigate whether performing immunohistochemical CD3 staining, in order to improve the detection of intra-epithelial lymphocytosis, has an additional value in the histological diagnosis of celiac disease.

**METHODS:** Biopsies stained by hematoxylin and eosin (HE) of 159 children were evaluated using the Marsh classification. Subsequently CD3 stains were evaluated separately and independently.

**RESULTS:** A difference in evaluation between the routine HE sections and the CD3 stains was present in 20 (12.6%) cases. In 10 (6.3%) patients the diagnosis of Celiac disease (Marsh II and III) changed upon examination of the CD3 stains: in 9 case celiac disease had initially been missed on the HE sections while 1 patient had been over-diagnosed on the routine sections. In all patients the final diagnosis based on the CD3 stains was concordant with serological results, but was not so previously.In the other 10 (12.3%) patients the detection of sole intra-epithelial lymphocytosis (Marsh I) improved. Nine patients turned out to have Marsh I on CD3 sections, but this had been missed on routine sections. Interestingly, the only patient with negative serology had Giardiasis. Finally, in 1 patient with negative serology, in whom Marsh I was suspected on HE sections, this diagnosis was withdrawn after evaluation of the CD3 sections.

**CONCLUSION:** Staining for CD3 has an additional value in the histological detection of Celiac disease lesions, with CD3 stains to be performed whenever there is a discrepancy between serology and the diagnosis made on HE sections.

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**Key words**: Celiac disease; CD3 staining; Diagnosis; Intra-epithelial lymphocytosis; Histology; Marsh classification

**Core tip:**  Intra-epithelial lymphocytosis is considered to be the most important histological finding in celiac disease and therefore provides the key to a correct diagnosis. However, when evaluating the number of intra-epithelial lymphocytes on hematoxylin and eosin stained sections, the lack of contrast between the cells might cause some diagnostic difficulties. In this study we showed that performing CD3 stains improves the histological diagnosis of celiac disease. In fact, this study demonstrated that CD3 staining should be performed whenever there is a discrepancy between serology and the histological evaluation on routine sections.

Mubarak A, Wolters VM, Houwen RHJ; ten Kate FJW. Immunohistochemical CD3 staining detects additional patients with celiac disease. *World J Gastroenterol* 2015; In press

**INTRODUCTION**

Celiac disease is a permanent intolerance to gluten, a storage protein in wheat and the related grain species barley and rye[1]. Ingesting these grain species in genetically susceptible individuals causes inflammation of the small intestine, which is reversible upon elimination of gluten from the diet[2,3].To screen for celiac disease, highly specific and sensitive antibodies are available, but until now in many cases a small intestinal biopsy is required for the diagnosis[4,5].

Typically, the triad of an increased density of intra-epithelial lymphocytes (IELs), hyperplasia of the crypts and villous atrophy are observed in patients with celiac disease[2]. However, villous atrophy can also be found in various other diseases such as Giardiasis, Whipple’s disease, Tropical Sprue *etc*[6].On the other hand according to most recent guidelines, villous atrophy is not necessary for the diagnosis of celiac disease, provided that intra-epithelial lymphocytosis and crypthyperplasia are present[5]. Crypt hyperplasia is a sign of increased intestinal turnover, and is thought to occur secondary to the villous destruction and inflammation. The presence of intra-epithelial lymphocytosis, although not pathognomonic for the disease, is considered to be the most important histological finding for celiac disease[7,8]. Therefore, in many cases detecting IELs provides the key to a correct diagnosis. The presence of IELs is usually evaluated on hematoxylin and eosin (HE) stains, but due to the lack of contrast between the cells, the presence of intra-epithelial lymphocytosis might not always be clear, especially when the number of IELs is only moderately increased. Because IELs are CD3 positive cells, performing immunohistochemical staining against CD3 might aid in estimating the number of IELs. The aim of this study was therefore to investigate whether CD3 staining should routinely be performed on all biopsies, or that it is only necessary in specific cases.

**MATERIALS AND METHODS**

***Patients***

Pediatric patients (53 girls; 106 boys) suspected with celiac disease who had undergone a small intestinal biopsy between March 2009 and October 2012 in the Wilhelmina Children’s Hospital, Utrecht, The Netherlands, were prospectively included in the study. Patients were referred to us because of celiac disease associated symptoms or because they carry a risk factor for celiac disease. All patients carried the disease associated HLA type. Patients were between 0.9 years and 17.8 years at the time of the biopsy. When a patient had undergone more than one biopsy session, only biopsies from the first session were included in the study.

Results of anti-endomysium antibodies (EMA) and anti-tissue transglutaminase antibodies (tTGA) as well as the clinical data of the patients were collected from the medical records. The study was performed according to the guidelines of the local medical ethical board.

***Histology***

Biopsies were obtained by upper endoscopy. Pediatric gastroenterologists were asked to take at least 4 biopsies from the distal duodenum and as of the end of 2009 at least 1 biopsy from the duodenal bulb.  In reality, 0 (in 33 cases) to 5 biopsies were obtained from the duodenal bulb with an average of 2.0 biopsies. From the distal duodenum 3.1 (range 1-7) biopsies were acquired on average. Biopsies were fixed in formalin (10% neutral buffered formalin) and then embedded in paraffin, and 4-um-thick sections were stained with HE, Periodic acid-Schiff and CD3 (Dako, Glostrup, Denmark; batchnumber 81639; dilution 1:50; pretreatment with EDTA).

All biopsies were evaluated by an experienced pathologist, specialized in gastro-intestinal diseases, who was blinded to the clinical and serological data of the patients. The pathologist first evaluated the HE stained sections. On a separate occasion the CD3 stains were evaluated independently from the HE stains.

Biopsy results were reported according to the Marsh classification, as modified by Oberhuber[2,9]. In case of patchy lesions, the final Marsh score was based on the worst affected site. Marsh I lesions are defined as an increased number of IELs. On the HE-stains this was determined by visual estimation. On the CD3 stains, ≥ 30 lymphocytes per 100 epithelial cells were considered as intra-epithelial lymphocytosis[10,11]. In Marsh II lesions crypt hyperplasia along with an increased number of IELs are found. Finally, Marsh III lesions include the findings in Marsh II, along with various grades of villous atrophy.

Marsh II and Marsh III lesion were considered to be diagnostic for celiac disease but were reported separately. Marsh I was reported as a separate entity. celiac disease was excluded in patients with a normal small intestine (Marsh 0) or abnormalities not diagnostic for Marsh II or III (*i.e.,* only crypt hyperplasia and/or villous atrophy without intra-epithelial lymphocytosis). In case of discrepancy between the HE and CD3 section, the final diagnosis was based on the serological data of the patients. For example, a patient with positive serology who has crypt hyperplasia and villous atrophy, but increased IEls only on the CD3 sections (so on the HE sections no diagnosis of celiac disease but on the CD3 sections a Marsh III lesion), was considered to have celiac disease (Figure 1A). Similarly, in patients with positive serology and a Marsh 0 on the HE section but a Marsh I on the CD3 sections, the final diagnosis was considered to be a Marsh 1 (Figure 1B).

***Statistical analysis***

Descriptive statistics using SPSS for Windows version 15.0 was used to compare the conclusion of the pathologist before and after performing the CD3 stains.

**RESULTS**

A diagnosis of Marsh III, based on the HE stains, could be made in 87 patients, but celiac disease was rejected in 1 (1.1%) patient with negative celiac disease serology after examination of the CD3 stains (Table 1). Only 1 patient had a Marsh II lesion on the HE sections which was also recognized on the CD3 stains.

On the HE stains, 6 patients were considered to have a Marsh I lesion, but in 2 patients the diagnosis of Marsh I changed after assessment of the CD3 stains. In 1 (16,7%) patient with negative celiac disease serology a Marsh 0 was seen instead and in the other one (16.7%) a Marsh III lesion was present. In the latter patient, who had positive tTGA and EMA, this could be explained by the fact that on the HE sections a Marsh I lesion was found in the bulb and crypt hyperplasia and villous atrophy (but without intra-epithelial lymphocytosis) were found in the distal duodenum. So, on the HE stains the most affected site seemed to be the duodenal bulb. However, on the CD3 stains an increased number of IELs was seen in both parts of the duodenum while the most affected site on the CD3 stains turned out to be the distal duodenum (Marsh III).

Celiac disease was excluded in 65 patients on the HE slides. However, celiac disease could be diagnosed after employing CD3 stains in 6 (9.2%) patients with Marsh III and 2 (3.1%) patients with Marsh II histology. All of them had positive celiac disease serology. Finally, after evaluation of the CD3 stains Marsh I lesions were identified in another 9 (13.8%) patients. Eight of them had positive Celiac disease antibodies whereas 1 patient was negative for tTGA and EMA. Interestingly, the patient with negative serology and Marsh I had Giardiasis.

 In summary, a difference in assessment between the HE slides and the CD3 sections was found in 20 (12.6%) patients. In 9 (5.7%) patients a Marsh I was found and in 1 (0.6%) patient a Marsh I was rejected when evaluating the CD3 sections. Most importantly, in 10 (6.3%) patients the diagnosis of celiac disease (Marsh II and Marsh III) changed: on the CD3 stains 1 (0.6%) patient turned out to have no celiac disease, 2 (1.3%) patients turned out to have Marsh II lesions and 7 (4.4%) patients had Marsh III histology.

**DISCUSSION**

Even after a recent update of the ESPGHAN guidelines for the diagnosis of celiac disease, which states that a biopsy can be omitted in symptomatic cases with very high tTGA levels, positive EMA and the disease related human leukocyte antigen types, for most patients histological assessment of duodenal biopsies is still necessary for the diagnosis. In this respect, apart from grading villous atrophy and crypt hyperplasia, judging intra-epithelial lymphocytosis is essential[5]. We evaluated whether performing CD3 stains improves the histological evaluation of celiac disease.

Our results show that compared to HE stains alone CD3 stains did lead to a different assessment in 12.6% (20/159) of the patients. More importantly, almost 10% (9/96) of the patients with celiac disease (Marsh II and III) in the current study would have been missed if a CD3 stain had not been performed. It is highly unlikely that these patients were over-diagnosed as all of them had positive celiac disease serology. They probably would not have started a gluten free diet or would unnecessarily have had subsequent biopsies. On the other hand, when the diagnosis of celiac disease is already made on the HE slides, the chance that celiac disease will be ruled out on subsequent CD3 stains is small. Yet, without a CD3 stain 1 of the 48 patients with apparent celiac disease on the HE stains would have been misdiagnosed with the disease, and would therefore unnecessarily have carried the burden of following the gluten free diet. Interestingly, in this over-diagnosed patient, celiac disease serology was negative. Therefore, in order to catch all Marsh II and Marsh III lesions and at the same time not over-diagnose any patient with celiac disease, CD3 staining should be performed in all cases of villous atrophy and/or crypt hyperplasia, when the initial conclusion made on the HE stains is discrepant with the serology results.

In addition, performing CD3 staining, also leads to an improved detection of Marsh I lesions. In fact, in almost 14% (9/65) of the patients in whom on the HE slides celiac disease was excluded, a Marsh I lesion was found. Interestingly, only 1 of these 9 patients had negative serology, but the Marsh I in this patient could be explained by a Giardiasis infection.  In addition without CD3 staining, another patient with negative serology would have been over-diagnosed with Marsh I. Therefore, in order to catch al Marsh I lesions, that are unexplained by other conditions, and at the same time not over-diagnose any patient with Marsh I, CD3 staining should again be performed whenever there is discrepancy between serology and histology.

The implication of finding lymphocytic enteritis (Marsh I) is unclear however, because this lesion does not occur exclusively in celiac disease, as was also seen in our patient with Giardiasis[5,12,13]. Nevertheless there is some evidence that a Marsh I lesion is clinically important and should therefore be detected, especially in patients with positive serology. First of all, Marsh I abnormalities may be an early stage of Celiac disease and may thus develop in some patients into active celiac disease over time[14-21].In addition, a gluten challenge seems to cause mucosal deterioration and a diagnosis of celiac disease in some patients with Marsh I[22].Finally, various studies have shown that patients with Marsh I lesions might benefit from the gluten free diet, at least on the short term[17-21].

In conclusion, immunohistochemical staining for CD3 has an additional role in the histological detection of celiac disease lesions. In order to make an appropriate diagnosis of the total spectrum of celiac disease associated lesions, CD3 staining should be performed in all cases of discrepancy between serology and the histological conclusion on the routine sections.

**COMMENTS**

***Background***

Histological lesions in celiac disease are characterized by intra-epithelial lymphocytosis, crypt hyperplasia and in many cases villous atrophy (Marsh II and III). In addition, the existence of solely intra-epithelial lymphocytosis (Marsh I), is also associated with (the development of) the disease. The presence of these lymphocytes in the epithelium is usually evaluated on hematoxylin and eosin (HE) stains, but the lack of contrast between the cells can make evaluation difficult, which can hinder a correct diagnosis. Because intra-epithelial lymphocytes are CD3 positive cells, performing immunohistochemical staining against CD3 might aid in estimating the number of intra-epithelial lymphocytes.

***Research frontiers***

The additional value of performing CD3 staining in the diagnosis of celiac disease has not been studied before. Therefore in this study we prospectively compared the results of HE sections to the results of CD3 sections in pediatric patients suspected with celiac disease. In case of discrepancy between the two sections, the final diagnosis was based on the clinical and serological data of the patient.

***Innovations and breakthroughs***

Performing CD3 staining, leads to an additional diagnosis of Marsh I in 5% of the studied patients, while in 0.6% of the patients the diagnosis could be withdrawn after assessment of the CD3 stains. More importantly, in 5.7% of the patients the diagnosis of celiac disease was missed on routine sections, but was detected with the addition of CD3 staining. Finally, in 0.6% of the cases the diagnosis of celiac disease could be rejected after evaluation of the CD3 sections.

***Applications***

In order to make an appropriate diagnosis of the total spectrum of celiac disease associated lesions, CD3 staining should be performed in all cases of discrepancy between serology and the conclusion of the pathologist based on the routine sections.

***Terminology***

Intra-epithelial lymphocytes: CD3 positive lymphocytes present in the epithelium of the small intestine. An increased number of intra-epithelial lymphocytes is mandatory to make a diagnosis of celiac disease. In addition, these lymphocytes are thought to play an important role in the pathophysiology of the disease.

***Peer review***

Mubarak *et al* have assessed the diagnostic improvement of celiac disease by adding immunohistochemical CD3 staining for duodenal mucosal biopsy samples.**REFERENCES**

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**Table 1 Marsh classification of duodenal biopsies on hematoxylin and eosin stains versus CD3 stains *n* (%)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Evaluation of CD3 stains** | | | | | |
| Evaluation  of hestains |  | Positive1 | | | Negative |
| Marsh III  (*n* = 93) | Marsh II  (*n* = 3) | Marsh I  (*n* = 13) | No CD  (*n* = 50) |
| Marsh III  (*n* = 87) | 86 (98.9) | - | - | 1 (1.1) |
| Marsh II  (*n* = 1) | - | 1 (100) | - | - |
| Marsh I  (*n* = 6) | 1 (16.7) | - | 4 (66.7) | 1 (16.7) |
| No CD  (*n* = 65) | 6 (9.2) | 2 (3.1) | 9 (13.8) | 48 (73.8) |

1≥ 30 intraepithelial lymphocytes per 100 epithelial cells. HE: Hematoxylin and eosin; CD: Celiac disease.

**Figure 1 Examples of histological hematoxylin and eosin and CD3 sections.** A: The HE sections (left) show partial villous atrophy and crypt hyperplasia but no intra-epithelial lymphocytosis, so the HE sections do not reveal celiac disease. However, on the CD3 sections (right) the intra-epithelial lymphocytosis becomes clear and the diagnosis of celiac disease can be made (Marsh IIIa). The patient had clinical symptoms associated with Celiac disease, positive serology and also responded well to the diet. The final diagnosis is Celiac disease. B: The HE sections (left) show a Marsh 0, while the CD3 sections (right) show a Marsh I lesion. The patient had clinical symptoms associated with celiac disease, positive serology and also responded well to the diet. The final diagnosis is Marsh I.

C:\Users\User\AppData\Local\Temp\_PA462\coeliakie T11-1092 HE.tif C:\Users\User\AppData\Local\Temp\_PA256\coeliakie T11-1092 CD3.tif

A

C:\Users\User\AppData\Local\Temp\_PA215\coeliakie T11-6565 He.tif C:\Users\User\AppData\Local\Temp\_PA687\coeliakie T11-6565 CD3.tif

B