Evaluation of crypt apoptotic bodies and apoptotic indices in pediatric celiac disease by routine staining and H2AX immunostaining

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Abstract

Celiac disease (CD) is an immune-mediated disorder with premature apoptosis occurring along the entire crypt-villous axis. H2AX is the end product of the intrinsic apoptotic pathway. This is the first study to assess apoptotic body counts (ABC) by H&E and apoptotic indices (AI) by immunohistochemistry (IHC) in pediatric CD. The aim of the current study was to evaluate ABC in pediatric patients with CD prior to and following institution of a gluten free diet (GFD). Sixty-three pediatric endoscopic duodenal samples were assessed and divided into three groups. A total of 21 samples from treatment naïve CD patients, 21 from the same patients after instituting a GFD, and 21 from non-celiac patients as a control group. Histopathological evaluation of ABC by H&E, and immunohistochemistry assessment of apoptotic indices (AI) by H2AX antibody were performed. The mean maximum ABC and AI were significantly higher in treatment naïve CD than in GFD and control samples. These values were also significantly higher in treatment naïve Marsh 3C (flat) than in Marsh 1, 2, 3A, and 3B (non-flat) CD cases. GFD samples with persistent flat lesions had significantly higher ABC and AI than GFD non-flat cases. ROC analysis of the mean maximum ABC and AI of treatment naïve CD cases had a statistically significant predictive potential for persistent villous atrophy at a cut-off level $\geqslant 6.61$ ($P = 0.008$) and $\geqslant 105.4$ ($P = 0.003$), respectively. Histopathological evaluation of crypt apoptotic bodies could provide predictive potential for continued villous atrophy following GFD.

Keywords

apoptosis, celiac disease, H2AX, immunohistochemistry, pediatric endoscopic evaluation

Date received: 30 January 2021; accepted: 2 June 2021

Introduction

Celiac disease (CD) is characterized by small intestinal mucosal damage and malabsorption due to an immunologic reaction to gluten, a group of proteins found predominantly in wheat, rye, and barley.[1] Villous atrophy is the histopathological hallmark of celiac disease on intestinal biopsy. The mechanism of villous atrophy is attributed to an imbalance between epithelial cell proliferation and cell death.[2] Under physiological conditions, apoptosis plays an important role in maintaining intestinal epithelial function by controlling normal enterocyte turnover and limiting it to the tips of the small intestinal villi. However, in immune mediated disorders, such as CD, an increased number of enterocytes exhibit premature apoptosis throughout the length of the intestinal crypts and villi.[3]

The normal number of crypt apoptotic bodies in small intestinal mucosa has not been specifically determined. For diagnosing graft-versus-host disease

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(GVHD), several values have been suggested. The liberal NIH criteria suggest a one or more crypt apoptotic bodies per biopsy specimen,[4] while the more restrictive criteria suggested by Lin et al. require 6 apoptotic bodies in 10 consecutive crypts.[5] However, the German-Austrian-Swiss GVHD Consortium suggested a middle value of two apoptotic bodies in one $10\times$ microscopic field. Moreover, they defined an apoptotic body as either condensed nuclear chromatin with eosinophilic cytoplasm or at least two fragments of nuclear, karyorrhectic debris with clearing and vacuolization.[6]

Despite this controversy over diagnostic threshold, increased crypt apoptosis is associated with some particular diseases such as GVHD and some medication reactions such as mycophenolic acid. Few studies have acknowledged the importance of crypt apoptotic body counts in CD as a useful histologic criterion for assessing the response to a gluten free diet (GFD).[7] Although in clinical practice immunohistochemistry (IHC) is not routinely used,[8] few immunohistochemical techniques have been used to demonstrate extrinsic, intrinsic, or common pathways of apoptosis in intestinal crypts.[9–11]

Only sparse immunohistochemical research has been conducted in pediatric celiac disease, and this is the first study to assess apoptotic body counts (ABC) by H&E and apoptotic indices (AI) by IHC in pediatric patients with CD. The aim of this study was to evaluate the crypt apoptotic count in pediatric patients with treatment naïve CD both by H&E and by IHC using H2AX before and after instituting a GFD and to compare the sensitivity of both techniques.

Material and methods

Tissue and patient data

The current study was a retrospective review of the endoscopic duodenal biopsies of 21 pediatric patients with newly diagnosed active CD. Biopsies were performed from January 2017 to December 2019 and were obtained from the archives of the Pathology Laboratory of Cairo University Hospitals. Inclusion criteria consisted of typical clinical manifestations of CD, positive serum immunoglobulin A (IgA) anti-tissue transglutaminase antibody (tTGA), and an abnormal biopsy of Marsh 2 or higher grade according to the modified Marsh-Oberhuber classification.[12] Two cases were

only modified Marsh 1 (only intraepithelial lymphocytosis), but were positive for both serum tTGA and endomysial autoantibodies. Therefore, based on the guidelines, they were likely to have CD and follow a GFD.[13] Each included case had a follow-up biopsy after initiation of a GFD. A total of 21 children without CD were selected as controls from the department archives. The controls had undergone endoscopic duodenal biopsies to evaluate gastric reflux. Neither endoscopic examination nor biopsies of the controls showed any abnormalities. All controls had normal levels of IgA tTGA.

Patients with CD showing any histopathological abnormality in the duodenal mucosa, and those with abnormal levels of tTGA antibodies, or where no report of tTGA values were available, were excluded from the control group.

Histopathology reports were reviewed to determine pertinent data including age, sex, clinical presentation, endoscopic findings, and results of serological tests. Additionally, files were reviewed for a history of small bowel transplantation or a history of exposure to apoptosis-inducing pharmacologic agents such as mycophenolate mofetil, methotrexate, and tumor necrosis factor alpha inhibitors.

Histopathologic examination of duodenal biopsy specimens using routine H&E staining

Hematoxylin and Eosin (H&E)-stained slides comprising at least 10 serial cuts per biopsy were examined by two pathologists (the authors) to verify the histopathologic diagnosis of celiac disease, assign its modified Marsh-Oberhuber grade,[12] and record the maximum number of apoptotic bodies in ten consecutive crypts. Discrepancies in assigning Marsh classification were resolved by consensus using a multi-head microscope. Discrepancies in the maximum number of apoptotic bodies were resolved by averaging the two results, and the outcome value was designated as the ABC. The pathologists were unaware of the medical histories of all cases. German-Austrian-Swiss Consortium criteria for an apoptotic body were used to identify an apoptotic body.[14] Only cases with enough tissue to examine 10 serial cuts were included in the analysis.

Originally, 43 cases of treatment-naïve pediatric CD were present in the archives during this period; 14 were excluded because their follow-up biopsies

were not performed at our hospital, and 8 cases for insufficient tissue to examine 10 serial cuts. Thus, the total number of treatment-naïve cases included in the current study was 21 cases.

Immunohistochemical staining and interpretation

Formalin-fixed and paraffin-embedded samples of duodenal endoscopic biopsies that were 4 µm in depth were prepared. Immunohistochemical staining was performed using rabbit monoclonal anti-Histone H2AX primary antibody, Catalogue Number: YMA1238; (Chongqing Biospes Co., Ltd, China), isotype IgG, diluted 1:50 at room temperature, according to the manufacturer's instructions on an automated Ventana immunostainer. After washing in phosphate buffered saline, the samples were incubated with a biotin conjugated secondary antibody and the Ultra view universal DAB-Ventana was used as the detection system.

Counter staining was performed using hematoxylin, washed in tap water, placed in two changes of 95% ethyl alcohol, and then two changes of absolute alcohol. Finally, the slides were dried, and cover slips were fixed by DPX (a mixture of distyrene, plasticizer, and xylene). Sections from tonsils were used as positive controls for the primary antibody. Negative control sections were incubated with normal mouse serum instead of the primary antibody.

Immunohistochemical slides were examined by two pathologists (the authors) without prior knowledge of the clinicopathological data or the results of H&E staining. Duodenal sections with three to four crypts arranged perpendicularly over the muscularis mucosa were analyzed for AI. AI is defined as the number of positive cells with positive nuclear staining among 100 examined enterocytes. The average of the counts by each pathologist was considered to be the AI for each case.[15]

Ethics statement

Written informed consent was obtained from legally authorized representatives (Parents or guardians of all patients) before the endoscopic biopsy was performed. The study was approved by the Research Ethics Committee of Faculty of Medicine, Cairo University (ID=N-55-2021).

Data management and analysis

Data were revised, coded, entered on a computer, and analyzed using SPSS package version number 20. Quantitative data were tested for normality with the Shapiro-Wilk test and described as mean, standard deviation (SD) or median interquartile range according to data distribution. The Student t-test and Mann Whitney test were used for comparing quantitative variables between the two study groups. The paired t-test and Wilcoxon signed rank test were used to compare continuous variables measured twice for the same group. A receiver operating characteristic (ROC) curve was used to evaluate the sensitivity and specificity of pretreatment H&E and IHC. Qualitative data were expressed as frequencies (n) and percentage (%). The Chi-square test was used to test the association between qualitative variables. A p-value ≤0.05 was considered significant. Interclass Coefficient was used to measure consistency or agreement for two quantitative variables within cases.

Results

A total of 63 samples were evaluated. A total of 21 samples were from treatment naïve CD, 21 post-treatment samples from the same cases, and 21 samples from children undergoing endoscopic biopsy for suspected reflux. The treatment naïve patients had a mean age of 5.17 ± 1.54 years) at the time of initial biopsy with 8 males and 13 females. The mean interval between initial diagnostic biopsy and post-GFD treatment biopsy was 2.8 years. The control group had a mean age of 5.83 ± 2.98 years with 10 males, and 11 females. There was no statistically significant difference in age or gender between the cases and controls.

The treatment naïve CD group included 15 (71.4%) patients with villous atrophy (modified Marsh 3 lesions), of whom 8 (38.1% of all cases) had complete villous atrophy (modified Marsh 3C) (Table 1). Among the patients with biopsies following GFD treatment, 4 had persistent villous atrophy (19%) while 17 had normal villous architecture. No control biopsies had any degree of villous atrophy.

The mean maximum ABC in treatment naïve biopsies with H&E was 5.44 ± 1.46 and 0.99 ± 0.86) in the GFD treatment group. This was highly significant ($P=0.001$). The mean maximum

Table 1. Modified Marsh classification of treatment naive cases.

Subgroup	N	%
Modified Marsh 1	2	9.5
Modified Marsh 2	4	19.0
Modified Marsh 3A	5	23.8
Modified Marsh 3B	2	9.5
Modified Marsh 3C	8	38.1

Table 2. Comparison of cases and controls for H&E and IHC staining.

	Group		P
	Cases	Control	
	Mean ± SD	Mean	
H&E AB count	5.44 ± 1.46	2.07 ± 0.38	0.001*
H&E AB count post treatment	0.99 ± 0.86	–	–
IHC AI count	97.97 ± 6.61	68.97 ± 7.60	0.001*
IHC AI count post treatment	58.24 ± 15.94	–	–

*Student t test.

Table 3. Comparison of ABC and AI counts before and after treatment.

	Mean ± SD	P
H&E AB count	5.44 ± 1.46	0.001*
H&E AB count post treatment	0.99 ± 0.86	
IHC AI count	97.97 ± 6.61	0.001**
IHC AI count post treatment	58.24 ± 15.94	

*Wilcoxon signed rank.
**Paired t test.

ABC for controls was 2.07 ± 0.38, which was significantly lower than the counts for treatment naïve CD and significantly higher than the counts for GFD treatment cases ($P = 0.001$ each). Using the AI to evaluate H2AX IHC staining, the AI of treatment naïve cases was 97.97 ± 6.61 which was significantly higher than the AI of post-treatment cases (58.24 ± 15.94, $P = 0.001$). The AI of controls was 68.97 ± 7.60 which was significantly lower than the AI of treatment naïve cases ($P = 0.001$) (Tables 2 and 3).

Modified Marsh 3C (flat) treatment naïve cases (Figures 1 and 2) had a significantly higher mean maximum ABC (6.82 ± 0.65; median, 7.00; IQR, 6.22–7.05) compared to modified Marsh 1, 2, 3A, 3B (Figure 3) (non-flat) treatment naïve cases (maximum mean ABC, 4.60 ± 1.13; median, 4.25; IQR, 3.94–4.80; $P = 0.001$). Moreover, those cases with persistent modified Marsh 3C (flat) classification following treatment had a significantly higher maximum ABC of 1.73 ± 0.99 (median, 1.70; IQR, 0.8–2.65) compared to post-treatment non-flat cases whose mean maximum ABC was 0.53 ± 0.25 (median, 0.6; IQR, 0.3–0.7; $P = 0.001$).

Using H2AX IHC, pre-treatment biopsies from modified Marsh 3C treatment naïve cases had a significantly higher AI of 105.6 ± 1.52 (median, 105.90; IQR, 104.50–106.75) when compared to the AI of modified Marsh 1 to 3B treatment naïve cases (93.28 ± 2.97; median, 93.00; IQR, 91.00–94.00; $P = 0.011$). Moreover, the AI of persistent modified Marsh 3C flat cases while on a GFD was 73.50 ± 11.53 (median, 78.00; IQR, 67.50–82.00) which is significantly higher than the AI of Marsh 1 to 3B cases while on a GFD (48.85 ± 9.75; median, 45.00; IQR, 43.00–50.00; $P = 0.001$) (Table 4).

Among the post-GFD treatment biopsies, 4 cases (19%) had persistent villous atrophy while the remaining 17 cases (81%) revealed restoration of normal villous architecture. Among the cases with restored villous architecture, there were highly significant relationships between the mean maximum crypt ABC and AI before and after treatment with a GFD ($P = 0.001$ for each). Additionally, there was a highly significant relationship between pre- and post-treatment mean maximum crypt ABC and AI for those cases with persistent villous flattening (Tables 5 and 6).

In the current study, the ROC curve for the mean maximum crypt ABC of treatment naïve cases showed a highly significant predictive potential for persistent villous atrophy at a cut-off level ≥6.61 ($P = 0.008$) (Figure 4a). Also, the ROC curve of AI in treatment naïve cases showed a highly significant predictive potential for persistent villous atrophy at a cut-off level ≥105.4 ($P = 0.003$) (Figure 4b). However, the ROC curve comparing the predictive potential of the mean maximal crypt ABC by H&E and that of the AI by H2AX IHC was statistically insignificant. This suggests that neither method is better than the other (Figure 4c).

The interobserver agreement between the two pathologists interpreting the slides was excellent. The Interclass Correlation Coefficient (ICC) between the two readers for crypt ABC by H&E in
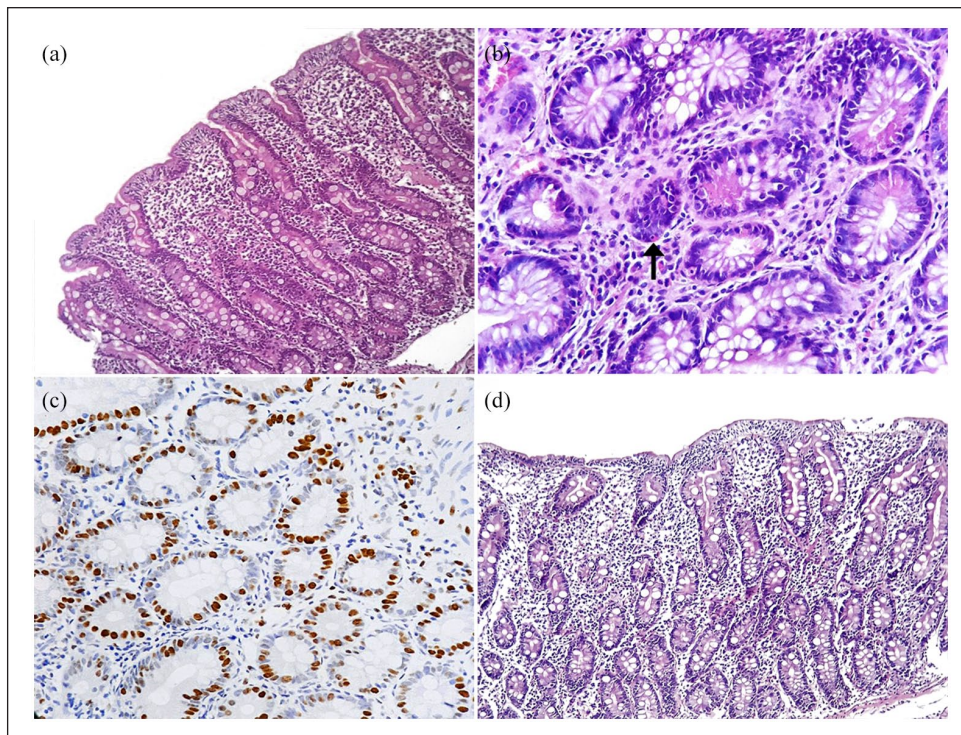
Figure 1. (a) A case of Marsh 3C celiac disease with complete villous atrophy (Marsh 3C) (H&E ×100). (b) A high power field with an arrow pointing at one of the apoptotic bodies that were readily detected in this case in several fields (H&E ×400). (c) High H2AX nuclear expression in crypt cells of the same case which showed high AI (IHC ×400). (d) Post-treatment (GFD) follow-up sample of the same case showing persistent flat lesion (H&E ×100).
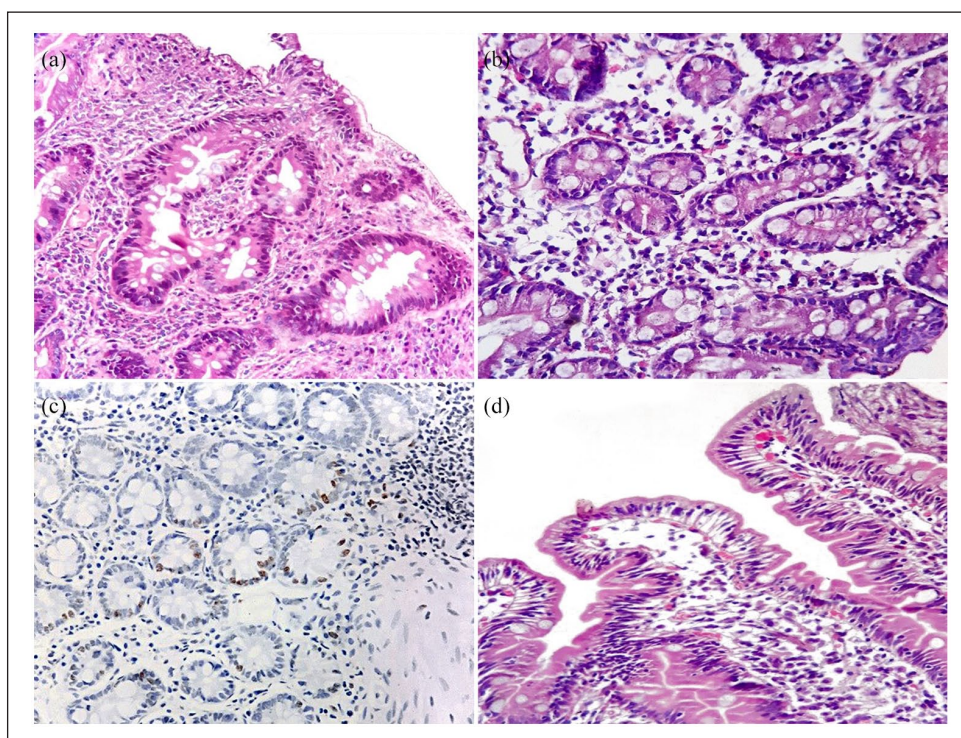

Figure 2. (a) Another case of Marsh 3C celiac disease with complete villous atrophy (Marsh 3C) (H&E ×200). (b) A high power field denoting absence of apoptotic bodies that were sparsely detected in this case (H&E ×400). (c) Minimal H2AX nuclear expression in crypt cells of the same case which showed low AI (IHC ×200). (d) Post-treatment (GFD) follow-up sample of the same case showing villi restored to a state of normalcy (H&E ×200).
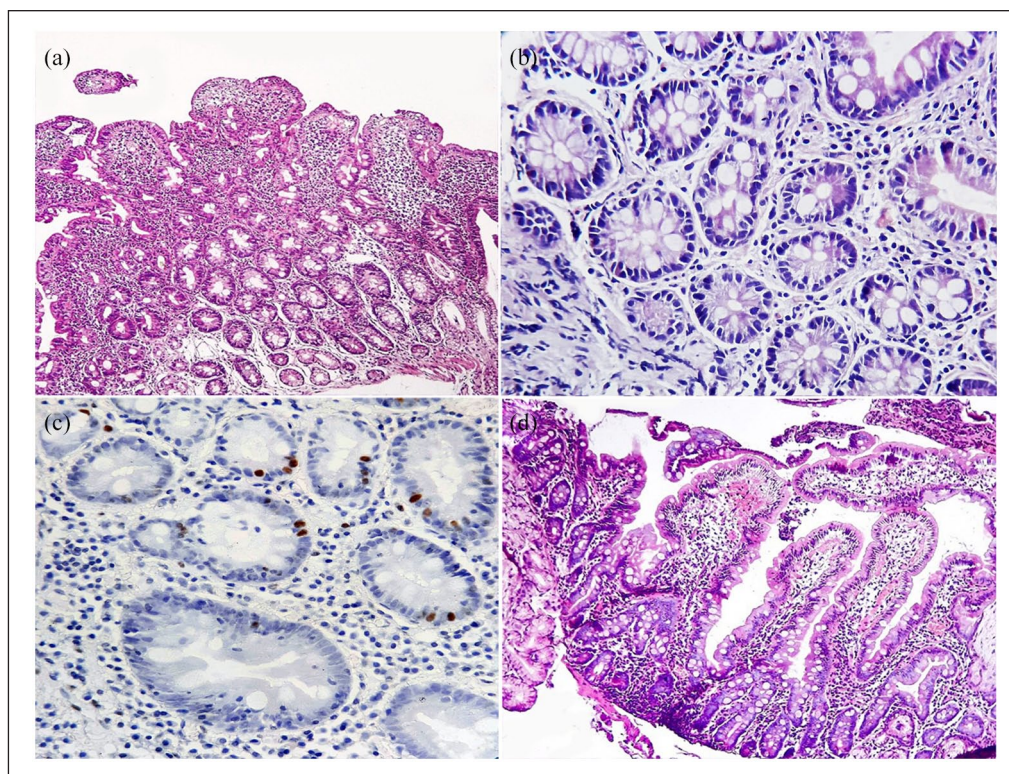
Figure 3. (a) A case of Marsh 3B celiac disease with incomplete villous atrophy (H&E ×100). (b) A high power field denoting absence of apoptotic bodies that were sparsely detected in this case (H&E ×400). (c) Minimal, focal H2AX nuclear expression in crypt cells of the same case which showed low AI (IHC ×400). (d) Post-treatment (GFD) follow-up sample of the same case showing villi that are restored to a normal state (H&E ×100).

Table 4. Comparison of AB counts and AI between Marsh 3C (flat) and Marsh 1 to 3B (non-flat) cases before and after treatment.

	Marsh 1, 2, 3A, 3B (non-flat)			Marsh 3C (flat)			P
	Mean ± SD	Median	IQR	Mean ± SD	Median	IQR	
H&E AB count	4.60 ± 1.13	4.25	3.95–4.80	6.82 ± 0.65	7.00	6.22–7.05	0.001*
H&E post treatment	0.53 ± 0.25	0.60	0.30–0.70	1.73 ± 0.99	1.70	0.80–2.65	0.001**
IHC AI count	93.28 ± 2.97	93.00	91.00–94.00	105.60 ± 1.52	105.90–104.50	104.50	0.011*
IHC AI count Posttreatment	48.85 ± 9.75	45.00	43.00–50.00	73.50 ± 11.53	78.00–67.50	67.50	0.001*

*Student *t* test.
**Mann Whitney.

Table 5. Comparison of AB and AI counts before and after treatment among improved cases.

	Mean	P*
H&E AB count	5.01 ± 1.27	0.001
H&E AB count post treatment	0.59 ± 0.246	
IHC AI count	95.92 ± 5.56	0.001
IHC AI count post treatment	52.64 ± 11.95	

*Paired *t* test.

Table 6. Comparison between H&E AB count before and after treatment, and IHC AI count before and after treatment among non-improved cases.

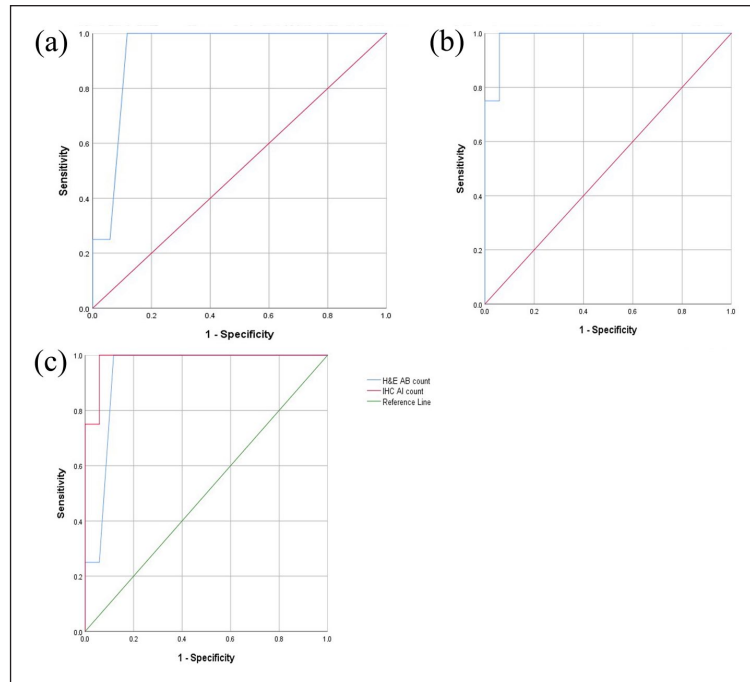|	Mean	P*
H&E AB count	7.25 ± 0.50	0.001
H&E AB count post treatment	2.65 ± 0.12	
IHC AI count	106.69 ± 0.71	0.001
IHC AI count post treatment	82.00 ± 0.81	

*Paired *t* test.

Figure 4. (a) ROC analysis curve of the mean maximum crypt ABC of treatment-naïve CD cases showing a highly statistically significant predictive potential of persistent villous atrophy at a cut-off level ≥6.61 (*P*=0.008). (b) ROC curve of apoptotic index of treatment-naïve CD cases showing a highly statistically significant predictive potential of persistent villous atrophy at a cut-off level ≥105.4 (*P*=0.003). (c) ROC analysis curve comparing the predictive potential of mean maximum crypt ABC by H& E and that of apoptotic index by H2AX IHC is statistically insignificant with no privilege of one technique over the other.

the control group was *r*=0.953 (*P*=0.001). The ICC for crypt ABC by H&E for treatment naïve patients was *r*=0.999 (*P*=0.001), while the ICC for crypt ABC by H&E for the GFD treated group was *r*=0.995 (*P*=0.001).

Data on ABC by H&E and AI by H2AX in the villous epithelium of the three studied groups are noted in the Supplemental Material.

Discussion

Apoptosis is a critical process in the maintenance of intestinal mucosal function. In immune-mediated disorders such as CD, premature apoptosis occurs along the crypt-villous axis[16,17] and is not just confined to the tips of the small intestinal villi.[18] Not only are extrinsic apoptotic pathways involved in small intestinal apoptotic activity, but also intrinsic and common apoptotic pathways contribute to this process.[3] H2AX is the end product of the intrinsic apoptotic pathway.[10] It is a histone H2A variant formed from a central domain with N-terminal and C-terminal tails that possess sites for various post-translational modifications including phosphorylation by proteins that are members of PI3 kinase

family. Both the C- and N-terminal tails have been proposed as potential therapeutic targets. H2AX is involved in DNA double-strand breakage as well as playing a role in apoptosis.[19]

Lee et al.,[7] in a study of adults with CD, demonstrated a significantly higher crypt ABC among patients with treatment-naïve CD compared to GFD and control groups. These results were similar to the results of the current study which was conducted among pediatric patients with CD and showed a highly significant difference between the mean ABC of active cases before and after introduction of a GFD (*P*=0.001). Cupi et al.[20] also noted similar results. The current study noted a significantly higher AI among treatment naïve CD cases as estimated by H2AX IHC when compared to cases following introduction of a GFD and controls. This was partly in agreement with the findings of Shalimar et al.[10] and Monguzzi et al.[21] who found H2AX to be significantly higher among treatment naïve CD patients than in controls. However, their studies did not include patients following introduction of a GFD making comparisons of this group to ours impossible.

The current study demonstrated that modified Marsh 3C (flat), treatment naïve CD, and persistently flat GFD cases had significantly higher mean maximum ABCs when compared with modified Marsh 1 to 3B treatment naïve CD cases and patients on a GFD ($P=0.001$ for each). Moreover, this study noted similar findings with AI on H2AX staining. This was partly in agreement with Lee et al.[7] whose cases with completely flat lesions had a mean maximum ABC that was higher than that of non-flat lesions. On follow-up biopsies of patients on a GFD, the mean maximum ABC was higher in their patients with persistent villous atrophy than in those without. However, they did not assess AI by H2AX or any other IHC marker to detect apoptosis. This relationship between the level of apoptosis and the degree of tissue injury (a completely flat mucosal surface) could be partly attributed to the fact that when the degree of apoptosis exceeds the available scavenging ability of macrophages and dendritic cells, secondary necrosis occurs leading to an uncontrolled inflammatory reaction and persistent tissue injury.[20]

Although crypt apoptosis has been observed in CD with ancillary techniques, histologic evaluation of crypt ABC has only been reported in one study in adult CD,[7] and it was never assessed in pediatric patients, to the best of our knowledge. Moreover, this is the first study to assess crypt ABC in pediatric CD both before and after the initiation of a GFD using both H&E and IHC and then comparing the results of these techniques. Additionally, the current study assessed the predictive potential of both techniques. There was no statistically significant difference between the predictive potential of either H&E or IHC for assessing crypt ABC. Each of these two techniques, maximum ABC by H&E and AI by H2AX IHC, had a significant predictive potential for persistent villous atrophy following institution of a GFD with cut-off values of ≥6.61 ($P=0.008$ by H&E) and ≥105.4 ($P=0.003$) by IHC.

Crypt apoptotic bodies are morphological abnormalities that are usually present in celiac disease, but they are rarely included in routine histological reports, since they are usually difficult to assess histologically and require a thorough high-power evaluation at several levels. In addition, only a few diseases, such as acute cellular rejection of an intestinal allograft and reactions to some medications like mycophenolic acid, require their identification to reach a pathological diagnosis. Therefore, evaluation of crypt apoptotic bodies is usually overlooked by pathologists, which may inappropriately suggest a low prevalence.[8,22,23] However, the results of this study might imply that, in cases of treatment-naïve CD, identification of crypt apoptotic bodies might have a significant predictive potential. Despite being a subjective measurement, estimation of ABC by H&E had a strong ICC between the readings of the two pathologists denoting a high interobserver agreement.

Non-compliance with a GFD has been encountered for a variety of reasons, highlighting the need for new CD therapies that target its underlying mechanism. This approach might provide promising complementary therapeutic options to a GFD.[24] Promising approaches might include apoptotic bodies (ApoBDs) which constitute a specific type of extracellular vesicle released by cells undergoing apoptosis. Previous studies have shown that contents of the ApoBDs might be used to activate the immune system, recruit dying cells, and regenerate damaged tissues, thus demarcating a potential therapeutic role in regenerative therapies.[25–27] In this context, a better understanding of ApoBD biology in the future might be of benefit in CD management especially in cases with post GFD persistent villous atrophy. Other promising therapeutic targets whose validation are yet to be established include H2AX and PI3kinases.[19]

One limitation to this study was the relatively small number of patients. This was likely due to the difficulty of collecting pediatric CD cases with follow-up biopsies after instituting a GFD. Further studies with larger patient cohorts are necessary to validate the current results, since only multi-institutional prognostic studies encompassing a larger sample size would be able to confirm the prognostic and therapeutic impact of apoptotic bodies in this context.

Conclusions

This is the first study, to our knowledge, to assess ABC by H&E and AI by IHC in pediatric CD both in treatment naïve patients and following the institution of a GFD in those same patients. Histopathological evaluation of crypt apoptotic bodies in treatment naïve CD could have predictive potential if the results of this study are validated in future studies with a larger cohort. Additionally,

assessment of crypt apoptotic bodies by either H&E or IHC provides similar predictive results for the persistence of villous atrophy following institution of a GFD.

Author contributions

Declaration of conflicting interests

Funding

Ethics approval and consent to participate

Written informed consent was obtained from legally authorized representatives (Parents or guardians of all patients) before the endoscopic biopsy was performed. The study was approved by Research Ethics Committee of Faculty of Medicine, Cairo University. (ID = N-55-2021).

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Supplemental material

Supplemental material for this article is available online.

Availability of data and material

All data generated or analyzed during this study is included in this submitted article.

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