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Study of Expression of Calprotectin and Myeloperoxidase in Clinically Diagnosed Inflammatory Bowel Disease

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ABSTRACT

Objective: To investigate the diagnostic role of calprotectin & myeloperoxidase expression in colonoscopic biopsies from patients suspected clinically to have inflammatory bowel disease (IBD) but with inconclusive diagnosis on histopathological examination.

Methods: Immunohistochemistry for Calprotectin and Myeloperoxidase was performed in colonoscopic biopsies obtained from fifty patients diagnosed clinically and/or endoscopically as IBD but with inconclusive pathological diagnosis and 10 positive control cases proved clinically, endoscopically & histopathologically as IBD were similarly studied. Epithelial calprotectin immunostaining was assessed using image analysis.

Results: Epithelial expression of calprotectin was encountered in 19 inconclusive cases (38%) and 6 positive control cases (60%). Image optical density of epithelial calprotectin immunostaining was not significantly different between both confirmed IBD cases and histopathologically inconclusive cases (p=0.459); and most of image optical density values for histopathologically inconclusive cases fell within the range of these of the confirmed cases of IBD. Myeloperoxidase score showed a significant difference (p=0.001) between the two studied groups as a whole, whereas it showed no significant difference (p=0.127) between the two groups demonstrating epithelial calprotectin staining in which most of the values of myeloperoxidase score in the histopathologically inconclusive cases fell within the range of the confirmed IBD cases.

Conclusion: Regarding epithelial calprotectin expression, cases that were inconclusive by histopathological examination demonstrated a similarity to – rather than a difference from- the confirmed cases of IBD. Therefore, epithelial expression of calprotectin immunostaining in tissue biopsy is helpful in the diagnosis of IBD as it was able to point to the cases with inconclusive histopathological diagnosis that showed agreement with the confirmed IBD cases.

Keywords: Inflammatory Bowel Disease; Myeloperoxidase

Introduction

Inflammatory bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis, are chronic relapsing disorders of the gastrointestinal tract that are characterized pathologically by intestinal inflammation and epithelial injury. [1,2] Inflammatory bowel diseases are lifelong disorders that are predominantly observed in developed countries. [3] Recently, reviews about the incidence of IBD revealed that as developing countries become more westernized the incidence of IBD increases, first UC followed by CD. [4]

The diagnosis of IBD requires a multidisciplinary approach. The diagnosis is reached by combining clinical data and laboratory findings together with typical endoscopic, pathologic and radiological findings. Thus, the histopathological examination of either endoscopic biopsies or resection specimens remains a cornerstone in the diagnosis of IBD and also in differential diagnosis, particularly in the differentiation of UC from CD and other non-IBD related colitides.[5,6]

Despite the golden role of pathological examination of colonic biopsies in the diagnosis of IBD, the difficulties facing the pathologists to reach an accurate final diagnosis still hinder the proper management of the affected patients. These difficulties may be due to the lack of awareness of the range of normal colorectal histology and the minimal features which indicate clinically important inflammation, the wide overlap in the pathological changes of most large bowel inflammatory diseases, and the absence of standard terminology for pathological description and diagnosis. Thus the issue of variability of reporting style and terms such as mild inflammatory change and non-specific colitis/proctitis may hide pathologists' diagnostic uncertainty and so confuse clinical management. [7]

Calprotectin is a proinflammatory factor of innate immunity.[8] It belongs to the family of S100 calcium binding proteins.[9] The Calprotectin molecule is a calcium and zinc binding heterodimeric molecule of 36.5 kDa, having two subunits MRP-8(S100A8) and MRP-14(S100A9).[10] MRP8 is the active subunit of MRP8/14 and MRP14 acts as the regulatory subunit preventing early degradation of MRP8.[11] MRP8 and MRP14 are are mainly expressed in cells of myeloid origin, such as granulocytes, monocytes and early stages of macrophages, but not in resident tissue macrophages.[12] Expression of these proteins can also be induced in keratinocytes and epithelial cells but only under inflammatory conditions.[13]

Fecal calprotectin plays a role in the screening and diagnosis of IBD cases. A previous meta-analysis of 13 studies reported that calprotectin had a pooled sensitivity of 93% and a pooled specificity of 96% to diagnose IBD in adults. [14]

Myeloperoxidases are lysosomal proteins which are released by activated neutrophils during inflammation. Only a few authors examined the role of MPOs as a diagnostic fecal marker of IBD. It has been reported that the diagnostic accuracy of MPOs to detect patients with IBD was inferior to calprotectin and polymorphonuclear elastase.[15]

There appear to be few published data on the immunohistochemical expression of calprotectin in intestinal mucosal biopsies. Therefore, the aim of our study was to investigate the diagnostic role of calprotectin immunohistochemical expression in patients suspected clinically to have IBD but with inconclusive diagnosis on histopathological examination. Also we aimed to study the relation between calprotectin and myeloperoxidase immunohistochemical expression in colonic mucosal biopsies in such patients.

Materials and Methods

Patients

This study was conducted on colonoscopic mucosal biopsies of IBD patients submitted to the Department of Pathology, Faculty of Medicine, Alexandria University during the years 2013-2015. Fifty cases were diagnosed clinically and/or endoscopically as IBD but the pathological diagnosis was not conclusive. The age of the patients ranged from 15 to 65 years (mean= 35.80). Thirty (60%) were males and 20 (40%) were females.

In addition, 10 positive control cases proved clinically, endoscopically & histopathologically as inflammatory bowel disease were studied.

Immunohistochemistry for Calprotectin and Myeloperoxidase

Two serial sections from each case were cut at 5 µm thick sections, mounted on positively charged, coated slides. Sections were deparaffinized in standard xyline (two changes, 10 minutes each). Then they were rehydrated in a graded alcohol series (100% to 70%) and finally brought to water for another five minutes. Sections were not allowed to dry from this point on. For both Calprotectin and Myeloperoxidase, heat induced antigen retrieval was done in a microwave oven for ten min twice in sodium citrate buffer. A monoclonal antibody against Calprotectin (Clone 27E10) and a polyclonal antibody against Myeloperoxidase were applied at a dilution of 1/100 and incubated overnight at +4°C in a humidified chamber. Bound antibodies were detected by the polymer-based system using Ultravision One Detection System, HRP Polymer & DAB Plus Chromogen (Ready-To-Use) that was provided by Lab Vision-Corporation (Fremont, USA).

In each immunohistochemistry run, tissue sections of acute suppurative appendicitis and tonsils were used as a positive control for Calprotectin and Myeloperoxidase respectively. Negative controls were obtained by omitting the step of addition of the primary antibody.

Analysis of staining

1-Calprotectin immunostaining

The pattern of distribution of immunostaining was evaluated under light microscope at X100 & X400 magnification. The observed patterns of distribution included: neutrophils (in the lamina propria, crypt abscesses, cryptitis), epithelial staining, extracellular material (within the lamina propria or subepithelial) and exudate.

Image analysis was resorted to for assessment of the epithelial staining. This is because epithelial staining -despite being significant positive cytoplasmic staining- was focal and the foci were too widely separated to be estimated by picking hot spots for semi quantitative method. [16]

2-Myeloperoxidase immunostaining

Using an Olympus light microscope (CX21), the slides were first screened at low power (X100 magnification) to identify the hot spots of myeloperoxidase immunostaining.

Then, within the hot spots, the number of myeloperoxidase positive neutrophils was calculated in 3 high power fields (X400 magnification) and their mean was calculated and termed “myeloperoxidase score”.

The positively stained neutrophils in the mucosal surface, crypts and tissue in between were counted. The neutrophils within the blood vessels were excluded at counting. [17]

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level.

Results

Immunostaining of Calprotectin in inconclusive and definitive cases

Four patterns of calprotectin immunostaining were observed: ***Cytoplasmic staining within the neutrophils*** either in the lamina prorpia, cryptitis, crypt abscess or within the surface epithelium (Figure 1a), epi***thelial cytoplasmic staining*** either surface mucosal

epithelium or crypt epithelium. It was significant but focal staining (Figure 1b&c), extracellular material within the lamina propria or subepithelial (Figure 1d) and exudate material (Figure 1e).

In the 50 histopathologically inconclusive cases under the study, 42 cases (84%) were positive for calprotectin IHC. Thirty four cases (68%) showed positivity within the neutrophils, 19 cases (38%) showed epithelial positivity, 7cases (14%) showed extracellular positivity and 7 cases (14%) showed exudate positivity.

All of the 10 positive control cases (100%) were positive for calprotectin IHC. Nine cases (90%) showed positivity within the neurophils, 6 cases (60%) showed epithelial positivity and a single case (10%) showed exudate positivity.

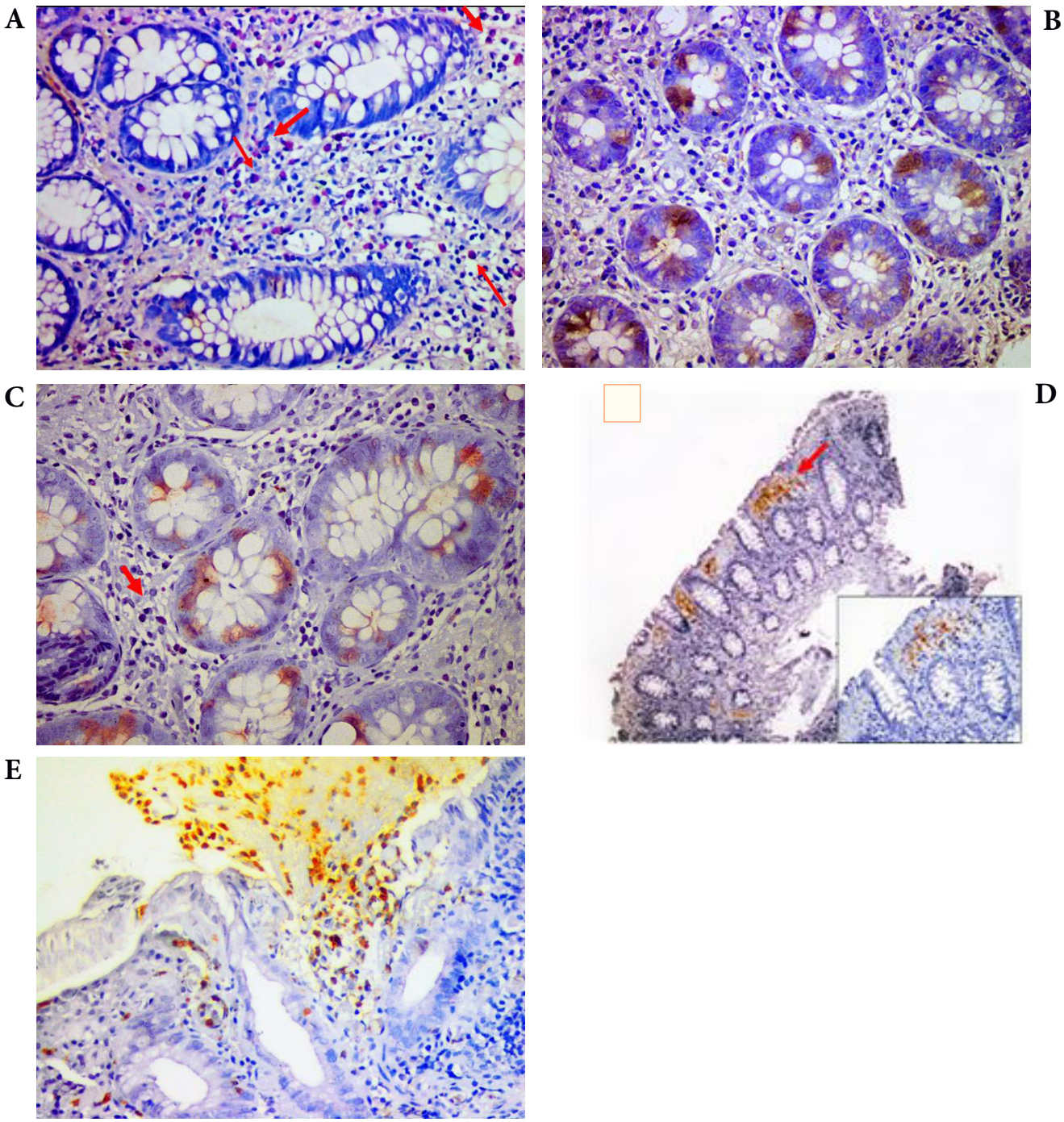

Figure 1: Colonoscopic biopsies showing: (A) Calprotectin positive neutrophils in the lamina propria (red arrows). (anti-calprotectin, X 400) (B&C) Focal calprotectin positivity in the crypt epithelial cells and positive neutrophils within lamina propria (red arrow). (anti-calprotectin, X 400) (D) Calprotectin positive subepithelial extracellular material (red arrow). (anti-calprotectin, X 100, inset X 400) (E) Positive neutrophils within exudate (anti-calprotectin, X 400).

The image optical density (IOD) of epithelial staining by calprotectin IHC in the 19 histopathologically inconclusive cases ranged between 78.65 and 158.92 (mean=116.09, median= 115.18, SD= ±23.20), while in the 6 positive control cases it ranged between 68.12 and 155.63 (mean=106.94, median=112.59, SD=± 33.96).

Immunostaining of Myeloperoxidase in inconclusive and definitive cases

Neutrophils exhibited positive cytoplasmic myeloperoxidase immunostaining. Neutrophils positive for myeloperoxidase were located in the lamina propria, cyptitis, crypt abscess, between epithelial surface mucosal cells and within the blood vessels. (Figure 2 a&b)

In the 50 histopathologically inconclusive cases, the myeloperoxidase score ranged between 15 and 314 (mean=112.91, median=101, SD=67.49) while in the 10 control cases, it ranged between 50 and 557 (mean=268.90, medina=194.50, SD=168.55).

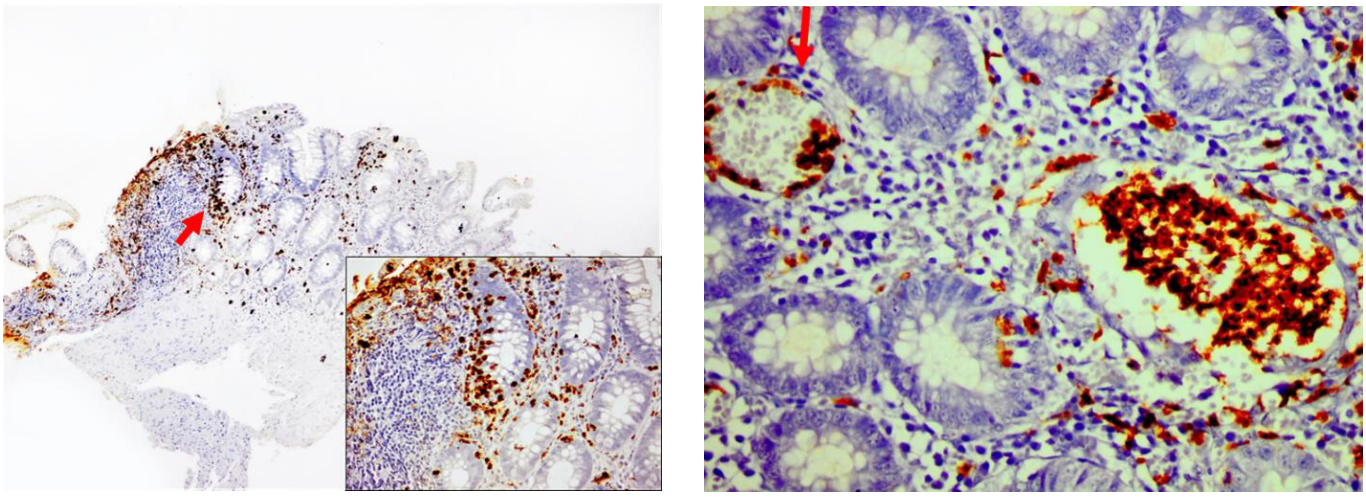

Figure 2: Colonoscopic biopsies showing (A) Myeloperoxidase positive neutrophils in the lamina propria and within crypt epithelium (cryptitis) (red arrow) (anti-myeloperoxidase, X100, inset X400). (B) Positive stained neutrophils in the lamina propria, within the crypts lumen (crypt abscess) and inside blood vessel which is excluded from the count (arrow) (anti-myeloperoxidase, X400).

Relation between Myeloperoxidase Score with Calprotectin IHC in inconclusive cases

Myeloperoxidase immunostaining score was found to be significantly higher in cases with positive calprotectin immunostaining staining (MW = 2.131, p= 0.033). (Table I)

	No.	Myeloperoxidase Score			MW	p
		Min. – Max.	Mean ± SD.	Median		
Calprotectin IHC						
Negative	**8**	15.0 – 113.0	68.50 ± 29.27	76.50	2.131*	0.033*
Positive	**42**	22.0 – 314.0	121.37 ± 69.57	113.50		

MW: Mann Whitney test

p: p value for comparing between the two groups

*: Statistically significant at p ≤ 0.05

Table 1: Relation between myeloperoxidase score with calprotectin expression in histopathologically inconclusive cases

Further assessment of cases with positive epithelial staining for calprotectin

The cases with positive epithelial staining for calprotectin were selected for further evaluation and correlation based on the concept that calprotectin is believed to be activated and secreted by the mucosal epithelial cells in IBD which may not be the case in other inflammatory conditions in the colon whereas calprotectin expression in neutrophils could be present in other inflammatory conditions. [13,18]

Results of both calprotectin immunostaining (as assessed by image analysis) and myeloperoxidase immunostaining score of cases showing epithelial staining for calprotectin in the two studied groups were compared to evaluate the significance and value of epithelial staining in reaching a diagnosis in the histopathologically inconclusive cases.

(a) Results of calprotectin immunostaining (as assessed by image analysis): (Table II)

No significant difference was observed between the image optical density of epithelial calprotectin immunostaining of both confirmed IBD caese and histopathologically inconclusive cases (t=0.754, p=0.459). In addition, most of the values of image optical density of calprotectin immunostaining of the histopathologically inconclusive cases fall within the range of image optical density of calprotectin immunostaining of the confirmed cases of IBD. These two observations suggest that the cases that were inconclusive by histopathological examination demonstrate a similarity to – rather than a difference from- the confirmed cases of IBD.

	Histopathologically inconclusive Cases (n = 19)	**Confirmed IBD cases (n = 6)**	**Test of Sig.**	**P**
Image optical density of epithelial staining by calprotectin				
Min. – Max.	78.65 – 158.92	68.12 – 155.63	t = 0.754	0.459
Mean ± SD.	116.09 ± 23.20	106.94 ± 33.96		
Median	115.18	112.59		

t: Student t-test

p: p value for comparing between the two groups

Table II: Comparison between the two studied groups as regards image analysis results of cases with epithelial staining for calprotectin

a. *Results* of myeloperoxidase immunostaining: (Tables III&IV)

	Histopathologically inconclusive cases (n = 50)	**Confirmed IBD cases (n = 10)**	**Test of Sig.**	**p**
Myeloperoxidase Score				
Min. – Max.	15.0 – 314.0	50.0 – 557.0	MW= 3.234*	0.001*
Mean ± SD.	112.91 ± 67.49	268.90 ± 168.55		
Median	101.0	194.50		

MW: Mann Whitney test

p: p value for comparing between the two groups

*: Statistically significant at $p \leq 0.05$

Table III: Comparison between the two studied groups according to myeloperoxidase immunostaining score

	Histopathologically inconclusive Cases (n = 19)	Confirmed IBD cases (n = 6)	MW	p
Myeloperoxidase Score				
Min. – Max.	22.0 – 314.0	50.0 – 546.0	1.527	0.127
Mean ± SD.	135.34 ± 81.85	239.17 ± 170.44		
Median	126.0	179.50		

MW: Mann Whitney test

p: p value for comparing between the two groups

Table IV: Comparison between the two studied groups as regards myeloperoxidse score in cases with epithelial staining for calprotectin

It was found that most of the values of myeloperoxidase score in the histopathologically inconclusive cases that showed positive epithelial staining for calprotectin fall within the range of the confirmed IBD cases that showed positive epithelial staining for calprotectin. In addition, there was no significant difference between the myeloperoxidase score of both groups (MW=1.527, p=0.127). These two observations suggest that the cases that were inconclusive by histopathological examination with positive epithelial staining for calprotectin probably belong to the category of IBD.

As Myeloperoxidase score showed a significant difference between the two studied groups as a whole, whereas it showed no significant difference between the two groups demonstrating epithelial calprotectin staining, the epithelial staining of calprotectin was able to choose the histopathologically inconclusive cases that are closer to the confirmed cases of IBD and so could be added to the confirmed cases.

Discussion

To the knowledge of the authors, this is the first study to address the value of epithelial immunostaining for Calprotectin- in colonoscopic biopsies- in the diagnosis of IBD.

The well recognized difficulties in interpretation of the histopathological findings in IBD may be due the wide overlap in the pathological changes of most large bowel inflammatory diseases and lack of awareness of the accuracy and reproducibility of many changes used in diagnosis. This result in variability of reporting style and terms such as mild inflammatory change and nonspecific proctitis/colitis which may hide pathologists' difficulties with diagnostic uncertainty and confuse clinical management. [19]

Therefore, the main aim of this study was reassessment of cases diagnosed clinically and endoscopically as inflammatory bowel disease and were histologically inconclusive for IBD. A marker that demonstrates comparable results in both the histopathologically inconclusive cases and confirmed IBD controls is supposed to accomplish the aim of this study.

In the present study, all the 50 studied histopathologically inconclusive cases and the 10 positive control confirmed cases of IBD were immunostained by both calprotectin and myeloperoxidase to assess their diagnostic value as markers of IBD.

Calprotectin IHC was chosen based on the fact that fecal calprotectin plays a role in the screening and diagnosis of IBD cases. We could not identify studies describing the immunohistochemical distribution of calprotectin in colonoscopic biopsies of IBD patients except for a single study conducted by Lügering et al in 1995. [20] This study was performed on confirmed cases of Crohn`s disease, without reference to cases with nonspecific colitis.

Myeloperoxidase was chosen in the current study being a lysosomal protein which is also released by activated neutrophils during inflammation. Only a few authors examined the role of MPO in stool as a diagnostic marker of IBD [21]. Silberer and colleagues found that the diagnostic accuracy of MPOs to detect patients with IBD was inferior to calprotectin and PMN elastase. [15]

To the best of our knowledge the present study is the first to address the cases that were inconclusive by histopathological examination as chronic nonspecific colitis and other ambiguous terms that reflect the uncertainty about the diagnosis of IBD by histopathological examination, with respect to calprotectin and myeloperoxidase immunostaining.

In the present study calprotectin IHC was positive in 42(84%) of the histopathologically inconclusive studied cases and all the 10(100%) positive control cases. Calprotectin immunostaining positivity showed different patterns: neutrophils, epithelial, extracellular and exudate.

Neutrophils showed positive cytoplasmic staining for calprotectin. The positive neutrophils were located in the lamina propria, within the crypts in the form of cryptitis or crypt abscess and infiltrating the surface mucosal epithelium. This pattern was observed in both colonic and ileal biopsies. It was found in 34 (68%) of the studied histopathologically inconclusive cases and 9 (90%) of the positive control confirmed cases of IBD.

Also both the colonic and ileal epithelial mucosal cells -either crypt or surface- showed a significant cytoplasmic staining in 19 (38%) of the studied histopathologically inconclusive cases and 6 (60%) of the positive control confirmed cases of IBD.

The calprotectin also showed positivity as extracellular material within the lamina propria or submucosa reflecting secretion of the protein. This pattern was observed in 7 (14%) of the histopathologically inconclusive studied cases and none (0%) of the positive control confirmed cases.

The exudate showed positive staining both as extracellular material and also in the neutrophils within the exudate. This pattern was observed in 7(14%) of the histopathologically inconclusive studied cases and only one (10%) of the positive control confirmed cases.

On comparing both groups regarding the localization of calprotectin expression, there was no significant difference between the two groups.

A significant association was found between calprotectin expression and basal plamacytosis. This finding suggests that the presence of basal plasmacytosis on histological examination may predict positivity for calprotectin by IHC, and that cases demonstrating basal plasmacytosis should have the priority in performing calprotectin IHC.

Lügering et al [20] reported that calprotectin (27E10) could be immunolocalized in inflamed tissues of CD patients in the majority of granulocytes and macrophages near the lesions. Also it showed strong immunoreactivity in epithelial cells near the inflammatory reactions. They also reported that calprotectin immunohistochemical expression showed no difference in the distribution pattern between small and large bowel biopsies.

Foell et al [18] reported that staining for calprotectin (MRP8/14) was not only found in infiltrating phagocytes but also in epithelial cells in ileal CD, which may point to the fact that small bowel epithelium can directly contribute to calprotectin (MRP8/14) release.

Leach et al [22], reported that serum and mucosal calprotectin and S100A12 levels were increased in children with IBD as compared with non-IBD controls. S100A8, S100A9 and S100A12 were found to be abundantly expressed throughout the lamina propria and epithelium in inflamed mucosa, whereas these proteins were present in the lamina propria, but not the epithelium, in non-inflamed mucosa.

Foell et al [18] found that cultured inflamed intestinal tissue samples from Crohn's disease and ulcerative colitis patients spontaneously release considerably more calprotectin than non-inflamed or healthy controls.

It has been proposed that mucosal epithelial cells can only under inflammatory conditions as IBD be induced to secrete calprotectin. [13, 118] Arijs et al [23] stated that the expression of S100A8 and S100A9 is substantially upregulated at mRNA level in inflamed ileal and colonic mucosa of Crohn`s patients. In ulcerative colitis mRNA levels are even higher in the colonic mucosa. The epithelial

calprotectin immunostaining was chosen in the current study for further evaluation based on the concept that it may render more specificity for IBD than the inflammatory cells that could be found in different proportions in other inflammatory conditions.

Thus, in the present work, image analysis was used to assess calprotectin epithelial staining because epithelial staining although being significant was focal and patchy and the positive foci were widely separated [16], which renders evaluation of immunostaining by semi quantitative methods not practical.

In the 50 histopathologically inconclusive cases under the study, 19 cases (38%) showed epithelial positivity, while in the 10 positive control cases, 6 cases (60%) showed epithelial positivity. The image optical density (IOD) of epithelial staining by calprotectin IHC in the 19 histopathologically inconclusive cases ranged between 78.65 and 158.92 (mean=116.09, median= 115.18, SD= ±23.20), while in the 6 positive control cases it ranged between 68.12 and 155.63 (mean=106.94, median=112.59, SD=± 33.96).

Myeloperoxidase score was significantly higher in confirmed cases of IBD than in histopathologically inconclusive cases; and correlated significantly with both crypt distortion and crypt abscess. Thus, a significant correlation was found between myeloperoxidase score and not only acute inflammatory features i.e activity (which is explained by the presence of neutrophils during activity that secrete myeloperoxidase), but also and one of the features of chronicity which is crypt distortion. This finding may suggest that myeloperoxidase protein may not only serve to detect the activity of IBD but also may help to the establish diagnosis of IBD.

Regarding the relation between calprotectin IHC and myeloperoxidase scores in the 50 studied histopathologically inconclusive cases, it was found that myeloperoxidase score was significantly higher in calprotectin positive cases. This may be explained by the fact that both proteins are secreted by neutrophils although calprotectin is also secreted by monocytes and epithelial cells. [21]

Both groups of patients (the histopathologically inconclusive cases & the confirmed cases of IBD) were compared as regards the epithelial calprotectin staining using image analysis and the myeloperoxidase score.

Regarding the comparison between the cases that showed epithelial staining in the 2 studied groups (histopathologically inconclusive cases with epithelial staining=19, confirmed IBD cases with epithelial staining=6) according to the image optical density, there was no significant difference between the image optical density of both groups and most of the values of the image optical density of the histopathologically inconclusive cases fell within the ranges of the image optical density of the confirmed IBD cases.

These observations suggest that the cases that were inconclusive by histopathological examination demonstrate a similarity to- rather than a difference from- the confirmed cases of IBD.

Based on these findings it could be suggested that histopathologically inconclusive cases that demonstrate positive epithelial staining for calprotectin probably belong to the category of IBD.

Furthermore, based on the statistical findings of the current study, myeloperoxidase score showed a significant difference between the two studied groups as a whole, whereas it showed no significant difference between the two studied groups when choosing cases that demonstrated calprotectin epithelial staining; with most of the values of the myeloperoxidase score of the histopathologically inconclusive cases falling within the range of the myeloperoxidase score of the confirmed cases of IBD. This suggests that epithelial calprotectin staining was able to select the histopathologically inconclusive cases that showed agreement with and were closer to the confirmed IBD cases, and thus can serve as a valuable marker to suggest the diagnosis of IBD in cases that were inconclusive by histopathological examination.

In conclusion, immunohistochemical expression of calprotectin in tissue biopsy is helpful in the diagnosis of IBD as, specifically epithelial calprotectin immunostaining, was able to point to the cases with inconclusive histopathological diagnosis that showed agreement with the confirmed IBD cases.

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