

Neutrophil Extracellular Traps in Colonic Mucosa: Biomarker Potential for Assessing Disease Activity in Pediatric Crohn's Disease

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Abstract

Objective

To investigate the expression of neutrophil extracellular traps (NETs) in colonic tissues of pediatric Crohn's disease (CD) and to explore its correlation with pediatric CD.

Methods

Pediatric CD colonic tissues by colonoscopy biopsy were selected as the subjects. The Pediatric Crohn's disease Activity Index (PCDAI) was used to assess the disease activity of CD. Immunofluorescence staining was used to detect the expression of citrullinated histone H3 (CitH3), myeloperoxidase (MPO), and neutrophil elastase (NE), the core biomarker components of NETs in colonic tissues. Correlations between the expression of CitH3, MPO, and NE and pediatric CD were analyzed.

Results

32 CD cases were enrolled, with an average age of 12.06 ± 2.75 years, including 10 in the mild activity phase, 13 in the moderate activity phase, and 9 in the severe activity phase. The positive expression rates of CitH3, MPO, and NE in CD colonic tissues were significantly higher than those in control colonic tissues ($P < 0.05$). The relative expression levels of CitH3, MPO, and NE were significantly increased in the CD group compared with the control group ($P < 0.001$). The expression of CitH3, MPO, and NE increased progressively with higher disease activity grades ($P < 0.05$). The expression levels of CitH3, MPO, and NE were all significantly positively correlated with PCDAI scores ($r = 0.652, 0.753, \text{ and } 0.757$, respectively; all $P < 0.001$).

Conclusion

NETs may participate in the pathogenesis of colonic epithelial injury in CD and are correlated with the severity of CD.

Introduction

Crohn's disease (CD) is a subtype of inflammatory bowel disease (IBD), characterized by chronic, non-specific, relapsing intestinal inflammation with skip lesions and transmural involvement that can affect the entire gastrointestinal tract [1]. In recent years, the incidence of CD in children has risen markedly [2, 3]. Pediatric CD often has an insidious onset and lacks specific early clinical manifestations, leading to frequent misdiagnosis or delayed diagnosis. Its protracted course and diverse complications pose considerable challenges for early diagnosis and standardized management [4, 5]. The precise etiology of CD remains incompletely understood. However, accumulating evidence suggests that genetic

susceptibility, microbial infections, intestinal immune dysregulation, environmental exposures, and dietary factors collectively contribute to its development and progression [6, 7]. Neutrophil extracellular traps (NETs) are web-like structures released by neutrophils upon exposure to endogenous or exogenous stimuli. They are primarily composed of DNA–histone complexes decorated with effector proteins, including citrullinated histone H3 (CitH3), myeloperoxidase (MPO), and neutrophil elastase (NE) [8]. NETs play a critical protective role in host defense by capturing, immobilizing, and neutralizing bacteria, viruses, and other pathogens. However, excessive NETs formation can trigger detrimental inflammatory cascades and cause tissue injury, thus exerting both protective and damaging effects on the host [9, 10].

This is an exploratory study aimed at investigating the expression of NETs in pediatric CD. This study used immunofluorescence staining to detect the expression of CitH3, MPO, and NE in colonic tissues of pediatric CD, analyzing the correlation between their expression and the disease activity of CD, and providing a potential biomarker for the clinical assessment of disease activity in patients with CD.

1. Materials and Methods

1.1 Study subjects

A total of 32 pediatric CD cases first diagnosed at Jiangxi Province Children's Hospital between September 2021 and December 2025 were selected. 30 normal colonic tissues by colonoscopy biopsy during the same period were included as the control group. The diagnostic criteria for CD followed the 2019 Chinese *expert consensus on the diagnosis and management of pediatric inflammatory bowel disease* [11]. Inclusion criteria: (1) meeting the diagnostic criteria for CD; (2) age < 18 years, regardless of sex; (3) well-preserved colonic tissue by colonoscopy biopsy; (4) no prior treatment with aminosalicylates, corticosteroids, biologics, or immunosuppressants before colonoscopy. Exclusion criteria: (1) inability to diagnose CD; (2) presence of other chronic infectious intestinal diseases or intestinal malignancies; (3) incomplete clinical data. This study was reviewed and approved by the Jiangxi Province Children's Hospital ethics committee (JXSETYY-YXKY-20240205), with a waiver of informed consent for retrospective studies.

1.2 PCDAI scoring and grouping

The Pediatric Crohn's disease Activity Index (PCDAI) was used to evaluate CD disease activity in accordance with the guideline *Evaluation of Children with Inflammatory Bowel Disease* [12]. PCDAI scores were based on factors such as abdominal pain, stool characteristics, general condition, body weight, height, abdominal signs, perianal disease, extraintestinal manifestations, hematocrit, erythrocyte sedimentation rate, and albumin. According to PCDAI scores, patients were classified as: clinical remission phase (< 10.0), mild active phase (10.0 ~ 27.5), moderate active phase (30.0 ~ 37.5), and severe active phase (40.0 ~ 100.0) [13].

1.3 Experimental methods

1.3.1 Specimen collection

Colonic tissue by colonoscopy biopsy from CD cases first diagnosed at the pathology department of Jiangxi Province Children's Hospital were collected, along with normal colonic tissue by colonoscopy biopsy during the same period. All colonic specimens used in this study were the remaining wax block tissues after clinical pathological examination.

1.3.2 Immunofluorescence staining

Colonic tissue specimens were collected from cases who met the inclusion and exclusion criteria. Paraffin sections were deparaffinized in xylene and graded ethanol series, then subjected to antigen retrieval using EDTA (pH 9.0) or citrate buffer (pH 6.0) under high pressure. Endogenous peroxidase was blocked with 3% H₂O₂. After three washes with PBST (phosphate-buffered saline with Tween 20), sections were blocked with 5% BSA for 1 h, then incubated with primary antibodies against MPO and NE (1:100, Boster) overnight at 4°C. After PBST washes, sections were incubated with FITC-conjugated secondary antibodies (1:60, Boster) for 1 h in the dark. Following PBST washes and another blocking step, sections were incubated with anti-citH3 antibody (1:2000, Abcam) overnight at 4°C, then with TRITC-conjugated secondary antibody (1:60, Boster) for 1 h. Nuclei were counterstained with DAPI-containing mounting medium, and images were acquired under a fluorescence microscope.

1.3.3 Quantification of immunofluorescence

A quantitative approach was used to evaluate the fluorescence signal intensities of CitH3, MPO, and NE in colonic tissues across all groups. According to the evaluation criteria established by Sater et al [14], two pathologists independently reviewed the slides and reached a final consensus. Quantitative analysis of the immunofluorescence images was performed using ImageJ software, which enabled precise calculation and measurement of three parameters: positive area, mean fluorescence intensity (MFI), and integrated density (IntDen). The experimental procedure was as follows: three fields of view were randomly selected from different locations within each section using a fluorescence microscopy imaging system, and the arithmetic mean ($\bar{x}$) of the MFI values from these three fields was used to represent the relative expression level of CitH3, MPO, and NE in that particular section.

1.4 Statistical analysis

Statistical analyses were performed using SPSS 27.0, and graphs were created with GraphPad Prism 10.0. Categorical data were shown as counts and percentages, with group comparisons using the χ^2 test or Fisher's exact test. Continuous variables were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$) and compared among multiple groups using one-way analysis of variance. Correlations were assessed by Pearson's correlation analysis. A *P*-value < 0.05 was considered statistically significant.

2. Results

2.1 Baseline characteristics and disease activity

A total of 32 pediatric CD cases were enrolled, including 20 males and 12 females, with an average age of 12.06 ± 2.75 years. Normal colon tissues were obtained from 30 cases, including 19 males and 11 females, with an average age of 9.19 ± 2.66 years. According to the PCDAI, the classification of disease activity levels for pediatric CD is shown in Table 1. No statistically significant differences in gender and age were observed between the CD group and the control group ($P > 0.05$).

Table 1 General characteristics of the cases and the PCDAI subgroup [n (%)]

Group		CD group (n = 32)	Control group (n = 30)
Gender [n (%)]	Male	20 (62.5)	19 (63.3)
	Female	12 (37.5)	11 (36.7)
Age ($\bar{x} \pm s$ years)		12.06 ± 2.75	9.19 ± 2.66
PCDAI Subgroup [n (%)]	Clinical remission phase	0	--
	Mild active phase	10 (31.3)	--
	Moderate active phase	13 (40.6)	--
	Severe active phase	9 (28.1)	--

2.2 HE staining of colonic tissues

Hematoxylin and Eosin (HE) staining results are shown in Figures 1A–C. Histological damage worsened with increasing disease activity ($\times 20$). CD in mild active phase: intact epithelium; scattered lymphocytes, plasma cells, neutrophils, and eosinophils in the lamina propria; no crypt abscesses, erosions, ulcers, or granulomas; CD in moderate active phase: intact epithelium; increased lymphocytes, with few eosinophils, neutrophils, and mast cells; no glandular atypia or metaplasia; CD in severe active phase: intact epithelium, numerous lymphocytes and plasma cells, with some neutrophils and eosinophils, cryptitis and crypt abscesses observed.

2.3 Expression of CitH3, MPO, and NE in colonic tissues from patients with CD and controls

Positive immunofluorescence signals for CitH3, MPO, and NE appeared as red or green fluorescence, consistent with the respective fluorophores. Representative staining images of colonic tissues from patients with mild, moderate, and severe active CD and control colonic tissues are shown in Figures 2 and 3. The MFI values for each marker across groups are shown in Figures 4–6. The positive expression rates of CitH3, MPO, and NE in CD colonic tissues were significantly higher than those in control colonic tissues ($P < 0.05$).

2.4 Comparison of the relative expression levels of CitH3, MPO, and NE in colonic tissues between the CD group and the control group

Figure 7 shows the relative expression levels of CitH3, MPO, and NE in the CD group and the control group. Relative expression levels were determined based on the mean MFI of positive immunofluorescence signals, and the values for each group are summarized in Table 2. The relative expression levels of CitH3, MPO, and NE were significantly increased in the CD group compared with the control group ($P < 0.001$).

Table 2 Comparison of the relative expression levels of CitH3, MPO, and NE between the CD group and the control group

Group	n	CitH3		MPO		NE	
		MFI	<i>P</i>	MFI	<i>P</i>	MFI	<i>P</i>
CD group	32	42.05 ± 11.75	<0.001	45.54 ± 9.70	<0.001	42.66 ± 10.84	<0.001
Control group	30	23.19 ± 2.66		23.91 ± 2.40		24.73 ± 2.80	

2.5 Correlation of CitH3, MPO, and NE with PCDAI

The expression levels of CitH3, MPO, and NE in colonic tissues of CD patients stratified by disease activity grade are shown in Table 3. According to PCDAI scores, CD patients were classified into clinical remission phase, mild active phase, moderate active, and severe active phase. The expression of CitH3, MPO, and NE increased progressively with higher disease activity grades ($P < 0.05$). Pearson correlation analysis showed that the expression levels of CitH3, MPO, and NE were all significantly positively correlated with PCDAI scores. The correlation coefficients were as follows: CitH3 ($r = 0.652$, $P < 0.001$) (Figure 8), MPO ($r = 0.753$, $P < 0.001$) (Figure 9), and NE ($r = 0.757$, $P < 0.001$) (Figure 10).

Table 3 The expression levels of CitH3, MPO, and NE in CD groups stratified by disease activity grade

CD group	n	CitH3	MPO	NE
Clinical remission phase	--	--	--	--
Mild active phase	10	30.23 ± 11.52	35.41 ± 6.80	30.95 ± 10.44
Moderate active phase	13	44.71 ± 5.70	46.50 ± 4.69	45.36 ± 5.24
Severe active phase	9	51.35 ± 7.32	55.40 ± 6.40	51.77 ± 3.92

3. Discussion

CD has an insidious onset, a complicated and protracted course, and marked inter-individual variability in clinical symptoms. Disease activity, lesion location, and histopathological changes are closely linked to disease progression [15]. In children, the immune system is still maturing and the intestinal mucosal barrier is relatively fragile, so CD tends to progress rapidly. Long standing inflammation can lead to growth retardation, malnutrition, strictures, fistulas, and other serious complications that severely affect development and quality of life [16, 17]. Current evidence indicates that CD results from the interplay of multiple factors, among which neutrophil mediated inflammation plays a pivotal role in mucosal injury and chronic inflammation [6, 18]. Neutrophils can release NETs upon various stimuli. These structures exert antimicrobial defense by trapping and eliminating pathogens extracellularly. However, excessive NETs formation can perpetuate inflammation and tissue damage, contributing to several diseases, including IBD [19–21]. Under physiological conditions, the intestinal mucosa produces low amounts of NETs to defend against microbial invasion and maintain gut homeostasis. In CD, dysbiosis and pathogenic stimuli activate intestinal neutrophils abnormally, leading to excessive NETs release. Excess NETs, on the one hand, damage intestinal epithelial cells by their associated proteases, disrupting mucosal barrier integrity; on the other hand, they promote the release of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-8, thereby driving chronic intestinal inflammation and ulceration [22, 23]. In this study, we enrolled 32 first diagnosed pediatric CD patients, including 10 in the mild activity phase, 13 in the moderate activity phase, and 9 in the severe activity phase. Using immunofluorescence staining, we detected the expression of CitH3, MPO, and NE in colonic tissues by colonoscopy biopsy and analyzed their correlations with CD, aiming to provide a potential biomarker for the clinical assessment of disease activity in patients with CD.

Our results demonstrated that the positive expression rates of CitH3, MPO, and NE in CD colonic tissues were significantly higher than those in control colonic tissues ($P < 0.05$), and the relative expression levels of CitH3, MPO, and NE were significantly increased in the CD group compared with the control group ($P < 0.001$), suggesting that NETs may participate in the pathogenesis of colonic epithelial injury in CD. Neutrophil infiltration is a hallmark of intestinal inflammation in pediatric CD, and as critical indicators of neutrophil activation, the expression of NETs components including CitH3, MPO, and NE directly reflect the extent of NETs formation. Our immunofluorescence results demonstrated that the fluorescence signal of CitH3, MPO, and NE were significantly elevated in colonic tissues from patients with CD; these signals were detected not only in the neutrophil cytoplasm but also extensively within the extracellular space. In contrast, in normal colonic tissues from the control group, the fluorescence signal for these proteins were weak and punctate, confined to the cytoplasm of a few neutrophils in the lamina propria. NETs play a significant role in the intestinal inflammatory process of CD and may contribute to disease development and progression through multiple mechanisms. First, NETs can release MPO and NE, which disrupt the integrity of the intestinal mucosal barrier, allowing luminal antigens and microorganisms to invade the mucosal layer and thereby aggravate intestinal inflammatory injury [24]. Second, within the inflammatory microenvironment of CD, cytokine enrichment and exacerbated oxidative stress promote neutrophil activation and NETs release. In turn, NETs can activate macrophages, leading to massive cytokine production and further amplifying the inflammatory cascade

[21, 25]. Furthermore, decreased deoxyribonuclease activity and aberrant expression of anti-NETs autoantibodies in CD patients impair NETs clearance, resulting in persistent NETs accumulation and exacerbated intestinal inflammation [26]. Our results showed that the positive expression rates of CitH3, MPO, and NE in colonic tissues from patients with CD were all significantly increased, indicating that NETs formation had surpassed the physiological protective threshold and shifted from a protective to a detrimental effect, thereby becoming a key factor in sustaining and exacerbating chronic inflammation. MPO can generate large amounts of reactive oxygen species through oxidative stress, directly causing oxidative damage to intestinal epithelial cells. NE can degrade tight junction proteins and extracellular matrix components of the intestinal mucosa, disrupting the mucosal barrier and amplifying inflammatory signals. Increased CitH3 expression drives sustained NETs formation, and the synergistic effect of these three molecules may be involved in the pathological processes of inflammatory infiltration, erosion, and ulceration in the intestinal mucosa of CD [27].

To investigate the correlation between NETs and disease activity in CD, we stratified pediatric CD patients according to PCDAI scores into clinical remission phase, mild active phase, moderate active phase, and severe active phase. Our results demonstrated that the expression of CitH3, MPO, and NE increased progressively with higher disease activity grades ($P < 0.05$), and the expression levels of these markers were all significantly positively correlated with PCDAI scores ($P < 0.001$). These findings indicate that the expression levels of CitH3, MPO, and NE can effectively reflect disease activity in CD. Lin et al. [28] reported that NETs can damage colonic epithelial cells, disrupt intestinal barrier function, and promote the development of 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. During the pathogenesis of CD, pathogenic microorganisms, inflammatory cytokines, and damage-associated molecular patterns can activate intestinal neutrophils, triggering NETs release and consequently increasing the expression of CitH3, MPO, and NE [29]. The PCDAI is an important scoring tool for assessing disease activity in pediatric CD, integrating multiple parameters including clinical symptoms, hematocrit, erythrocyte sedimentation rate, and serum albumin. However, this scoring system has certain limitations: the subjective nature of symptom assessment and the lack of specificity of laboratory parameters make it difficult to precisely reflect the degree of intestinal lesion activity [30]. CitH3, as a specific marker of NET formation, shows a significant positive correlation with disease activity and can reflect the extent of intestinal NETs formation. MPO and NE, as core functional proteins of NETs, also exhibit significant positive correlations with disease activity; their increased expression levels may directly indicate the disease activity of intestinal lesions. A study on TNBS-induced CD mouse models demonstrated that as disease activity increased, the expression levels of CitH3, MPO, and NE in colonic tissues rose significantly and were positively correlated with intestinal mucosal injury scores [31]. During the active phase of CD, intestinal inflammation intensifies continuously, with massive neutrophil infiltration and aberrant activation at inflammatory sites, which initiates NETs formation and releases large amounts of NETs. This leads to accumulation of CitH3, MPO, and NE in intestinal tissues, further exacerbating mucosal damage and driving disease activity to higher levels [32]. Conversely, during remission, intestinal inflammation subsides, neutrophil activation decreases, NETs formation is reduced, and the expression levels of CitH3, MPO, and NE correspondingly decline. Taken together, our

findings indicate that the expression levels of CitH3, MPO, and NE are correlated with disease activity in CD, providing a theoretical basis for the clinical assessment of disease progression and guidance of treatment through detection of these markers.

The present study has several limitations. All study subjects were pediatric CD patients admitted to our hospital, and the expression levels of CitH3, MPO, and NE were detected only in colonic tissues. Due to the single center source and relatively small sample size, as well as the absence of simultaneous detection of NETs levels in peripheral blood, further multicenter, large sample clinical studies are warranted to validate our conclusions. In addition, this study only analyzed the differences in CitH3, MPO, and NE expression between colonic tissues from CD patients and normal controls, without comparing their expression in colonic tissues from CD patients versus those with other non-inflammatory gastrointestinal diseases. In future work, prospective studies with longitudinal follow-up could be designed to dynamically monitor changes in CitH3, MPO, and NE expression at different stages of the disease in the same patient, and to analyze their correlations with varying disease activity phases, thereby providing a more robust theoretical basis for the clinical diagnosis and management of CD.

In summary, NETs are essential for host defense, but their excessive formation can aggravate intestinal inflammation and compromise the mucosal barrier, highlighting their dual role. Our study confirms that NETs may participate in the pathogenesis of colonic epithelial injury in pediatric CD and are correlated with disease severity of CD, offering a theoretical foundation for further exploring the value of NETs in assessing disease activity in patients with CD.

Declarations

Conflicts of interest

There are no conflicts of interest.

Author Contribution

Shuanghong Zhang , Xiaoxiao He , and Jingxing Fan wrote the main manuscript text . Chunzhi Kang , and Xinyan Zhao prepared figures 1-10 and table 1-3. All authors reviewed the manuscript.

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Figures

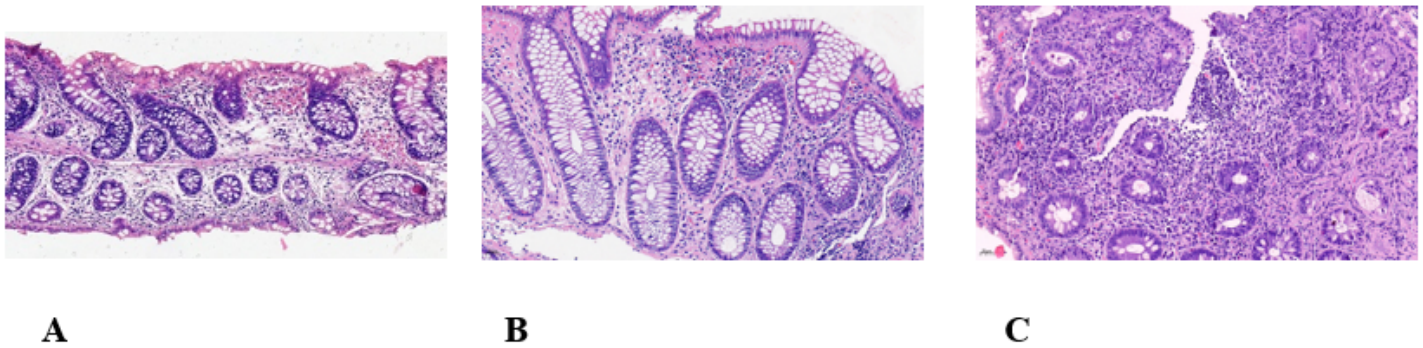


Figure 1

Colonic histopathological changes in pediatric CD stratified by disease activity grade. (A) HE staining ($\times 20$) of colonic tissue for CD in mild active phase. (B) HE staining ($\times 20$) of colonic tissue for CD in moderate active phase. (C) HE staining ($\times 20$) of colonic tissue for CD in severe active phase.

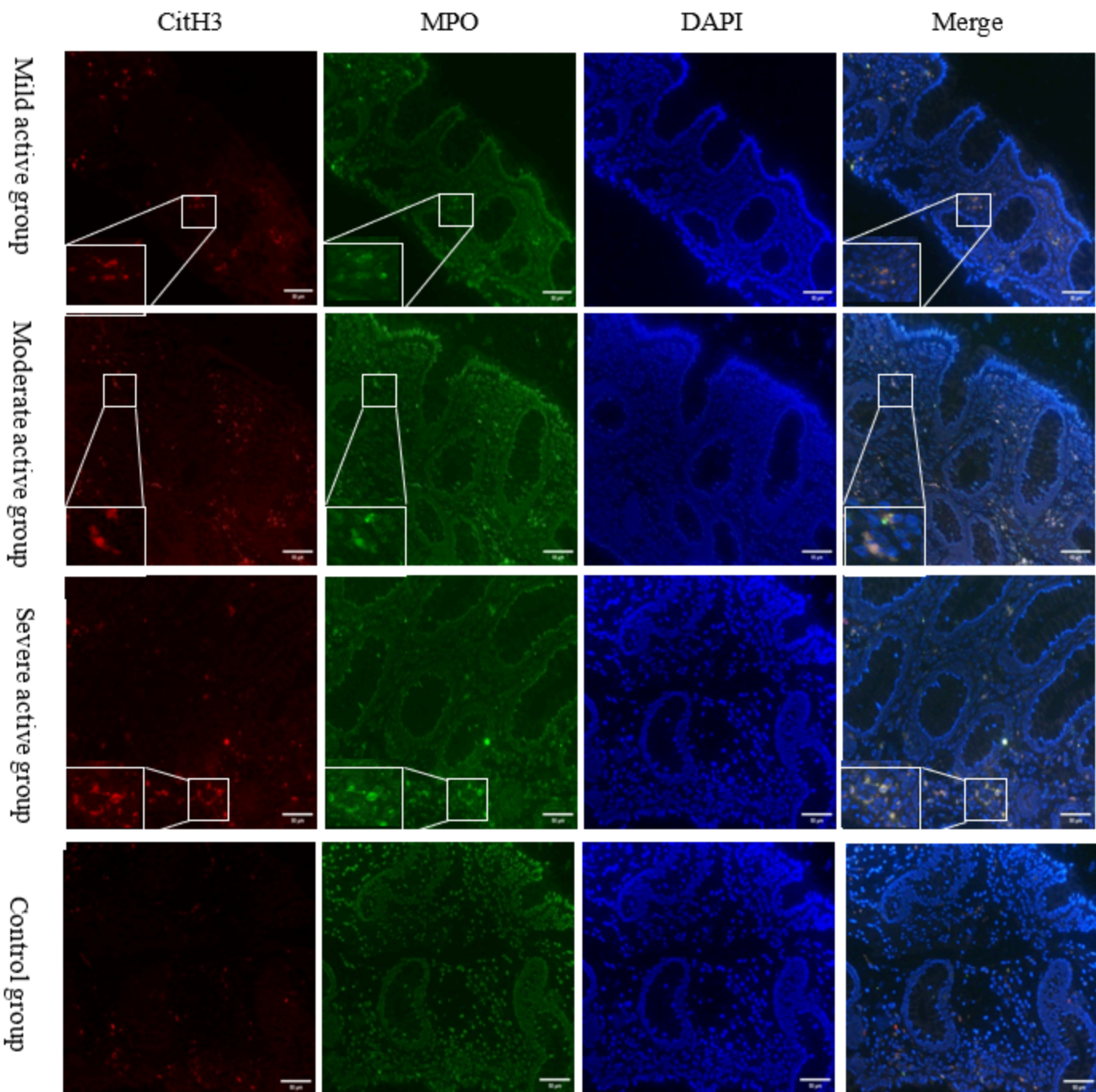


Figure 2

Immunofluorescence staining for CitH3 and MPO in CD and control groups (In CD colonic tissues, CitH3 and MPO immunofluorescence signals were observed not only in neutrophils within the lamina propria but also extensively in the extracellular space. In normal colon tissues, weak punctate signals were confined to the cytoplasm of a few neutrophils in the lamina propria. Main image $\times 20$; white box indicates magnified area $\times 40$.)

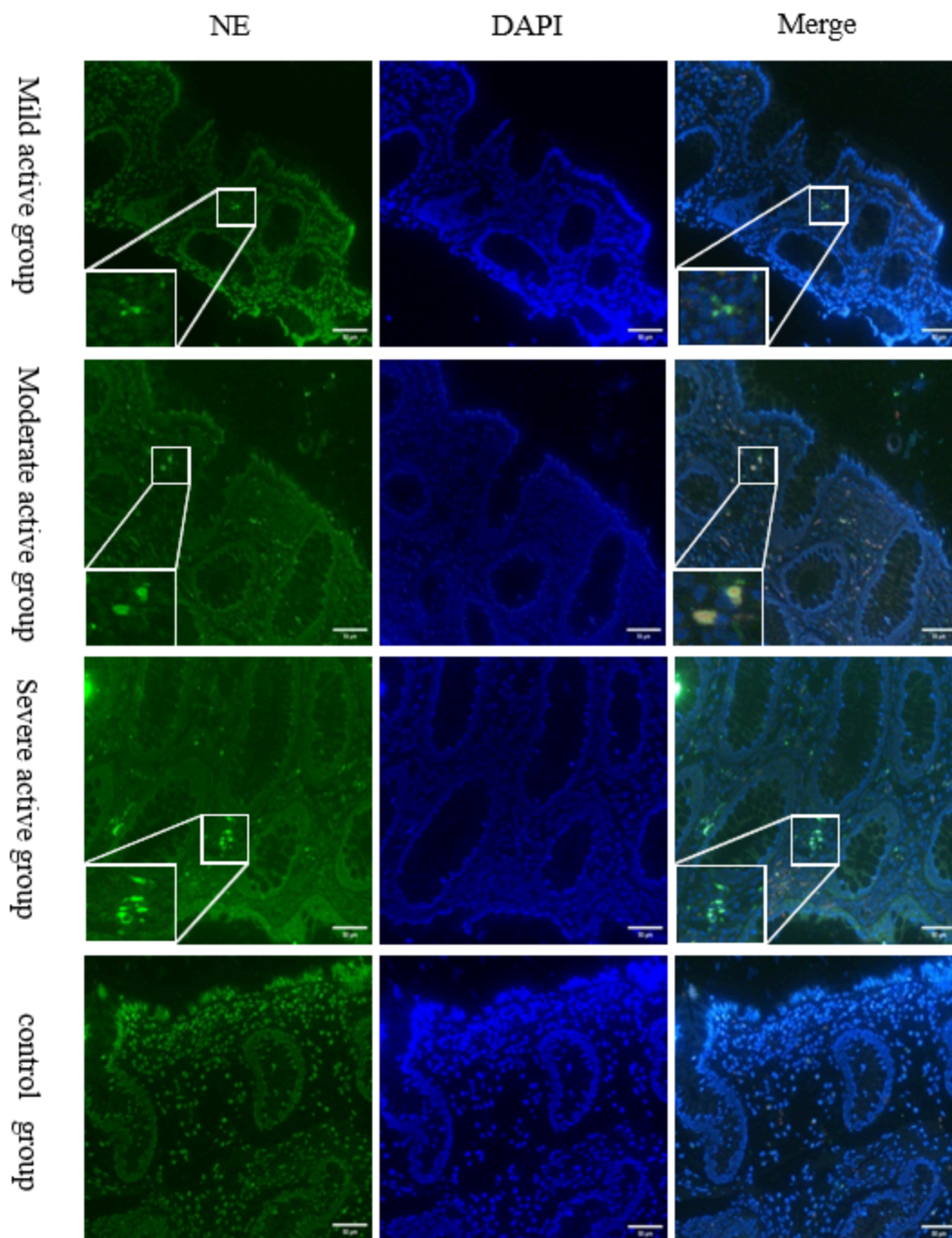


Figure 3

Immunofluorescence staining for NE in CD and control groups (In CD colonic tissues, NE immunofluorescence signals were observed not only in neutrophils within the lamina propria but also extensively in the extracellular space. In normal colon tissues, weak punctate signals were confined to the cytoplasm of a few neutrophils in the lamina propria. Main image $\times 20$; white box indicates magnified area $\times 40$.)

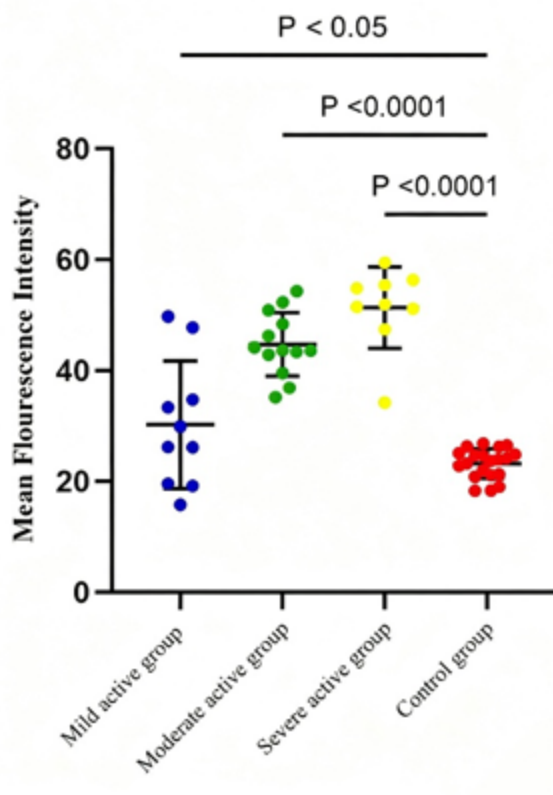


Figure 4

Mean Fluorescence Intensity of CitiH3 in the CD group stratified by disease activity grade and the control group

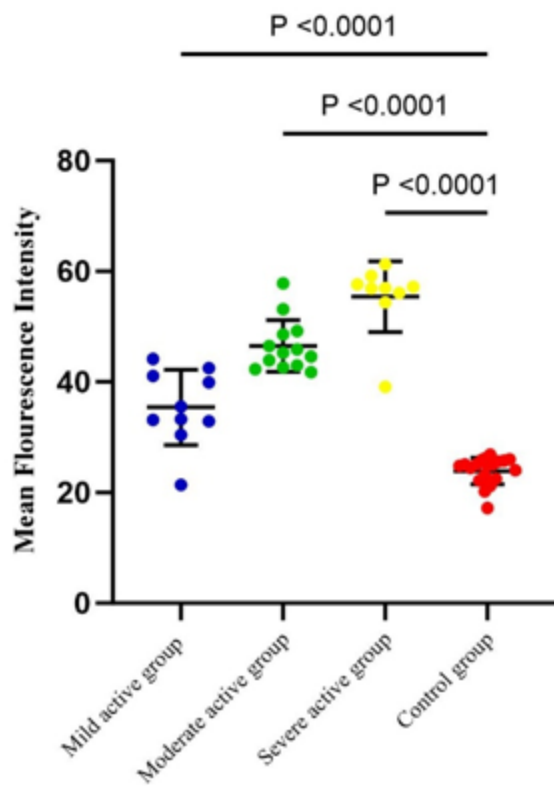


Figure 5

Mean Fluorescence Intensity of MPO in the CD group stratified by disease activity grade and the control group

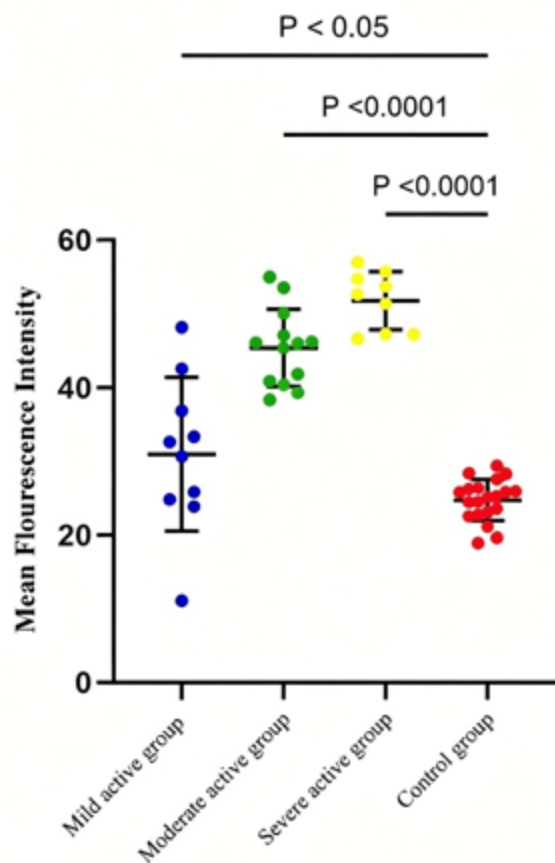


Figure 6

Mean Fluorescence Intensity of NE in the CD group stratified by disease activity grade and the control group

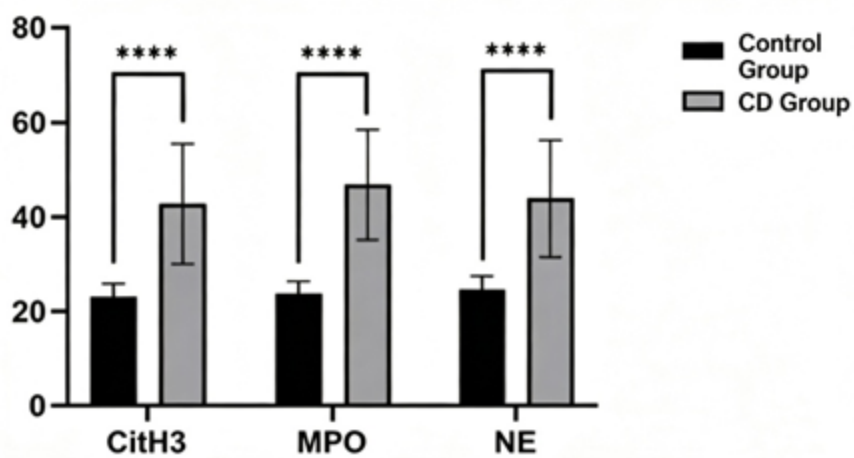


Figure 7

Comparison of the relative expression levels of CitH3, MPO, and NE between the CD group and the control group

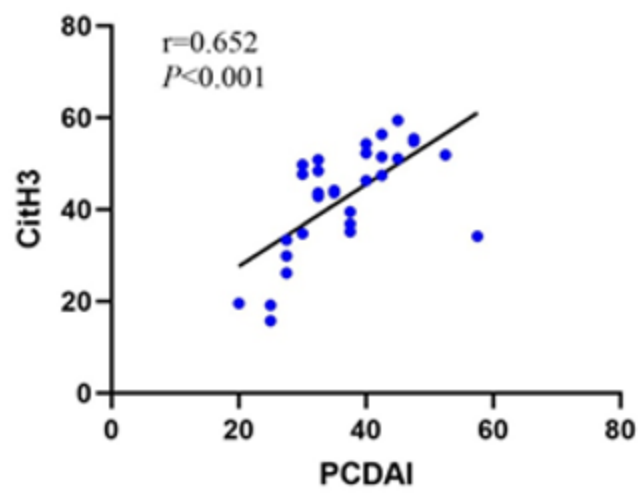


Figure 8
Correlation analysis between CitH3 and PCDAI

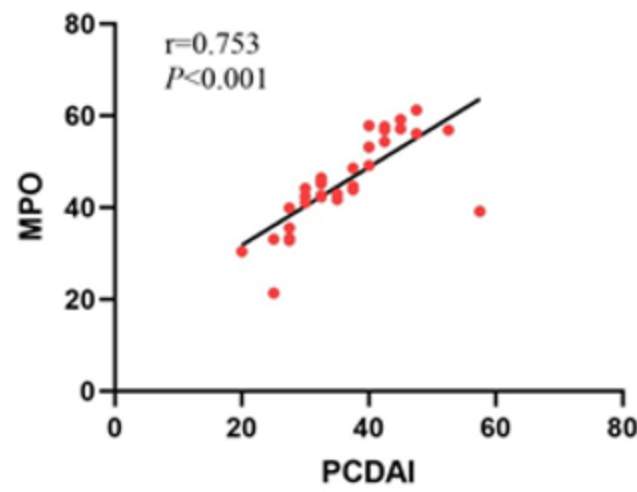


Figure 9
Correlation analysis between MPO and PCDAI

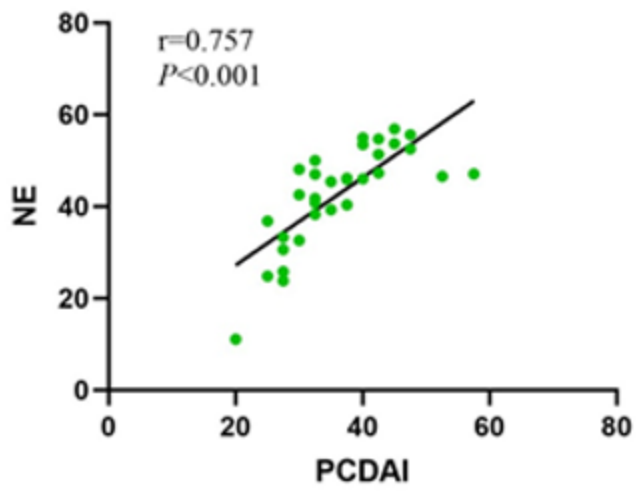


Figure 10

Correlation analysis between NE and PCDAI