Elevated caspase 3 expression correlates with severe inflammation in Crohn's disease

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ABSTRACT

Caspase 3 is a key executioner of apoptotic cell death and contributes to intestinal epithelial homeodynamics. Apoptotic dysregulation has been implicated in Crohn's disease, yet data on caspase 3 expression across disease activity states remain limited. This study analyzed caspase 3 expression in intestinal biopsies from Crohn's disease patients. Paraffin-embedded biopsies from 289 individuals were examined, including active disease (mild and severe inflammation), upper gastrointestinal involvement, remission and non-inflamed tissue. Expression in epithelial and immune cells was assessed by immunohistochemistry and scored using the Remmele immunoreactive score (IRS). Caspase 3 expression levels in epithelial cells increased in cases of severe inflammation (p=0.012), and immune cells exhibited even more pronounced expression levels (p<0.001). These findings suggest that caspase 3 expression in epithelial and immune cells may help to distinguish between mild and severe inflammation.

Key words: apoptotic cell death; gastrointestinal; immunohistochemistry; lamina propria.

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Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease characterized by segmental, transmural inflammation that can affect any part of the gastrointestinal tract, most commonly the terminal ileum and proximal colon.[1] The global incidence of CD is estimated at approximately 4 cases per 100,000 person-years and is increasing in many regions worldwide, particularly in recently industrialized countries, highlighting its growing global disease burden.[2,3] CD follows a relapsing-remitting course and arises from a complex interplay between epithelial barrier dysfunction and dysregulated immune responses.[1]

Impaired epithelial barrier function is considered an early event in CD pathogenesis, leading to increased permeability and enhanced exposure to luminal antigens. In the terminal ileum, Paneth cell dysfunction, associated with genetic variants such as Nucleotide-binding oligomerization domain-containing 2 (NOD2) and autophagy related genes including Autophagy-related 16-Like 1 (ATG16L1), compromises antimicrobial defense and facilitates bacterial translocation. This triggers a sustained mucosal immune response characterized by inflammation driven by T helper cells (Th1 and Th17) and reduced regulatory T cell activity.[4,5]

Chronic inflammation in CD is maintained by persistent immune activation and impaired tissue repair. Proinflammatory signaling pathways, including nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) and NOD-like receptor protein 3 (NLRP3) inflammasome activation, promote cytokine production and immune cell recruitment, whereas regenerative mechanisms contribute to epithelial restitution.[1,6,7] The maintenance of intestinal homeodynamics critically depends on a tightly regulated balance between cellular proliferation and apoptosis.[8,9] Disruption of this balance contributes to disease progression, with excessive epithelial apoptosis impairing barrier integrity and insufficient apoptosis of immune cells including lymphocytes (predominantly T cells), macrophages, plasma cells, eosinophils and neutrophils, promoting prolonged inflammation.[5,10] At the molecular level, apoptosis is executed by caspases, a family of cysteine-dependent aspartate-directed proteases that can be classified according to their position and function within the apoptotic cascade. Among them, caspase 3 represents the principal executioner caspase and a central mediator of apoptotic cell death. It serves as a central convergence point for both intrinsic and extrinsic apoptotic pathways, being activated downstream of mitochondrial cytochrome c-dependent caspase 9 signaling as well as death receptor-mediated caspase 8 activation.[11] Once activated, caspase 3 cleaves a broad spectrum of structural and regulatory substrates, thereby irreversibly committing cells to apoptosis. Consistent with this central role, basal caspase 3 expression in healthy tissues is essential for maintaining controlled apoptotic turnover, including the efficient elimination of damaged or dysfunctional epithelial cells.[12,13] Dysregulation of caspase 3 has therefore been implicated in a wide range of pathological conditions, encompassing chronic inflammatory diseases, neurodegeneration and cancer.[14,15]

Collectively, caspase 3 has a central role as a potential key regulator at the interface between physiological tissue homeodynamics and pathological cell damage. However, its expression in CD has not yet been systematically investigated in a cell type-specific and disease activity-dependent manner. A correlation between caspase 3 expression and disease activity may help to distinguish mild and severe inflammation. Therefore, the aim of this study was to systematically analyze caspase 3 expression in intestinal biopsies from patients with CD across different stages of inflammatory activity and to compare these findings with non-inflamed tissue.

Materials and Methods

Tissue samples and data acquisition

This retrospective study analyzed archived intestinal biopsy specimens from patients with CD obtained at the Institute of Pathology (Klinikum Bayreuth, Germany).

Biopsies collected between 2018 and 2025 as part of routine diagnostic procedures were retrieved from the institutional archive and eligible cases were randomly selected. Inclusion criteria comprised a histologically confirmed diagnosis of CD and the availability of adequate formalin-fixed, paraffin-embedded biopsy material of colon and ileum. Specimens with dysplasia or neoplasia, incomplete medical records, or insufficient material for immunohistochemical staining were excluded. Control samples consisted of intestinal biopsies from healthy individuals without histological signs of inflammation or neoplasia. All included cases represented endoscopic biopsies. Histological evaluation was performed on hematoxylin and eosin-stained sections. Based on established histopathological criteria for CD activity, 289 specimens were classified into the following subgroups: mild inflammatory activity, severe inflammatory activity, histological remission and CD with upper gastrointestinal tract involvement (duodenum and/or stomach).

Clinicopathological data including age, sex, and disease subgroup were retrieved from the pathology information system (dcpathos) and anonymized prior to analysis (Table 1).

Immunohistochemistry

All samples were fixed in 4 % neutral-buffered formalin for 24-48 h and routinely processed and paraffin-embedded according to standard histopathological protocols.[16] Sections (3 µm) were cut and stained with hematoxylin and eosin using automated staining systems. Paraffin-embedded tissue sections were prepared using a rotary microtome (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) and mounted on coated glass slides for immunohistochemical analysis. Immunohistochemistry (IHC) was performed on whole tissue sections to assess protein expression intensity and cellular localization. For each case, one section from the routine diagnostic workflow was stained with hematoxylin and eosin, while an adjacent section was immunohistochemically processed to evaluate total caspase 3 expression.

Sections were deparaffinized and stained with the automated Leica Bond III (Leica Mikrosysteme Vertrieb GmbH). Antigen retrieval was performed using Ethylenediaminetetraacetic acid (EDTA) buffer for 20 minutes. Sections were incubated with a monoclonal anti-caspase 3 antibody (clone 3CSP01 (7.1.44), Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a dilution of 1:100. The immunogen used for antibody production was recombinant full-length human caspase 3 protein and the antibody was validated according to the manufacturer's technical documentation. Primary antibody incubation was carried out for 15 min at room temperature.

Immunodetection was performed using the Bond Refine Detection Kit according to the manufacturer's instructions (Leica Biosystems, Nußloch, Germany). Human intestinal tissue served as positive control, while omission of the primary antibody was used as a negative control to exclude non-specific staining. Whole slide scanning and image acquisition was performed with Hamamatsu Nanozoomer S360 scanner and associated software (Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany), featuring a 20 × (NA 0.75) objective lens at a scanning resolution of 0.23 µm/pixel.

Evaluation of immunostaining

Immunohistochemically stained tissue sections were independently evaluated by two experienced pathologists blinded to clinical data. To ensure scoring consistency, a subset of randomly selected cases was reassessed, confirming concordant inter-observer scoring.

Digital image evaluation was performed using NDP.view2 image viewing software (Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany). Staining was assessed using the IRS as originally described by Remmele and Stegner.[17,18]

This semiquantitative scoring system integrates staining intensity and the proportion of positively stained cells, resulting in a composite score ranging from 0 to 12. Staining intensity was graded on a four-tier scale (0 = no detectable staining, 1 = weak, 2 = moderate, 3 = strong). The proportion of positively stained cells was scored on a scale from 0-4 (0 = no positive cells, 1 = less than 10%, 2 = 10-50%, 3 = 51-80%, 4 = more than 80%). The final IRS was calculated by multiplying both scores. Based on the resulting IRS values, samples were divided into four groups: negative (0-1), weakly positive (2-3), moderately positive (4-8), or strongly positive (9-12). No samples were classified as negative (IRS 0-1).

Caspase 3 expression was evaluated separately in intestinal epithelial cells at × 20 magnification using representative high-quality areas, avoiding necrosis and artifacts of each histological section. In immune cells of the lamina propria a × 40 effective magnification was used.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Mac, version 30.0 (IBM Corp., Armonk, NY, USA). As IRS values represent ordinal data, non-parametric tests were applied. Each disease group was compared directly with the non-inflamed control group, resulting in four comparisons indicated by the adjusted *p*-values (Table 1).

Group differences were assessed using two-sided Mann-Whitney U tests for independent samples. All tests were two-tailed and a *p*-value <0.05 was considered statistically significant. To correct for multiple testing, *p*-values were adjusted separately for epithelial as well as immune cells using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) at 5%.[19] Only adjusted p-values are presented and interpreted (Table 1).

Results and Discussion

Caspase 3 expression is ameliorated in epithelial cells of CD patients with severe inflammation

Caspase 3 expression was assessed by a semiquantitative IRS separately in intestinal epithelial cells and immune cells of the lamina propria, enabling compartment-specific analysis.

In non-inflamed intestinal tissue, caspase 3 expression in epithelial cells was predominantly of moderate intensity, with moderate IRS scores observed in 60.5 % of cases, whereas weak expression was detected in 34.2 % and strong expression in only 5.3 % of samples (Table 1). Representative immunohistochemical staining demonstrated a predominantly cytoplasmic staining pattern with additional nuclear positivity in epithelial cells, distributed diffusely along both the crypt and surface epithelium (Figure 1a). There were no notable differences in staining intensity between the deeper glandular epithelium and the surface epithelium. Tissue with mild inflammation (Figure 1b) as well as tissue from the upper gastrointestinal tract (Figure 1c) exhibited caspase 3 expression levels comparable to non-inflamed tissue, resulting in no statistically significant differences (p=0.169, mild inflammation; p=0.155, upper gastrointestinal tract).

A clear disease activity-dependent pattern of caspase 3 expression emerged in samples from patients with severe inflammation compared with non-inflamed tissue (p=0.012), characterized by a shift toward higher proportions of moderate (80.0%) and strong (9.1%) immunoreactivity (Table 1, Figure 1d) and a predominance of moderate staining, accompanied by an increased strong expression.

This observation is consistent with established concepts of acute mucosal injury in CD, in which pronounced inflammatory flares are accompanied by increased epithelial cell death, ulceration and disruption of epithelial barrier integrity.[20–22] Although the present study analyzed only the expression of the proenzyme in the tissue, this measure still provides valuable insight into the molecular regulation of apoptotic cell death. Accumulating evidence shows that total caspase 3 expression has pathological relevance. For example, immunohistochemical expression of caspases 3, 6 and 8 strongly correlated with an increased apoptotic index in aggressive breast lesions.[23,24] Consistently, a statistical correlation between caspase 3 expression and the apoptotic index in interface

Table 1. Clinical characteristics of the study cohort and caspase 3 immunoreactivity. The table summarizes the number of cases, age range and sex distribution, as well as the categorical classification of caspase 3 expression in intestinal epithelial cells and immune cells. Caspase 3 immunoreactivity was assessed by IHC and categorized as weak, moderate, or strong staining in non-pathological and pathological tissue samples. Statistical significance was assessed using the Mann-Whitney U test in comparison to non-inflamed control tissue (p-values). Asterisks mark statistically significant (*) or very significant (**) differences.

Diagnosis	Number	Age (years)	Sex		Staining intensities: epithelial (E) and immune cells (I)			*p*
			M	W	Weak	Moderate	Strong	
Non-inflamed control tissue	76	15-37	39	37	34.2% (E) 67.1% (I)	60.5% (E) 31.6% (I)	5.3% (E) 1.3% (I)	
Mild inflammation	54	27-90	25	29	27.8% (E) 53.7% (I)	57.4% (E) 40.7% (I)	14.8% (E) 5.6% (I)	0.169 (E) 0.190 (I)
Upper gastrointestinal involvement	51	21-74	26	25	47.1% (E) 56.9% (I)	51.0% (E) 43.1% (I)	2.0% (E) 0.0% (I)	0.155 (E) 0.489 (I)
Severe inflammation	55	20-79	30	25	10.9% (E) 1.8% (I)	80.0% (E) 41.8% (I)	9.1% (E) 56.4% (I)	0.012* (E) <0.001** (I)
Remission	53	24-81	22	31	54.7% (E) 58.5% (I)	41.5% (E) 30.2% (I)	3.8% (E) 11.3% (I)	0.052 (E) 0.237 (I)

membranes of aseptically loosened hip prostheses was observed.[25]

During remission, epithelial caspase 3 expression was reduced (p=0.052) compared with non-inflamed tissue. This decrease was reflected by a dominance of weak (54.7 %) and moderate (41.5%) staining intensities and a markedly low proportion of strong immunoreactivity (3.8%) (Table 1, Figure 1e). Figure 2 provides a comprehensive overview of the IRS values for all tissue types. Remission might not merely be characterized by a return to physiological baseline levels of apoptotic activity, but rather by an active suppression of caspase 3 expression in the context of mucosal healing and restoration of epithelial barrier function. This interpretation is supported by experimental and translational data demonstrating that the inflammatory microenvironment can induce adaptive, anti-apoptotic mechanisms within the intestinal epithelium.[26,27] In particular, inflammation-associated downregulation of the tight junction protein occludin was linked to suppression of CASP3 transcription and reduced epithelial apoptosis.[27] Promoter analyses revealed that occludin positively regulates CASP3 transcription and decreased occludin expression in biopsies from patients with CD or ulcerative colitis correlated with lower caspase levels. This mechanism has been interpreted as a protective adaptation aimed at limiting excessive epithelial cell loss under conditions of chronic inflammatory stress, even after resolution of overt inflammation.[28-30]

In summary, the increased caspase 3 immunoreactivity observed in intestinal epithelial cells within our severely inflamed samples most likely reflects enhanced apoptotic signaling within the intestinal mucosa of CD patients, which may directly contribute to barrier disruption in severe disease stages. However, the inflammatory microenvironment exerts the capability to induce

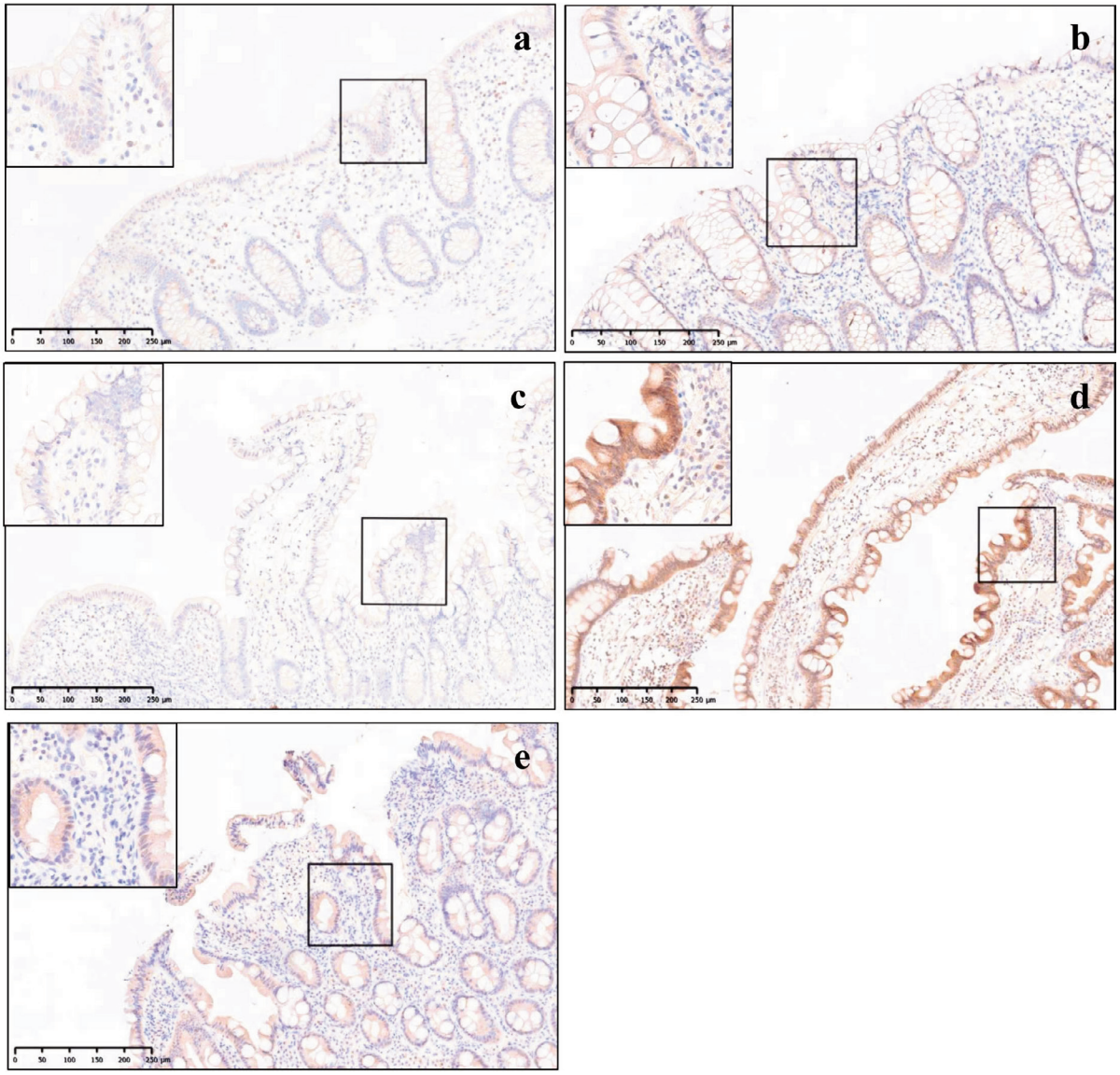
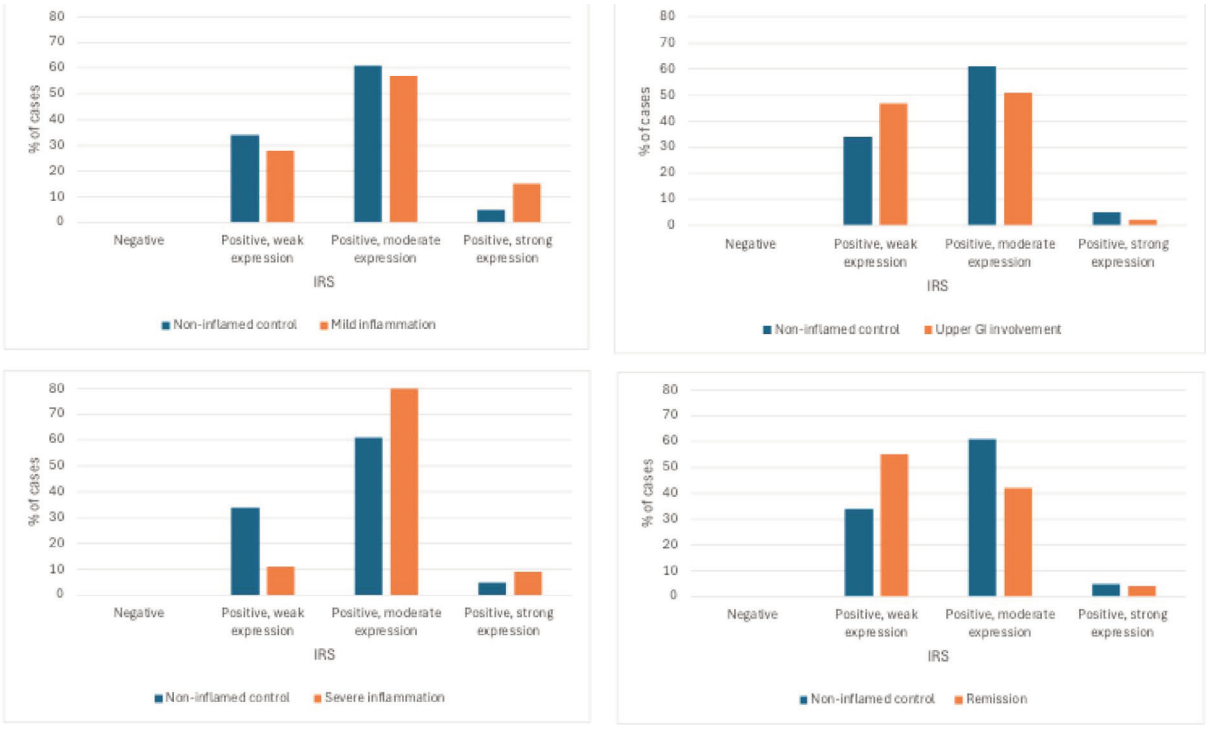


Figure 1. IHC of caspase 3 expression in intestinal epithelial cells in non-inflamed and pathological intestinal tissue. Representative images show: (**a**) weak expression in non-inflamed samples; (**b**) moderate expression in mild inflammation; (**c**) moderate expression in the upper GI tract; (**d**) strong expression in severe inflammation; (**e**) weak expression in remission. Scale bar: 50 µm.



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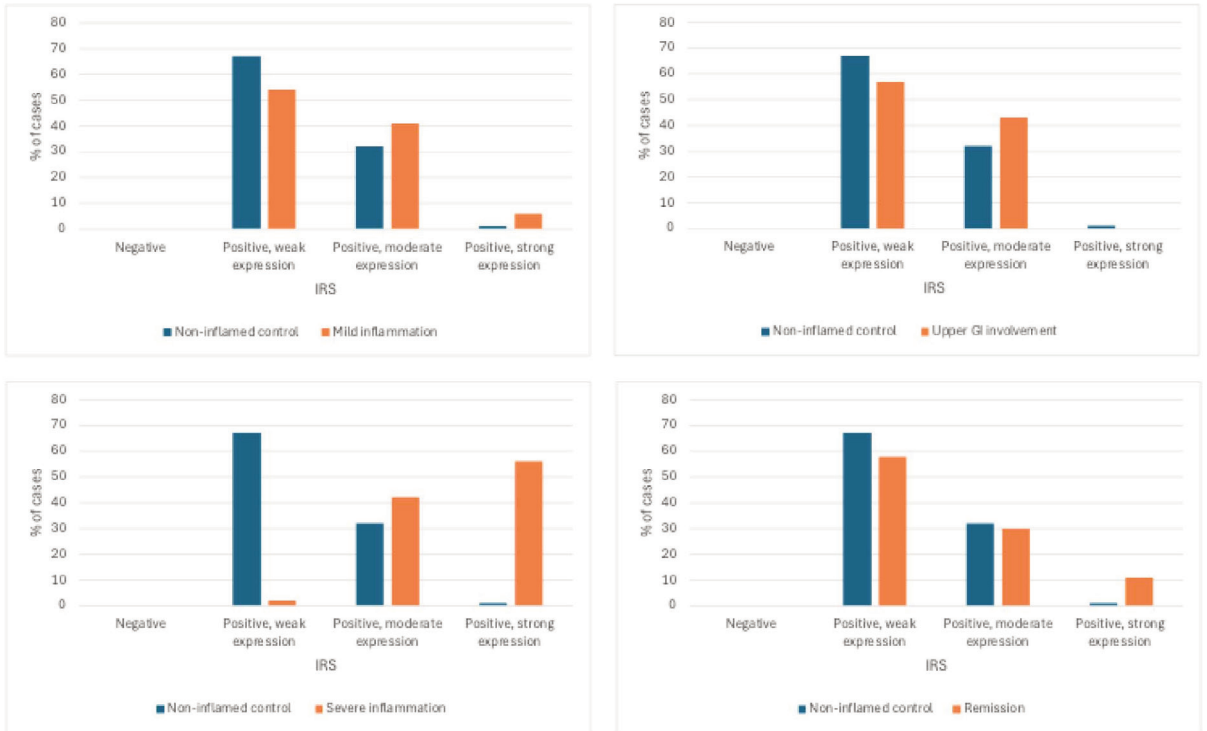

Figure 2. IRS distribution of caspase 3 in Crohn's disease-associated tissue. Comparison of the percentage of cases across different disease subgroups, including mild inflammation, upper gastrointestinal involvement, severe inflammation and remission, relative to non-inflamed control tissue (n=77). Each panel illustrates the distribution of cases according to immunoreactivity score (IRS) categories: Negative, positive weak, positive moderate and positive strong. Caspase-3 expression is shown separately for (**a**) intestinal epithelial cells and (**b**) immune cells of the lamina propria. Data correspond to epithelial cell staining (E) and immune cell staining (I) as shown in Table 1.

adaptive, anti-apoptotic mechanisms within the intestinal epithelium with active suppression of caspase 3 expression.

Caspase 3 expression is increased in immune cells of the lamina propria during severe CD

In non-inflamed intestinal tissue, caspase 3 expression in immune cells was predominantly of weak intensity, with weak IRS scores observed in 67.1% of cases, while moderate expression was present in 31.6% and strong expression in only 1.3% of samples (Table 1). Representative immunohistochemical staining (Figure 1a) showed weak staining in scattered immune cells of the lamina propria. In samples with severe inflammation, caspase 3 immunoreactivity in immune cells was significantly increased ($p<0.001$) compared with non-inflamed control tissue. This increase was reflected by a predominance of moderate (41.8%) and strong (56.4%) staining intensities (Table 1). By contrast, immune cell caspase 3 expression in mild inflammation ($p=0.190$), the upper gastrointestinal tract ($p=0.489$) and remission ($p=0.237$) did not differ significantly from non-inflamed tissue and was primarily characterized by weak to moderate staining (Table 1, Figure 1 b-e).

The pathogenesis of CD is characterized by an exaggerated and persistent activation of immune cells, resulting in a sustained pro-inflammatory response that contributes to ongoing tissue damage. Although the significantly increased expression of caspase 3 observed in severely inflamed tissue suggests enhanced apoptotic potential, accumulating evidence indicates that immune cells in CD exhibit increased resistance to apoptosis.[31-34] Several mechanisms have been proposed to explain this apparent discrepancy. Enhanced S-nitrosylation of caspase 3 has been shown to inhibit its enzymatic activity, leading to the accumulation of the inactive form of the protein.[35] In addition, overexpression of decoy receptors (DcRs) such as DcR3 can block CD95L-mediated apoptotic signaling, thereby suppressing caspase 3-dependent apoptosis in immune cells.[36,37] Furthermore, reduced catalase activity in T cells may decrease susceptibility to apoptosis and indirectly impair caspase 3 function.[38]

Our findings suggest that although caspase 3 is abundantly expressed and biologically available, its functional activity may be impaired, contributing to increased apoptosis resistance of immune cells. This resistance is considered a key mechanism underlying the prolonged survival of pro-inflammatory cell populations and the persistence of chronic inflammation in CD.

Due to the constraints of immunophenotypic IHC, immune cells could not be reliably assigned to specific subpopulations. However, the intestinal mucosa in CD is known to contain a complex mixture of innate and adaptive immune cells with disease-specific alterations in their relative proportions. Active disease is characterized by a predominance of Th1/Th17-driven responses, with increased IL-17-producing T cells and a relative reduction in regulatory T cells, resulting in an increased Th17/Treg ratio. In addition, the mucosa shows infiltration by neutrophils, macrophages, dendritic cells, B cells and plasma cells, contributing to a highly pro-inflammatory microenvironment.[1,5,39] Given this heterogeneous cellular composition, the observed caspase 3 expression likely represents a composite signal derived from multiple immune cell subsets rather than a single defined population.

In summary, our findings demonstrate that caspase 3 expression in CD is dynamically regulated in a disease activity- and cell type-dependent manner. Immune and epithelial cells displayed increased caspase 3 expression during severe active disease. However, the magnitude and distribution differ substantially. Immune cells exhibited a pronounced shift toward strong expression, whereas epithelial cells showed mainly moderate expression. In remission, immune and epithelial cells showed a reduced proportion of strong expression, but the reduction was more pronounced in epithelial cells, likely reflecting adaptive and potentially protective mechanisms aimed at preserving mucosal integrity. Future studies should include the analysis of other caspases and the activated (cleaved) form of caspase 3 and ideally integrate functional apoptosis assays. Such approaches will be helpful to more precisely define the contributing role of caspase 3 in epithelial and immune cell dysregulation and disease progression in CD. Furthermore, there is a need for future studies focusing on the functional activation of apoptotic signaling pathways. A more detailed understanding of these mechanisms may facilitate the identification of additional biomarkers beyond caspase 3 to improve the evaluation of disease progression in patients with CD.

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