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ORIGINAL ARTICLE

# Altered expression of inflammasomes in Hirschsprung's disease

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## Abstract

**Aim of the study** The pathogenesis of Hirschsprung's disease-associated enterocolitis (HAEC) is poorly understood. Inflammasomes are a large family of multiprotein complexes that act to mediate host immune responses to microbial infection and have a regulatory or conditioning influence on the composition of the microbiota. Inflammasomes and the apoptosis-associated speck-like protein (ASC) lead to caspase-1 activation. The activated caspase-1 promotes secretion of pro-inflammatory cytokines (IL-1β and IL-18) from their precursors (pro-IL-1β and pro-IL-18). Inflammasomes have been implicated in a host of inflammatory disorders. Among the inflammasomes, NLRP3, NLRP12 and NLRC4 are the most widely investigated. Knock-out mice models of inflammasomes NLRP3, NLRP12, NLRC4, caspase-1 and ASC are reported to have higher susceptibility to experimental colitis. The purpose of this study was to investigate the expression of NLRP3, NLRP12, NLRC4, caspase-1, ASC, pro-IL-1β and pro-IL-18 in the bowel specimens from patients with HSCR and controls.

**Methods** Pulled-through colonic specimens were collected from HSCR patients ($n=6$) and healthy controls from the proximal colostomy of children with anorectal malformations ($n=6$). The gene expression of NLRP3, NLRP12, NLRC4, caspase-1, ASC, pro-IL-1β and pro-IL-18 was assessed using qPCR. The protein distribution was assessed using immunofluorescence and confocal microscopy.

**Main results** qRT-PCR analysis revealed that NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β gene expressions was significantly downregulated in the aganglionic and ganglionic colon of patients with HSCR compared to controls. Confocal microscopy revealed a markedly decreased expression of NLRP3, NLRP12, NLRC4 and ASC protein in the colonic epithelium of aganglionic and ganglionic bowel of patients with HSCR compared to controls.

**Conclusions** To our knowledge, this is the first study analyzing NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β gene expressions in patients with HSCR. Decreased expression of NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β in the aganglionic and ganglionic bowel may increase susceptibility of HSCR patients to develop HAEC.

**Keywords** Inflammasome · Enterocolitis · Hirschsprung's disease

## Introduction

Hirschsprung's disease (HSCR) is a relatively common cause of functional bowel obstruction in the neonate [1]. Hirschsprung's disease-associated enterocolitis (HAEC) is the most serious cause of morbidity and mortality in HSCR. HAEC occurs in 17–50% of patients with HSCR and may occur before or after a properly performed pull-through operation [2]. The pathogenesis of HAEC is poorly understood. Several theories have been proposed including intestinal epithelial barrier dysfunction, abnormal innate immunity and disturbed microbiota composition.

Inflammasomes are a large family of multiprotein complexes recognizing pathogen-associated molecular pattern

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molecules (PAMPs) and damage-associated molecular patterns (DAMPs) [3]. Inflammasomes and the apoptosis-associated speck-like protein containing CARD (ASC) lead to caspase-1 activation, which promotes secretion of pro-inflammatory cytokines including IL-1β and IL-18, and activates a bactericidal immune response [3]. Activated caspase-1 cleaves IL-1β precursor (pro-IL-1β) and IL-18 precursor (pro-IL-18) to mature IL-1β and IL-18 [3]. Inflammasomes regulate the production and release of the pro-inflammatory cytokines IL-1β and IL-18. It is known that inflammasomes are major regulators of host innate immunity; in the gut, they may have a regulatory or conditioning influence on the composition of the microbiota [4]. Inflammasomes have been involved in the pathogenesis of a wide variety of auto-inflammatory conditions that are caused by the dysregulation of the IL-1 pathway.

Within the nucleotide oligomerization domain (NOD)-like receptor (NLR) family, there are several NLRP (NLR family, pyrin domain-containing) proteins that are involved in the formation of inflammasomes [5]. The NLRP3 inflammasome is the best characterized member of the family and has recently been implicated in gut homeostasis and determining the severity of IBD and inflammation-associated colorectal cancer [5]. The NLRP12 inflammasome is reported to contribute to the maintenance of intestinal homeostasis and modulation of the gut microbiota [5, 6]. NLRC4 (NLR family CARD domain-containing protein 4) is an NLR protein expressed in a variety of cells including phagocytes and intestinal epithelial cells [3, 7]. Similar to other inflammasomes, NLRC4 inflammasome formation leads to the activation of caspase-1, IL-1β, IL-18 and pyroptosis in response to Gram-negative bacteria [3, 7]. There are several reports of investigating the severity of intestinal inflammation in knock-out mice models of inflammasomes (NLRP3, NLRP12, NLRC4), caspase-1 and ASC, respectively. These reports showed higher susceptibility to experimental colitis and worsening intestinal inflammation and alterations in epithelial barrier [3, 5, 7].

We analyzed the expression of inflammasomes (NLRP3, NLRP12, NLRC4), caspase-1, ASC, pro-IL-1β and pro-IL-18 to test the hypothesis that the expression of various inflammasomes and inflammasome complex proteins (ASC and caspase-1) is downregulated in HSCR tissue samples.

## Materials and methods

### Tissue collection

Ethical approval for collection of specimens was in place from both the institutions participating in the study (Our Lady's Children's Hospital Ethics Committee, GEN292.12; Temple Street Children's University Hospital Research and Ethics Committee, 13.003). Full-length resected bowel specimens obtained during pull-through operation for HSCR were collected from six patients. Two of these patients had a history of preoperative HAEC. Resected tissue included aganglionic and ganglionic segments. Ganglionic specimens were taken from the most proximal margin of the resected pull-through specimen while aganglionic samples were taken from the most distal margin of the resected specimen. Healthy control colonic specimens were obtained from the proximal colostomy at the time of stoma closure in patients with imperforate anus. Tissue specimens were stored in three ways following collection. One segment of each specimen was fixed in formalin at room temperature, for paraffin embedding and immunochemistry. A second segment was snap frozen in a mould containing optimal cutting temperature (OCT) medium and stored at − 80 °C for further using immunofluorescence and confocal microscopy. The remaining segment was stored at − 80 °C for RNA extraction and protein extraction.

### RNA isolation

TRIzol reagent (Invitrogen) was used for the acid guanidinium–thiocyanate–phenol–chloroform extraction method to isolate total RNA from HSCR and control tissues ($n = 6$ for each group) according to the manufacturer's protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Scientific Fisher, Wilmington, USA). The RNA solution was stored at − 20 °C until further use.

### cDNA synthesis and quantitative polymerase chain reaction

Reverse transcription of total RNA was carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing) and at 92 °C for 10 min (reverse transcriptase inactivation) using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instruction. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 20 µl per well. The following gene-specific primer pairs were used: Human NLRP3 (Eurofins) sense primer 5′ AGA TGA TGT TGG ACT GGG CG and Human NLRP3 (Eurofins) antisense primer 5′ TCT GTG TCA CAA GGC TCA CC, Human NLRP12 (Eurofins) sense primer 5′ TGC AAA CTC CGA GTC CTC TG and Human NLRP12 (Eurofins) antisense primer 5′ AGA GCC AGC AGA TAG GAC CA, Human NLRC4 (Eurofins) sense primer 5′ GTG TCC AGC GTG AAT GAG GA and Human NLRC4 (Eurofins) antisense primer 5′ TCA CCT CCT CTG GCT CAT GA,

Human Caspase-1 (Eurofins) sense primer 5′ TGC CTG TTC CTG TGA TGT GG and Human Caspase-1 (Eurofins) antisense primer 5′ GCA TCT GCG CTC TAC CAT CT, Human ASC (Eurofins) sense primer 5′ GCC GAG GAG CTC AAG AAC TT and Human ASC (Eurofins) antisense primer 5′ AGC TTG TCG GTG AGG TCC AA, Human pro-IL-18 (Eurofins) sense primer 5′ CAT TGA CCA AGG AAA TCG GCC and Human pro-IL-18 (Eurofins) antisense primer 5′ TAA ATA TGG TCC GGG GTG CA, and Human pro-IL-1β (Eurofins) sense primer 5′ GCT TGG TGA TGT CTG GTC CA and Human pro-IL-1β (Eurofins) antisense primer 5′ TCA ACA CGC AGG ACA GGT AC. For normalization purposes, real-time RT-PCR was performed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH sense primer 5′ACA TCG CTG AGA CAC CAT GG and GAPDH antisense primer 5′ GAC GGT GCC ATG GAA TTT GC were used. After 5 min of initial denaturation at 95 °C, 55 cycles of amplification for each primer was carried out. Each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics) and the relative changes in gene expression level of interest were normalized against the level of GAPDH gene expression in each sample (DDCT method). Experiments were carried out in duplicates for each sample and primer.

### Immunofluorescence

Colonic sections were embedded in OCT compound [VWR, Ireland (361603E)] and snap frozen in liquid nitrogen. Twenty micron sections were cut and were fixed in 10% neutral buffered formalin [Sigma-Aldrich, Ireland (HT501128-4L)]. Cell membranes were permeabilized by rinsing in 1% w/v PBS with 1% Triton X-100. Sections were blocked in 10% BSA diluted in 1% w/v PBS with 0.05% PBST for 90 min at room temperature to prevent non-specific antibody binding. Samples were incubated simultaneously in primary antibodies: rabbit anti-NLRP3 (1:500, 5% BSA) (Abcam, Cambridge, UK, ab4207), rabbit anti-NALP12 (known as NLRP12) (1:500, 5% BSA) (Abcam, Cambridge, UK, ab100863), rabbit anti-CARD12 (known as NLRC4) (1:500, 5% BSA) (Abcam, Cambridge, UK, ab115537), rabbit anti-caspase-1 (1:500, 5% BSA) (Abcam, Cambridge, UK, ab1872), rabbit anti-TMS1 (known as ASC) (1:500, 5% BSA) (Abcam, Cambridge, UK, ab155970) and mouse anti-EpCAM (1:500, 5% BSA) (Santa Cruz, Heidelberg, Germany, sc-25308) at 4 °C overnight. Following incubation in primary antibody solution, samples were rinsed intensively in 0.05% PBST, following which they were incubated in a solution containing corresponding secondary antibodies (antirabbit Alexa Fluor488 (ab150073, GR226381), dilution 1:1000 and antimouse Alexa Fluor 584 (ab150116, GR232081), dilution 1:1000), (Abcam, Cambridge, UK) for 90 min at room temperature. After intensive rinsing in 0.05% PBST, samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain [Thermo Scientific, Ireland (EN62248)]. Sections were mounted with glass coverslips using Mowiol® 488 fluorescence mounting medium [Sigma-Aldrich, Ireland (81381-50G)], which was constituted according to manufacturer's specifications. Specimens were visualized using laser scanning confocal microscopy (LSM700 Confocal Microscope, Carl Zeiss MicroImaging GmbH, Jena, Germany). Resulting images were processed using ImageJ—an open-access software available from http://imagej.nih.gov/ij/.

### Statistical analyses

A one-way ANOVA and the Student's *t* test with Bonferroni correction were conducted to determine a statistically significance difference between aganglionic, ganglionic and healthy controls. A $p$ value $< 0.05$ was considered to be statistically significant.

Data are presented as mean ± standard error. Specimens were classified into three groups: aganglionic ($n = 6$), ganglionic ($n = 6$) and healthy controls ($n = 6$).

## Results

### Relative mRNA expression levels of NLRP3, NLRP12, NLRC4, caspase-1, ASC, pro-IL-18 and pro-IL-1β

The relative mRNA expression levels of NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β were significantly decreased in aganglionic and ganglionic (HSCR) specimens compared to normal controls ($p < 0.05$, Fig. 1).

The relative mRNA expression levels of caspase-1 and pro-IL-18 were similar between the three tissue types ($p > 0.05$).

### Immunofluorescence staining and confocal microscopy

NLRP3, NLRP12, NLRC4, caspase-1 and ASC could be detected in the colonic epithelium of the aganglionic and ganglionic HSCR specimens, and control samples. Compared with the control group, there were significantly decreased expression of NLRP3, NLRP12, NLRC4 and ASC in colonic epithelium of patients with HSCR (Fig. 2).

## Discussion

To the best of our knowledge, this is the first study reporting significantly decreased expression of NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β in the patients with HSCR.

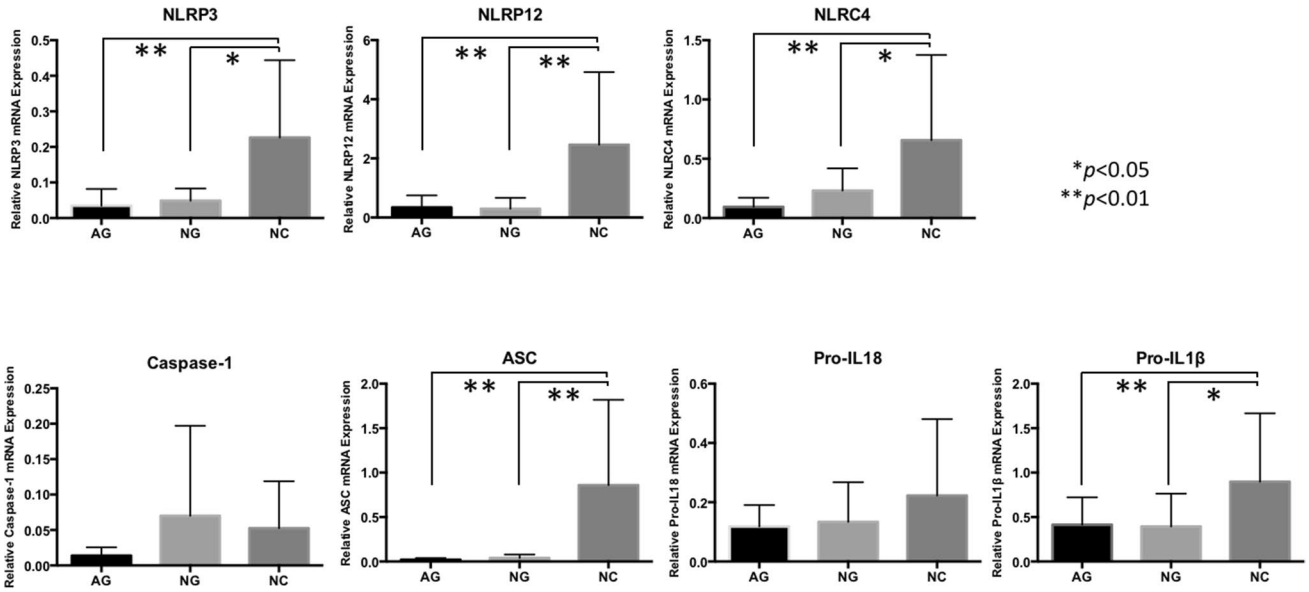


**Fig. 1** qRT-PCR revealed significantly decreased relative mRNA expression level of NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β in the aganglionic and ganglionic HSCR specimens ($n=6$) compared to normal control tissue ($n=6$). Results are presented as mean ± SEM (*$p<0.05$, **$p<0.01$). *AG* agnglionosis, *NG* normoganglionosis, *NC* normal control

Inflammasomes such as NLRP3, NLRP12, and NLRC4 are high-molecular weight (700 kDa) cytosolic multiprotein innate immune modulators found in cells of the innate immune system including macrophages, monocytes, dendritic cells and neutrophils [3, 8]. Inflammasomes detect various endogenous and environmental stimuli, and regulate homeostasis or damage control [3, 8]. In the gastrointestinal tract, inflammasomes have a critical role in maintaining intestinal homeostasis and providing protection against microbial signals and the initiation of inflammatory responses to invading pathogens [8].

The NLRP3 inflammasome which is by far the most studied inflammasome is expressed in both epithelial cells and in the gut immune system [8]. NLRP3 is an essential mediator of host immune responses through the activation of caspase-1, which converts the cytokine precursors pro-IL-1β and pro-IL-18 into mature and biologically active IL-1β and IL-18 [3]. NLRP3 can be activated by an array of stimuli including microorganisms such as viruses, fungi and bacteria [3, 9–12]. Some studies have reported that a deficiency of NLRP3 worsens colitis in a chemical-induced colitis mouse model, manifested by increased mortality, breached epithelial integrity, and enhanced translocation of commensal bacteria from the gut to the systemic circulation, as well as reduced production of cytokines, including IL-1β [8, 9, 13, 14]. In these studies, isolated macrophages from NLRP3 knock-out mice failed to mount an immune response to bacterial muramyl dipeptide, and neutrophils showed impaired chemotaxis and exacerbated apoptosis. Hirota et al. [13] reported that NLRP3-deficient mice showed an altered microbiome configuration compared to their wild-type counterparts. In infectious enteritis and colitis, NLRP3 has a pivotal role in protection from intestinal pathogens [8]. Apart from inducing the secretion of pro-inflammatory cytokines (IL-18 and IL-1β), it controls a preponderance of immune processes necessary to combat infections [15]. In the present study, NLRP3 expression was significantly downregulated in the aganglionic and ganglionic colon compared to controls.

Alongside the role of NLRP3, NLRP12 and NLRC4 have also been reported to be involved in colonic inflammation. The NLRP12 inflammasome has both inflammasome-dependent and inflammasome-independent properties, and is predominantly expressed by myeloid cells, where it plays an important role in gut homeostasis. NLRP12-deficient mice exhibit severe colitis upon DSS administration than mild-type controls [3, 6, 8]. Similarly, NLRC4 which is expressed in monocytes, macrophages and T cells in the small intestine and colon is mainly involved in promoting host defenses [3]. NLRC4-deficient mice develop severe colitis upon chemical challenge and upon infection with pathogenic bacteria [3, 8, 16–19].

HAEC is the most common cause of morbidity and mortality in HSCR [1, 20]. Post-pull-through HAEC has been reported in 5–44% of HSCR patients. Since HAEC can occur following a properly performed pull-through operation, it has been suggested that the presence of ganglia alone does not necessarily mean that the proximal ganglionic bowel in HSCR patients will function normally. In the present study, we have shown altered inflammasome expression in both aganglionic and ganglionic bowel in HSCR patients.

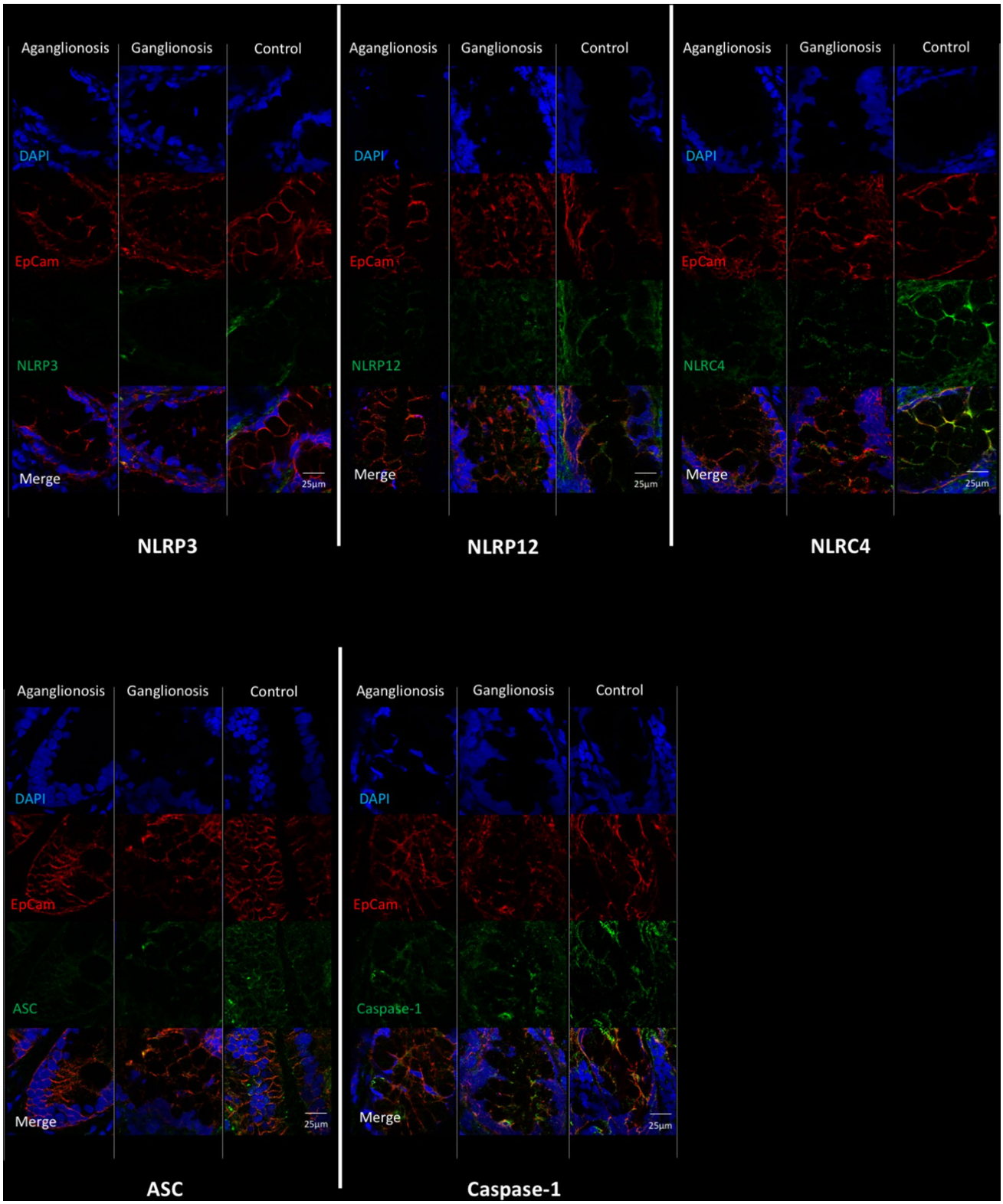


**Fig. 2** NLRP3, NLRP12, NLRC4, ASC and caspase-1 expressions (green) in the colonic epithelium (red) of HSCR specimens compared to normal controls. EpCAM (red) was used to identify colonic epithelium to show coexpression with NLRP3, NLRP12, NLRC4, ASC and caspase-1 (scale bar 25 μm, original magnification × 63). NLRP3, NLRP12, NLRC4, ASC and caspase-1 expressions (green) in the colonic epithelium (red) of HSCR compared to healthy controls. A total of six specimens were analyzed; only the most representative stainings are shown. Compared with the control group, there were significantly decreased expressions of NLRP3, NLRP12, NLRC4, and ASC in colonic epithelium of patients with HSCR

We speculate that decreased NLRP3, NLRP12, NLRC4, ASC and pro-IL-1β expressions observed in ganglionic and aganglionic bowel may result in disruption of the IL-1 pathway which fails to maintain homeostasis in the colon of the patients with HSCR and thus contribute to epithelial damage, including recurrent HAEC.

One major limitation of our study is the small number of patients. Future studies comprising of a large number of HSCR patients with preoperative history of HAEC should be investigated for the inflammasome pathway in the ganglionic and aganglionic bowel, and this should provide further insights into the pathogenesis of HAEC in HSCR patients.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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