RESEARCH ARTICLE

Interleukin-18 and tumor necrosis factor alpha cytokine levels in Hirschsprung-associated enterocolitis

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Hirschsprung-associated enterocolitis (HAEC) is a life-threatening condition that impacts children with Hirschsprung disease (HSCR). This condition can present both in the pre-operative and post-operative periods. Due to the interrelated mechanisms underlying the pathogenesis of HAEC, this study aimed to explore the potential contributions of interleukin-18 (IL-18) and tumor necrosis factor alpha (TNF-α) to its development. Fifteen patients were enrolled in the study and categorized into the non-HAEC group (n=7) and HAEC group (n=8). Blood and gut tissue samples including both ganglionic and aganglionic segments of the colon were collected during surgery, which was part of a transanal perineal pull-through (TPPT) procedure. IL-18 and TNF-α levels were measured using enzyme-linked immunosorbent assay (ELISA). A histological evaluation of inflammation was conducted on gut tissue samples stained with hematoxylin and eosin (HE). Independent t-tests were used to compare IL-18 and TNF-α levels between the two groups. The results showed that IL-18 levels were higher in aganglionic and blood samples from the HAEC group. In contrast, IL-18 levels were higher in the ganglionic segments of the non-HAEC group. TNF-α levels were uniformly higher across all samples from the HAEC group. Statistically significant differences in cytokine levels between the two groups were observed solely for TNF-α in the ganglionic segment (*P* = 0.0028). This study revealed a significant difference in TNF-α levels in the ganglionic segments between patients with and without HAEC, indicating the possible involvement of TNF-α in the pathogenesis of HAEC.

Keywords: enterocolitis; Hirschsprung; interleukin-18; tumor necrosis factor alpha.

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Introduction

Hirschsprung-associated enterocolitis (HAEC) is the predominant life-threatening condition among children burdened with Hirschsprung disease (HSCR). Its occurrence can span infancy to adulthood, and it is not influenced by the specific definitive surgical procedure for HSCR [1]. HAEC incidence varies widely with rates ranging from 6% - 60% before and 25% - 42% after pull-through surgery [2]. HAEC's clinical presentation comprises fever, diarrhea,

abdominal distention, leukocytosis, and edema of the small intestine and colon based on abdominal films [3]. The pathogenesis of HAEC remains unclear. Moreover, research advances have shown that HAEC pathogenesis involves genes, the enteric nervous system (ENS), the gut microbiome, the gut mucosal barrier, and the immune system [1, 2, 4]. Stasis resulting from impaired motility and ENS dysfunction in HSCR leads to gut microbiota dysbiosis. Additionally, compromised intestinal barrier function and abnormal mucosal immune responses contribute to the development of HAEC [1]. Subsequently, all these factors can trigger the inflammatory response and finally lead to the development of HAEC [2]. From a histological perspective, HAEC is characterized by crypt abscesses, severe inflammation, and neutrophil infiltration within the crypts [4, 5]. Notably, these histological findings are not confined to the aganglionic segment but extend to the ganglionic segment, suggesting a more complex mechanism beyond the absence of ganglion cells.

The release of cytokines is a hallmark of inflammation, yet the cytokine profile of HAEC has not been comprehensively assessed in humans. Several studies of enterocolitis in other contexts, such as inflammatory bowel disease (IBD) and necrotizing enterocolitis (NEC), have investigated the cytokine profile including interleukin-18 (IL-18) and tumor necrosis alpha (TNF-α) in humans and mouse models. IL-18, a constitutively expressed cytokine in intestinal epithelial cells (IECs), plays a critical role in maintaining epithelial integrity. Excessive IL-18 expression leads to a loss of mature goblet cells and increased susceptibility to inflammation. Conversely, IL-18 deficiency in IECs confers protection against dextran sulfate sodium (DSS)-induced colitis. Thus, IL-18 serves as a prime example of cytokine-mediated crosstalk between the intestinal epithelium and the immune system [6]. IL-18 plays a dual role in the early and late stages of colitis [7]. Increased IL-18 levels enhance inflammation and worsen colitis, whereas decreased IL-18 reduces the resulting damage [8]. Increased IL-18 secretion has been correlated with increased human IBD severity [9]. Conversely, IL-18 deficiency in a mouse model of NEC was associated with reduced disease severity [10]. TNF-α, a cytokine belonging to the TNF superfamily, has multiple functions and is involved in a wide range of biological processes, such as preserving intestinal integrity and homeostasis, modifying signaling pathways, controlling immune responses, and coordinating innate immunity-related inflammatory reactions, cell activation, proliferation, and necrosis. TNF-α, however, is also essential for the development of infections, wounds, and inflammatory illnesses such as IBD and tumors [11-13]. TNF-α allele polymorphisms are reported to be more frequent in IBD patients compared to healthy controls [14]. TNF-α is a key factor in intestinal permeability and is implicated in the control of intestinal tight junction integrity [15, 16].

The disturbance of intestinal integrity is consistent with one of the hypotheses regarding HAEC pathogenesis and lends credence to the theory that TNF-α and IL-18 may play a role therein. This work sought to assess the possible roles of TNF-α and IL-18 in the establishment of the inflammatory response in HAEC in light of the most recent update on HAEC pathogenesis, which initiates this response. The preclinical detection of HAEC is anticipated to be made possible by measuring cytokine profiles, allowing for the reduction of related morbidity and death. The enzyme-linked immunosorbent assay (ELISA) was used to measure cytokine levels in this study.

Materials and methods

Subjects and samples

A cross-sectional study was performed on all HSCR patients aged 0 - 12 months who had not undergone diversion or stoma procedures, conducted between January and December 2023 at Dr. Saiful Anwar General Hospital in Malang, East Java, Indonesia. A total of 15 patients participated in the study with 7 consented to both gut and blood sample collection, 4 consented only to gut sample collection, and 4

consented only to blood sample collection. All procedures in this study were approved by the Research Ethics Commission of Dr. Saiful Anwar General Hospital (Malang, East Java, Indonesia) (Approval number: 400/141/K.3/102.7/2023). All patients received suction rectal biopsies, which were performed by a single operator and interpreted by a single pathologist. The patients were subsequently divided into HSCR without enterocolitis (non-HAEC) (n = 7) and HSCR with enterocolitis (HAEC) (n = 8) groups. HSCR was diagnosed based on the histological examination of suction rectal biopsy specimens. HAEC was established if the HSCR patient had a HAEC score ≥ 4 according to the consensus-based HAEC scoring system for diagnosis established by Pastor *et al*. and Gunadi *et al*., which included patient history, physical examination, radiology findings, and laboratory findings [17, 18]. Gut samples, encompassing both ganglionic and aganglionic segments of the colon, were collected at the same time as surgery performance. Macroscopically, there was a collapse of the aganglionic segment, dilation of the ganglionic segment, and the transitional zone in between. The transanal perineal pull-through (TPPT) procedure was performed for each patient according to the method of Dasgupta *et al*. [19]. Briefly, after exposing the anal canal, an incision was made 0.5 cm above the dentate line circumferentially and continued across the full thickness of rectal wall dissection until the transition zone was reached. Frozen sections were prepared to ensure the presence of ganglion cells in this segment. Once the ganglion cells were identified by the histological examination, at least 2 cm above the biopsy sites of the colon was resected to make sure that the transition zone was removed along with the aganglionic segment followed by a coloanal anastomosis (Figure 1). The gut samples were promptly preserved at -40°C and fixed in 10% neutral buffered formalin (NBF) prior to paraffin block preparation. Blood samples were obtained either during the TPPT surgery or the patient's hospitalization, contingent upon patient consent for gut sampling. A total of 3 mL blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes, following which plasma was extracted and stored at −80°C.

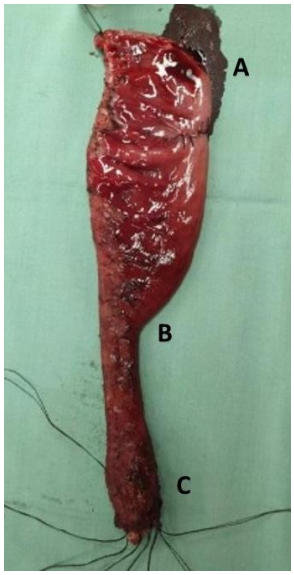

Figure 1. Gut tissue sample. **A.** Ganglion segment. **B.** Transitional zone segment. **C.** Aganglion segment.

Hematoxylin and Eosin staining

Hematoxylin and eosin (HE) staining was performed to assess the grade of inflammation in the gut based on the histological grading criteria for HAEC. The gut samples underwent macroscopic dissection, and optimal fixation was achieved by immersion sample in 10% NBF for a minimum duration of 4 - 6 hours. After fixation, the gut samples were processed in an automated tissue processor and embedded in paraffin blocks. The paraffin blocks were sectioned to a suitable thickness using a rotary microtome. The sections were floated in a water bath and then deparaffinized for 2 hours. Routine HE staining was performed to visualize the tissue morphology using Mayer's Hematoxylin Solution and Eosin Solution (Scytek Laboratories Inc., Logan, Utah, USA). The sections were initially stained with hematoxylin for 10 min, then were rinsed under running tap water, differentiated in acid alcohol, and neutralized with lithium carbonate before staining with eosin for 10 min. Subsequently, the sections were dehydrated through a graded series of alcohols, cleared in

xylene, and mounted with a permanent mounting medium.

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-18 and TNF-α in the ganglion, aganglion, and blood plasma samples were quantified using ELISA (Elabscience, Houston, Texas, USA) following the manufacturer's instructions. ELISA was performed concurrently for all collected samples with each sample assessed in duplicate. The color absorbance was measured at a 450 nm wavelength using an LMPR-A12 microplate reader (Labtron, Frimley, Surrey, England). The results were reported as optical density values, which were subsequently used to determine cytokine levels. The calculation of cytokine concentrations involved establishing a standard curve following the ELISA kit manufacturer's instructions with levels determined using the standard curve formula and adjusted for the dilution factor.

Statistical analysis

GraphPad Prism version 10.2.1 (GraphPad Software LLC, Boston, Massachusetts, USA) was utilized for all statistical analysis. Independent t-tests were employed to compare cytokine levels between the non-HAEC and HAEC groups. Additionally, one-way analysis of variance ANOVA was conducted to assess differences in cytokine levels among ganglion, aganglion, and blood samples. Data were presented as mean ± standard deviation. A *P* value of less than 0.05 was considered statistically significant.

Results and discussion

Patient characteristics

Among 15 patients, the average age of the patients was 13.34 ± 3.05 days old. 11 consented to the surgery with the average age of 3.82 ± 0.60 months. 93.3% patients were male. The patients exhibited the three classic symptoms of HSCR including delayed meconium passage (86.7%), vomiting (66.7%), and abdominal distention (60%).

Histological findings

Histological grading through HE staining demonstrated varying levels of inflammation, classified from grades I to IV. Grade I inflammation was marked by crypt dilatation and mucus retention, while grade II was identified by the presence of crypt abscesses and cryptitis. Grade III involved multiple crypt abscesses, and grade IV was characterized by fibrinopurulent debris accompanied by mucosal ulceration (Figure 2). These inflammatory characteristics were predominantly noted in the aganglionic segments of both the HAEC and non-HAEC groups.

Cytokine levels

ELISA was performed on blood plasma and gut tissue samples including both ganglionic and aganglionic segments with the results presented in Table 1. The comparison of IL-18 and TNF-α levels between the HAEC and non-HAEC groups was shown in Figure 3. IL-18 levels demonstrated divergent patterns between ganglionic and aganglionic segments across the two groups. In the non-HAEC group, IL-18 levels were higher in the ganglion compared to the aganglion, whereas the HAEC group showed higher IL-18 levels in the aganglionic segment. Despite these observations, no significant differences in IL-18 levels were found between the groups for either gut segment. Both groups exhibited a broad range of IL-18 levels in both segments. Notably, while there was no overall significant difference in blood plasma IL-18 levels between the groups, the HAEC group had higher IL-18 levels than that of the non-HAEC group, consistent with the pattern observed in the aganglionic segment. No significant differences in IL-18 levels were observed between the non-HAEC and HAEC groups in either ganglionic or aganglionic segments. IL-18 has a dual role in inflammatory processes, which initially acts as an anti-inflammatory agent by promoting the function and proliferation of goblet cells early in the disease but can later become proinflammatory by suppressing goblet cell function and numbers [7]. This study found that IL-18 levels in the ganglionic segment were higher in the non-HAEC

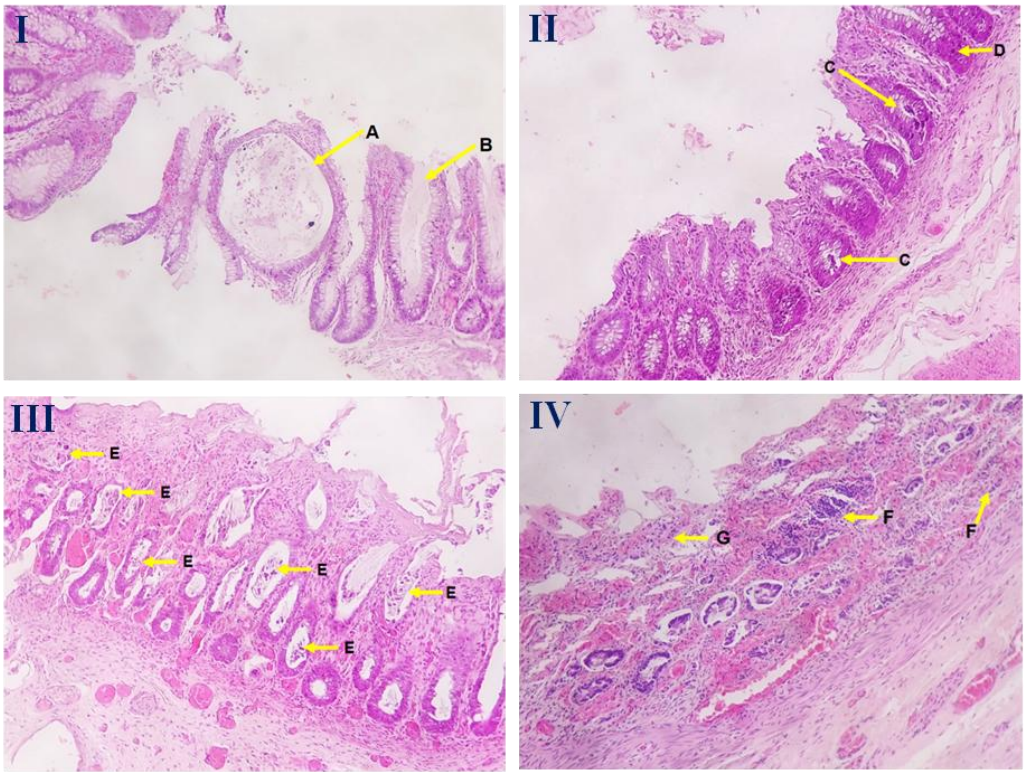

Figure 2. Histological grading of inflammation. Inflammation grade I: crypt dilatation (A), mucus retention (B). Inflammation grade II: crypt abscess (C), cryptitis (D). Inflammation grade III: multiple crypt abscess (E). Inflammation grade IV: fibrinopurulent debris (F), mucosal ulceration (G).

Table 1. Comparison of cytokines level between the groups.

	Mean ± SD (pg/mL)		*P* value
	non-HAEC	HAEC	
IL-18 ganglion	1,914.06 ± 528.54	1,141.63 ± 83.03	0.2223
IL-18 aganglion	1,436.03 ± 521.37	1,503.81 ± 266.28	0.9159
IL-18 blood plasma	1,685.04 ± 386.96	2,873.94 ± 449.34	0.1099
TNFα ganglion	209.75 ± 32.45	480.27 ± 61.99	0.0028
TNFα aganglion	223.27 ± 53.40	272.23 ± 44.83	0.5109
TNFα blood plasma	12.02 ± 4.82	74.40 ± 58.94	0.4549

group than in the HAEC group. This finding supported the notion that elevated IL-18 levels in the non-HAEC group might have a protective role against complications such as enterocolitis, aligning with the cytokine's anti-inflammatory effects in the early stages. In blood plasma, IL-18 levels were higher in the HAEC group compared to the non-HAEC group. Elevated IL-18 expression can result in the loss of mature goblet cells and heightened susceptibility to inflammation [6]. Although no studies have specifically addressed IL-18 in HAEC or non-HAEC thus far, research has shown a significant reduction in goblet cell populations in the aganglionic and ganglionic colon of HAEC patients compared to controls. Alterations in goblet cell number and function can disrupt the intestinal barrier and contribute to HAEC development [20]. This observation is consistent with the role of IL-18 in promoting inflammation during later stages. The elevated IL-18 levels in HAEC patients may thus be released into the

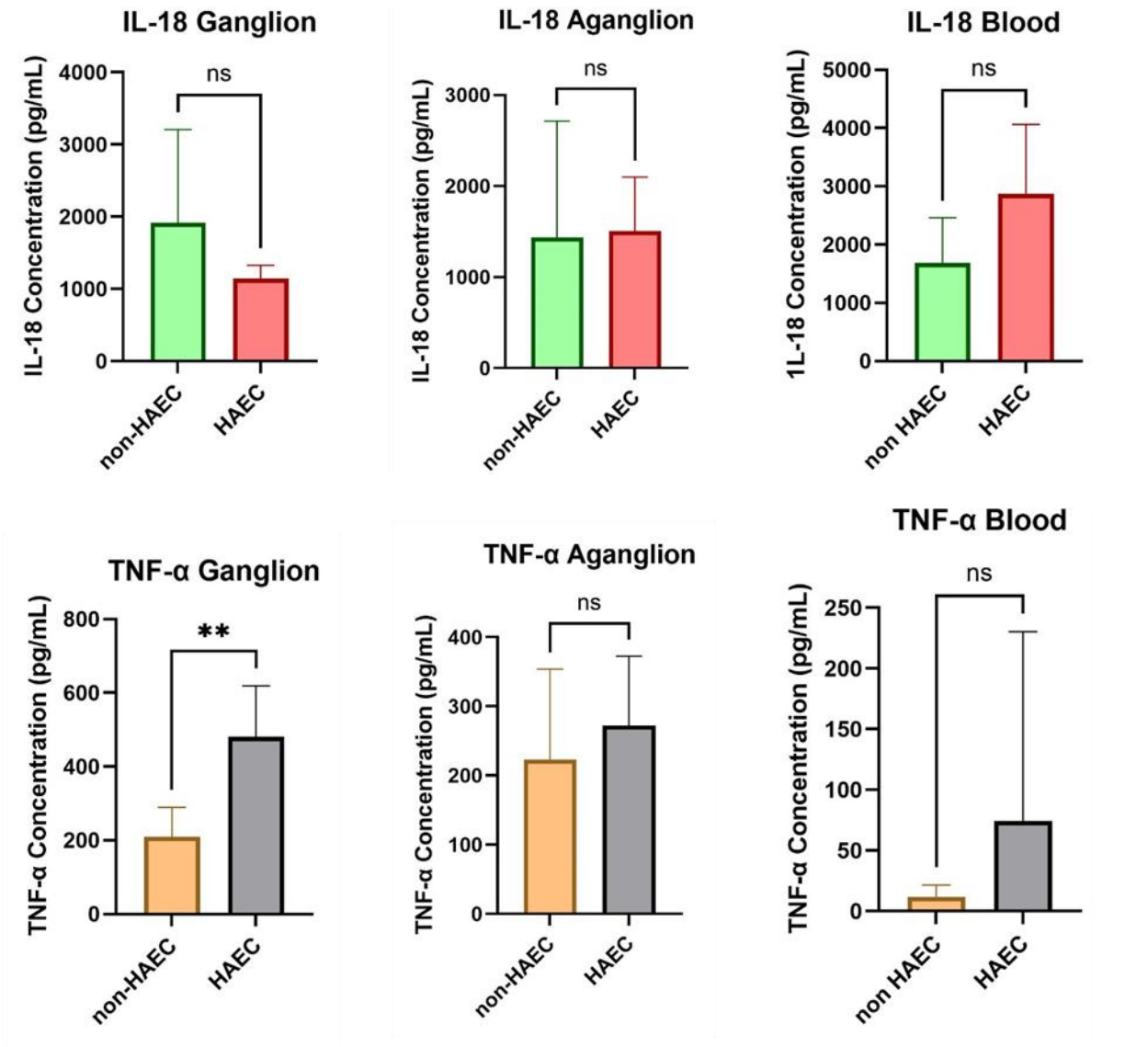

Figure 3. Blood IL-18 and TNFα levels. ns: no significant difference. **: significant difference.

bloodstream, resulting in increased blood IL-18 concentrations.

TNF-α levels were uniformly higher in the ganglion, aganglion, and blood plasma of the HAEC group compared to the non-HAEC group. Nevertheless, statistically significant differences between the two groups were observed exclusively in the ganglionic segment. The HAEC group demonstrated a wider range of TNF-α levels compared to the non-HAEC group. TNF-α is a protein produced by various cell types within the body, predominantly by monocyte-derived cells such as macrophages. As a proinflammatory cytokine, TNF-α plays a critical role in maintaining gut homeostasis and integrity, modulating signaling pathways and immune responses, and regulating inflammatory reactions. However, TNF-α is also implicated in the pathogenesis of various diseases including infections, injuries, and inflammation. Conditions associated with elevated TNF-α levels include IBD, infectious diseases, and tumors [11-13]. In this study, elevated TNF-α levels were observed in the HAEC group compared to the non-HAEC group across ganglion, aganglion, and blood plasma samples.

However, significant differences were noted only in the ganglionic segment. Chronic inflammation is associated with an increase in TNF-α-producing cells, which results in higher TNF-α levels. Elevated TNF-α levels can induce epithelial cell death in the intestine, potentially leading to barrier disruption and the invasion of harmful pathogens, thereby contributing to intestinal inflammation [11]. Furthermore, research indicates that the allelic polymorphism of TNF-α is significantly higher in patients with IBD compared to healthy individuals [14]. TNF-α released by polarized macrophages has been identified as a key factor disrupting the function of interstitial cells of Cajal, which impairs intestinal motility, exacerbates intestinal dysbiosis, and contributes to the development of HAEC [2].

This study evaluated cytokine levels in both gut and blood plasma samples. This assessment was exclusively conducted on paired samples, which means that the data were obtained from patients who provided both gut and blood samples. According to the results of a one-way ANOVA, significant differences in IL-18 (P = 0.014) and TNF-α (P = 0.047) levels among ganglion, aganglion, and blood plasma samples were observed only in the HAEC group.

Conclusion

This study found that IL-18 levels were elevated in aganglionic and blood samples from the HAEC group. Additionally, TNF-α levels were higher in all samples from the HAEC group compared to the non-HAEC group. While significant differences in TNF-α levels were observed only in the ganglionic segment between the HAEC and non-HAEC groups, the findings of this study might enhance the understanding of HAEC pathogenesis. However, this study had several limitations, which included the absence of a control group of healthy children due to ethical considerations prohibiting surgical procedures for gut sample collection, and the limitation of sample size due to personal reasons of the participants' families that affected their ability to contribute to the research.

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