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A retrospective study of the expression of TNF-alpha and IL-1bet in canine granulomatous colitis

Egy retrospektív tanulmány a TNF-alfa és az IL-1béta expressziójáról kutyák granulomatózus kolitiszében

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2024

Abstract

The goal of this retrospective study is to determine the expression of the cytokines TNF-alpha and IL-1beta in the intestinal mucosa, specifically in the colonic mucosa, of canine patients diagnosed with granulomatous colitis.

For the analysis, we utilized previously collected endoscopic biopsy samples from the colon, preserved as FFPE blocks, and employed the RNAscope method to quantify cytokine expression. Statistical comparisons were performed between dogs with GC and healthy controls, focusing on the cluster per area and spot per area expression of the cytokines in the colonic mucosa.

Our results show increased expression of TNF-alpha and IL-1beta in the colonic mucosa of dogs with GC compared to controls. IL-1beta showed a pronounced and consistent upregulation, indicating its central role in the inflammatory processes of GC. TNF-alpha expression was more variable but also elevated, suggesting its involvement in amplifying the inflammatory response. These findings highlight the importance of TNF-alpha and IL-1beta in the pathogenesis of GC and provide a foundation for further research into their potential as biomarkers.

Absztrakt

Ennek a retrospektív tanulmánynak a célja a TNF-alfa és az IL-1béta citokinek expressziójának meghatározása a bél nyálkahártyájában, különösen a vastagbél nyálkahártyájában, granulomatózus kolitiszben szenvedő kutyapácienseknél.

Az elemzéshez korábban begyűjtött, a vastagbélből származó endoszkópos biopsziás mintákat használtunk fel, amelyeket FFPE blokkokban konzerváltunk, és a citokinek expressziójának kvantifikálásához az RNAscope módszert alkalmaztuk. Statisztikai összehasonlításokat végeztünk a GC-s kutyák és az egészséges kontrollok között, különös tekintettel a nyálkahártyában mért klaszter/terület és pont/terület alapú citokinexpresszióra.

Eredményeink szerint a GC-s kutyák vastagbél nyálkahártyájában emelkedett volt a TNF-alfa és az IL-1béta expressziója a kontrollcsoporthoz képest. Az IL-1béta kifejezetten és következetesen fokozott expressziót mutatott, ami a GC gyulladásos folyamataiban betöltött központi szerepére utal. A TNF-alfa expressziója változóbb volt, de szintén emelkedett, ami azt jelzi, hogy szerepet játszik a gyulladásos válasz felerősítésében. Ezek az eredmények rávilágítanak a TNF-alfa és az IL-1béta fontosságára a GC patogenezisében, és alapot nyújtanak további kutatásokhoz, amelyek ezek biomarkerként való potenciális alkalmazhatóságát vizsgálják.

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2 List of abbreviations

BVL..... Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (eng.:Federal Office of Consumer Protection and Food Safety)
CD.....Crohn's disease
CFA..... Canis Familiaris
CIE.....chronic inflammatory enteropathy
DD.....death domain
E. coli ..... *Escherichia coli*
FFPE .....formalin-fixed, paraffin-embedded
FISH..... fluorescence in situ hybridization
FQs..... fluoroquinolones
GALT..... gut-associated lymphoid tissue
GC..... granulomatous colitis
H&E .....hematoxylin and eosin
HUC .....histiocytic ulcerative colitis
IBD..... inflammatory bowel disease
IELs..... intraepithelial lymphocytes
IFN-gamma .....interferon-gamma
IgA ..... immunoglobulin A
IL-1beta interleukin-1beta
IL-1Ra ..... interleukin-1 receptor antagonist
IL-2 .....interleukin-2
IQR..... interquartile range
ISH ..... in situ hybridization
LP.....lamina propria
MAPKs.....mitogen-activated protein kinases
mRNA ..... messenger-ribonucleic acid
NF-κB.....nuclear factor κB
PAMPs .....pathogen-associated molecular patterns
PAS .....periodic acid-Schiff stain
rDNA.....ribosomal DNA
RT-PCR.....reverse transcription polymerase chain reaction

SLAM.....Signaling Lymphocytic Activation Molecule
sTNF-alpha.....soluble form of TNF-alpha
TACE.....TNF-converting enzyme
TGF-beta.....transforming growth factor-beta
tmTNF-alpha.....transmembrane form of TNF-alpha
TNF-alpha.....tumor necrosis factor-alpha
TNFR.....tumor necrosis factor-receptor
TRADD.....TNFR1-associated death domain
UC.....ulcerative colitis
VEGF.....vascular endothelial growth factor

3 Introduction

Granulomatous colitis (GC) is a rare and chronic large intestinal disease in dogs that occurs primarily in Boxers and certain other predisposed breeds [1, 2]. GC was first recognized as an independent disease in the United States in 1965 [1]. In recent decades, research has linked the disease to invasive *Escherichia coli* infections of the intestinal mucosa [3], changing our understanding of the pathogenesis and expanding its importance in veterinary gastroenterology.

The clinical presentation of GC in dogs typically includes chronic diarrhea, weight loss, abdominal discomfort and other gastrointestinal signs that often respond poorly to therapy and can result in euthanasia of affected dogs [1, 4, 5]. The disease primarily affects the colon, where *E. coli* clusters are often found in macrophages, contributing to the inflammatory response [1, 6, 7]. During such an inflammatory response, macrophages and T cells infiltrate the lamina propria, producing large amounts of pro-inflammatory cytokines such as interleukin 1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) [8, 9]. Due to the persistence of symptoms and the risk of poor clinical outcome, GC represents a major health challenge for affected animals and a complex diagnostic and therapeutic problem for veterinarians.

In this retrospective study, we are analyzing formalin-fixed, paraffin-embedded (FFPE) -samples sampled endoscopically from the GI tract of five canine patients diagnosed with GC, and four negative control dogs. The analysis was performed by using the RNAscope method. The goal of this study is to determine the correlation between the disease group and the expression of TNF-alpha and IL-1beta in the intestinal mucosa of canine patients with GC.

4 Literature review and research

4.1 Introduction to granulomatous colitis in dogs: definition, classification and epidemiology

Granulomatous colitis (GC, formerly histiocytic ulcerative colitis (HUC) is an inflammatory disease of the bowel. This severe disease, the etiology of which is not yet fully understood, was first described clinically and pathologically in Boxers under four years of age in 1965 by Van Kruiningen et al. [1]. In recent years, GC has also been diagnosed in mixed breeds and related breeds such as French Bulldogs [10–12] and other breeds such as American Staffordshire Terriers [13], Mastiffs, Alaskan Malamutes, Doberman Pinschers [14] and Beagles [15].

Affected animals showed symptoms such as frequent bloody-mucous diarrhea, anemia, hypoalbuminemia, lethargy and weight loss [1, 6]. Colonoscopy reveals redness, thickening and ulceration of the colonic mucosa [1, 6, 7]. The predominant histologic features include loss of colonic epithelium and goblet cells and the accumulation of significant numbers of periodic acid-Schiff stain (PAS)-positive macrophages in the lamina propria (LP) of the colon and submucosa [3, 16].

To date, no precise data on prevalence are available, and many authors describe the disease only as “rare” [7, 14].

4.2 Pathophysiology of granulomatous colitis in dogs

4.2.1 Role of the intestinal immune system

The immune system of the intestinal tract is highly complex and the knowledge about it continuously evolving. It is primarily responsible for maintaining a balance between immune tolerance and active responses to pathogens. This balance involves tolerance to dietary antigens, protection against local pathogens, and the dissemination of activated lymphocytes both systemically and within the mucosa. The gut-associated lymphoid tissue (GALT) plays a central role in this process, with key components including mucosal lymphoid follicles, Peyer’s patches, and mesenteric lymph nodes [17].

The intestines possess both mechanical and immunological defenses to protect against ingested pathogens and manage the resident microbiota. Mechanical defenses include the mucosal epithelial barrier, intestinal motility, and mucus production, which, together with the resident bacterial flora, prevent pathogen adherence and penetration. Immunologically, the GALT contains a variety of immune cells, including B- and T-

lymphocytes, macrophages, and dendritic cells, which contribute to the immune response by processing antigens and producing immunoglobulins like immunoglobulin A (IgA) [17].

Intraepithelial lymphocytes (IELs) primarily consist of $CD8^+$ suppressor T-cells, which play a crucial role in maintaining immune tolerance and cytotoxic responses. In the lamina propria, IgA-producing B-cells dominate, supported by $CD4^+$ T-helper cells that aid in immunoregulation and inflammation control. IgA, bound to epithelial cells, is secreted into the gut lumen to neutralize antigens and prevent their attachment to the mucosa [17].

Upon antigen encounter, the immune system undergoes a coordinated response involving both cell-mediated and humoral immunity. T-helper-1 cells produce cytokines such as interleukin-2 (IL-2), interferon-gamma (IFN-gamma), and TNF-alpha, which enhance cell-mediated immune responses, whereas T-helper-2 cells secrete IL-4, IL-5, and IL-10, promoting antibody production [17, 18]. These processes are crucial in the body's defense mechanisms, with IgG, IgM, and IgE providing additional layers of humoral immunity through opsonization and inflammatory responses [17].

After an infection is cleared, the immune system transitions from an inflammatory state to tissue repair. Initially, macrophages adopt a proinflammatory role, activated by TNF-alpha to eliminate pathogens. As the infection resolves, these macrophages switch to an anti-inflammatory M2 phenotype, promoting healing and reducing inflammation. This shift is crucial for preventing chronic inflammation and tissue damage. The resolution of inflammation is also aided by specialized lipid mediators like resolvin E1 and protectin D1, which regulate immune cell activity and promote the clearance of apoptotic cells. Additionally, macrophages play a central role in repairing tissue by secreting vascular endothelial growth factor (VEGF) to stimulate blood vessel growth and transforming growth factor-beta (TGF-beta) to encourage tissue regeneration. If inflammation is not properly resolved, chronic conditions such as granulomas may form, characterized by the accumulation of immune cells and fibrous tissue around persistent irritants. These granulomas can cause significant tissue damage and contribute to the pathology of chronic inflammatory diseases [19].

Tolerance is a critical function of the intestinal immune system to prevent chronic inflammation from benign antigens. This is achieved through mechanisms such as T-cell deletion, anergy, and cytokine-mediated suppression, with IL-10 and TGF-beta playing significant roles in promoting tolerance. Failure of these tolerance mechanisms may

contribute to the pathogenesis of conditions like inflammatory bowel disease (IBD), where an inappropriate immune response leads to tissue damage and chronic inflammation [17].

4.2.2 Etiological factors of GC in dogs

The cause of GC is still uncertain. The pathogenesis of the disease is not yet fully understood.

Even in the first descriptions, bacterial infections were suspected [1, 20, 21]. In recent years, *Escherichia coli* was detected for the first time in macrophages of the lamina propria (LP) using fluorescence in situ hybridization (FISH) [3, 4, 10]. This so-called PAS-positive *E. coli*-associated GC with genetic predisposition in Boxers and French Bulldogs is linked to a region on Canis Familiaris (CFA) chromosome 38 that encodes genes of the Cluster of Differentiation 48/Signaling Lymphocytic Activation Molecule (SLAM) family [22, 23] and is involved in the recognition or killing of intracellular *E. coli* by macrophages [24]. This region also plays a role in IBD in humans [22].

4.2.3 Immunological mechanisms in granulomatous colitis of dogs

Some researchers proposed, that the bacteria found in the inflamed and ulcerated mucosa were just occasional inhabitants, suggesting a primary immune-mediated pathogenesis [25]. Immunohistochemistry analysis of the mucosa in GC revealed an increased number of specific immune cells, similar to those seen in human ulcerative colitis (UC) [25]. The standard treatment for GC until 2004 involved immunosuppression using corticosteroids and azathioprine, along with antibiotics and dietary modifications [26]. However, the response to treatment was generally poor, often leading to euthanasia and classifying GC as an incurable, immune-mediated disease [25–27].

The observation of elevated levels of immunoglobulin-producing plasma cells and cytokine-producing T cell subsets in dogs with IBD supports the hypothesis of impaired immune regulation [28]. It is hypothesized that the inflammation is due to inadequate cytokine production by diverse T cell subpopulations. The activation of the nuclear factor κB (NF-κB) indicates an important role of cytokines in the pathogenesis of the disease and has been demonstrated in various studies [29, 30].

A novel theory suggests that macrophages and T-helper lymphocytes (T cells) infiltrate the lamina propria and produce significant quantities of IL-1beta in response to an excessive immune reaction to commensal bacteria [8, 9].

4.3 Basics of IL-1beta and TNF-alpha in inflammatory processes

Cytokines, essential polypeptide molecules in cell communication, originate from diverse cell types and wield multifaceted effects on cellular behavior [31]. Operating mainly in autocrine and paracrine modes, they orchestrate rapid and dynamic responses, although excessive levels can trigger hazardous cytokine storms [32, 33]. Of particular significance in inflammatory and autoimmune processes, cytokines are targeted by therapeutic interventions [32].

Upon encountering pathogens or their pathogen-associated molecular patterns (PAMPs), macrophages release cytokines, including TNF-alpha, IL-1, and IL-6. TNF-alpha is produced early in the inflammatory response, increasing vascular permeability and facilitating the entry of immune components into tissues. This is followed by the release of IL-1, which activates the vascular endothelium and promotes tissue destruction [32, 34]. This complex interaction highlights the crucial role of cytokines in controlling immune responses and emphasizes their potential as targets for treating various diseases.

4.3.1 Biological functions of IL-1beta

Interleukin-1beta is produced by various cells, such as macrophages. IL-1beta plays a vital role in the initiation and amplification of the inflammatory response, similar to TNF-alpha [35]. It promotes adhesion of neutrophils to vascular endothelial cells, facilitating their migration to sites of infection. During severe infections, IL-1beta can enter the bloodstream, where it collaborates with TNF-alpha to induce systemic effects, including fever, lethargy, and loss of appetite, collectively referred to as sickness behavior. Furthermore, IL-1beta influences muscle cells by mobilizing amino acids, leading to pain and fatigue, and stimulates liver cells to produce acute-phase proteins that bolster the body's defenses [34, 36].

The activity of IL-1beta is regulated by its receptors, CD121a and CD121b. CD121a, also known as interleukin-1 receptor 1, is responsible for signaling and mediating the proinflammatory effects of IL-1beta, while CD121b, also referred to as interleukin-1 receptor 2, acts as a decoy receptor, inhibiting these effects [34]. The IL-1 receptor antagonist (IL-1Ra), an endogenous inhibitor of IL-1beta, competes for binding to IL-1 receptor 1, thereby blocking IL-1-mediated signaling and acting as a crucial regulator of inflammation [36].

4.3.2 Biological functions of TNF-alpha

Tumor necrosis factor-alpha is a multifunctional cytokine that influences various cell types and is recognized as a key player in the regulation of inflammatory responses. It is involved in the pathophysiology of several inflammatory and autoimmune disorders [37]. This cytokine is primarily synthesized by activated macrophages, T-lymphocytes and natural killer cells. It is a homotrimeric protein consisting of 157 amino acids [37]. Functionally, TNF-alpha stimulates the production of numerous inflammatory mediators, including other cytokines and chemokines. TNF-alpha exists in both soluble and transmembrane forms [38, 39]. The transmembrane form (tmTNF-alpha) is initially produced and converted into the active soluble form (sTNF-alpha) by the TNF-converting enzyme (TACE), a membrane-associated metalloproteinase [39]. The resulting sTNF-alpha binds to tumor-necrosis-factor-receptor-1 (TNFR1) and tumor-necrosis-factor-receptor-2 (TNFR2) [38], facilitating various cellular activities, including the release of chemokines and cytokines from nearby cells, and promoting the adherence, migration, attraction, and activation of leukocytes [34]. Although both TNFR1 and TNFR2 can be activated by tmTNF-alpha, the latter is primarily associated with signaling through TNFR2 [38].

In dogs, TNFR1, which is a principal signaling receptor for TNF-alpha, is expressed in all tissues and is a crucial mediator within inflammatory signaling pathways. TNFR2 expression is usually restricted to immune cells, triggering a narrower range of biological effects. TNF-alpha transmits signals for processes such as inflammation and apoptosis by binding to TNFR1 and TNFR2. Both soluble and transmembrane forms of TNF-alpha can activate TNFR1, which contains a death domain (DD) that interacts with the adaptor protein TNFR1-associated death domain (TRADD), which subsequently recruits other proteins. This signaling pathway leads to the activation of NF-κB and mitogen-activated protein kinases (MAPKs), promoting inflammation, cell survival, proliferation, and immune defense [37].

In combination with Interleukin-1, TNF-alpha induces changes in the endothelial cells of small blood vessels [40].

4.4 Significance of IL-1beta and TNF-alpha in the pathogenesis of intestinal diseases

In contrast to human medicine, the role of cytokines in inflammatory processes has not been studied as extensively in veterinary medicine. While the importance of the synthesis and activity of proinflammatory cytokines in human IBD such as Crohn's disease (CD) and ulcerative colitis (UC) is well documented, this also suggests a similar relevance in dogs [41].

In Crohn's disease, a complex and chronic intestinal disorder, TNF-alpha plays a critical role as a key mediator in the final stages of the inflammatory cascade. This proinflammatory cytokine sustains chronic inflammation by activating and recruiting lymphocytes and granulocytes, promoting the expression of adhesion molecules on inflamed endothelial cells, and stimulating the secretion of other inflammatory mediators such as IL-6 and IFN. The success of TNF-alpha blockers in treating CD, evidenced by mucosal healing and reduced need for surgeries, highlights the significant role of TNF-alpha in the disease. Moreover, increased TNF-alpha immunoreactivity in the lamina propria of intestinal samples from patients with CD and UC further underlining its importance in human IBD. Studies have also shown that TNF-alpha blockers like infliximab can induce remission in patients with CD and, to a lesser extent, in UC [42, 43].

Additionally, research has indicated an imbalance between IL-1beta and its natural inhibitor, IL-1Ra, in both human and canine IBD. Elevated ratios of IL-1beta to IL-1Ra have been observed in the intestinal mucosa of patients with CD, UC and in dogs with IBD [8, 35].

Several studies on chronic inflammatory enteropathy (CIE) in dogs have indeed demonstrated elevated levels of cytokines in the intestinal mucosa [44]. In one study, De Majo et al. found elevated TNF-alpha levels in dogs with GC [41]. This observation may be due to the significant damage to the mucosa of the colon or the presence of cells involved in immunological responses (e.g. macrophages) [44].

A new concept suggests that T cells infiltrating the lamina propria may produce high levels of the cytokine IL-1beta. IL-1beta is an important component of the innate immune system and activates the inflammasome, a protein complex involved in the control of inflammatory responses [18].

4.5 Diagnostic methods for granulomatous colitis in dogs

Diagnosing granulomatous colitis in dogs requires a multifaceted approach. Several factors are critical in reaching a definitive diagnosis, including clinical symptoms, blood tests, parasitological analysis, and imaging techniques to rule out other potential causes of the clinical signs. Therefore, the definitive diagnosis of GC is achieved through the exclusion of other diseases presenting with similar symptoms, combined with histological examination of biopsies from the colonic mucosa.

4.5.1 Clinical symptoms

The condition manifests with a variety of clinical symptoms, primarily severe large bowel diarrhea characterized by frequent, bloody, and mucus-laden stools, tenesmus, lethargy, and weight loss [1, 6]. The severity of these symptoms can range widely, from dogs that appear clinically normal and afebrile to those with severe illness and profound lethargy [26].

Differential diagnoses to consider include IBD, neoplastic diseases, dietary sensitivities, parasites in the enteric tract, and infections such as *Giardia*, *Cryptosporidium*, *Campylobacter*, and *Salmonella* [26].

4.5.2 Bloodwork

In dogs with GC, blood tests may show no significant changes [10, 45, 46]. However, some studies have reported findings such as hypoalbuminemia [12] and mild to moderate anemia [10, 13]. The anemia observed may be associated with advanced disease [6] or significant blood loss due to severe hematochezia [26]. In rare cases, prolonged blood loss can lead to iron deficiency anemia, which is characterized by microcytosis and hypochromia [26]. Hypoalbuminemia can result from hemorrhage, protein loss through ulcerated mucosa, anorexia, and inflammation, as albumin levels typically decrease during the acute phase response [26].

4.5.3 Cytologic Evaluation based on Rectal Scraping Samples

A recent study from 2022 has demonstrated the utility of cytologic evaluation based on rectal scraping samples in diagnosing GC in dogs. Notably, a presumptive diagnosis of GC has been successfully made using rectal scraping samples, marking a significant development in the diagnostic process. This approach involves identifying abundant PAS-positive macrophages during cytologic evaluation [12].

This method offers a less invasive alternative to traditional biopsy methods,

providing a rapid and potentially more accessible diagnostic tool for veterinarians. For clinicians assessing susceptible breeds presenting clinical signs consistent with GC, incorporating rectal scrape cytology into the diagnostic workflow is recommended [12].

4.5.4 Histopathological Examination

For an adequate diagnosis, colonic mucosal pinch biopsies are essential [26]. Histopathological examination using hematoxylin and eosin (H&E) and PAS stains reveals several characteristic features. There is a loss of goblet cells, multifocal ulceration, and erosion of the mucosa, along with dense cellular infiltration with macrophages, neutrophils and lymphocytes involving the colonic mucosa, lamina propria and sometimes submucosa [1, 25, 27]. Infiltrating macrophages contain a granular, eosinophilic cytoplasm that is PAS-positive [1].

4.5.5 Microbial Analysis

16S ribosomal DNA (rDNA) sequencing and FISH have revealed valuable insights into the bacterial factors influencing the inflammatory response in IBD [3]. These techniques show an increased quantity of mucosa-associated bacteria coupled with reduced microbial diversity in patients [3]. In case of GC, the identification of invasive *E. coli* using FISH is now crucial for accurate diagnosis and effective disease management [3]. FISH analysis is conducted on formalin-fixed, paraffin-embedded colonic mucosal biopsy samples using an *E. coli*-specific probe alongside a eubacterial probe [26]. Other bacterial preparations mounted on microscope slides, including *Salmonella, Proteus, Klebsiella, Enterococcus, Staphylococcus, Streptococcus*, and *Clostridia*, are used to ensure probe specificity [26, 47]. A negative FISH result does not entirely rule out *E. coli* invasion due to the potential for patchy distribution; therefore, collecting at least 10 mucosal biopsies is advised [26, 47]. False-negative results may also occur due to factors such as dead or dying bacteria, low bacterial counts, overfixation, or sulfasalazine treatment [26, 47].

4.6 Treatment for granulomatous colitis in dogs

Treatment with fluoroquinolones (FQs) can achieve positive results [3]. Due to their pronounced lipophilicity, FQs can achieve high intracellular concentrations, making them particularly effective against *E. coli* residing within macrophages [45]. However, due to widespread antimicrobial resistance to FQs in *E. coli* associated with GC, isolation and susceptibility testing of colonic mucosal *E. coli* is strongly recommended to adjust initial treatment accordingly [5, 11, 48]. It is generally advised that antibiotic treatment, specifically with enrofloxacin at a dosage of 5-10 mg/kg every 24 hours, should be administered for a total duration of eight weeks to ensure effective management of the infection [4, 10, 46]. Dogs with FQ-resistant *E. coli* showed improvement after treatment with meropenem [48].

4.7 Prognosis of granulomatous colitis in dogs

In a study from 2009, clinical remission in Boxers with GC following enrofloxacin therapy was shown to be closely associated with the eradication of invasive *E. coli*, with complete histologic healing of the intestinal mucosa potentially delayed due to the longer regeneration time required by the mucosal tissue [4].

In cases of enrofloxacin resistance, where clinical relapse has been observed [4], the prognosis has been reported as poor, with a higher risk of requiring euthanasia [4, 5].

Additionally, antimicrobial treatment guided by susceptibility testing, whether or not combined with empirical symptomatic therapy, has been associated with positive long-term clinical outcomes in over 80% of cases [48].

4.8 Parallels with human diseases

The suspected presence of *Escherichia coli* within macrophage clusters and a marked sensitivity to fluoroquinolones show similarities to Crohn's disease in humans [3]. GC has macroscopic features, regional distributions and immunopathologic characteristics that resemble ulcerative colitis [3, 27]. In addition, GC is reminiscent of Whipple's disease [1, 27], which is characterized by the presence of PAS-positive macrophages and bacteria in macrophages [3].

These diseases in humans cannot be definitively diagnosed with a single test; similar to GC, clinical signs, laboratory results, and endoscopic, histologic findings are interpreted collectively, with additional use of radiologic imaging and ultrasonography [49, 50].

In CD and UC patients, inhibiting effector cytokines such as TNF-alpha can reduce harmful inflammatory effects [51, 52], suggesting that targeting similar cytokine pathways in GC in dogs could potentially offer therapeutic benefits.

5 Objectives

This retrospective study aims to investigate the correlation between the expression of the cytokines TNF-alpha and IL-1beta in granulomatous colitis. As part of the study, we used GI biopsy samples that were endoscopically taken from 5 patients over the past years and stored in the pathology laboratory. This study is a pilot study, as the number of samples examined is very small. By examining past data and patient records, the study seeks to determine whether TNF-alpha and/ or IL-1beta can serve as histological biomarkers for diagnosing granulomatous colitis, guiding therapy decisions, and assessing prognosis, while also providing insights into the disease's pathogenesis and identifying potential therapeutic targets.

6 Material and Method

6.1 Patients

In this retrospective study, five privately owned dogs diagnosed with granulomatous colitis were selected. These dogs were referred to the Small Animal Clinic of the University of Veterinary Medicine Budapest for gastrointestinal endoscopy between 2017 and 2021, and biopsies collected during the procedures were submitted for routine histopathological analysis. Since dogs with GC often present symptoms such as large bowel diarrhea, hematochezia, and tenesmus, lower GI endoscopies were performed in all five patients. The biopsy samples were stored in the pathology department. Further details about the dogs are provided in Table 1.

Table 1 *Patient information*

NUMBER DOGS	ID NUMBER	BREED	AGE	GENDER
1	275330	French Bulldog	6 years	Male
2	287936	Boxer	4 years	Female
3	289332	French Bulldog	5 years	Male (neutered)
4	281408	French Bulldog	5 years	Female
5	271448	French Bulldog	7 years	Male (neutered)

The four negative control samples were obtained from the control group of a separate study (license number: PE/EA/000286-7/2021). Four Beagles, all 8–9 months old, including two males and two females, were euthanized for sample collection. Using an endoscopic biopsy forceps, samples were taken from the stomach, duodenum, ileum, and colon within five minutes post-mortem. Since the pathological changes in dogs with GC are predominantly located in the large intestine, this study focuses exclusively on the data from the colon.

6.2 RNAscope

RNAscope is a cutting-edge RNA in situ hybridization (ISH) technique that utilizes an innovative probe design to achieve single-molecule resolution while preserving tissue morphology. This method can employ traditional chromogenic dyes for bright-field microscopy or fluorescent dyes for multiplex analysis on standard FFPE tissue samples. Unlike grind-and-bind RNA analysis methods such as real-time reverse transcription polymerase chain reaction (RT-PCR), RNAscope offers the advantage of in situ RNA biomarker analysis, enabling the rapid development of RNA ISH-based molecular diagnostic assays [53, 54].

6.2.1 Description of the RNAscope method

During the procedure, 4 μm thick sections were cut from FFPE samples and mounted on positively charged glass slides (SuperFrost, Thermo Scientific).

During pretreatment, formaldehyde-induced cross-links were broken down to expose nucleic acids. Sections were first incubated at 60°C for one hour, followed by two 5-minute rinses in xylene under a fume hood, two 2-minute dehydrations in 96% ethanol, and air-drying for 5 minutes at room temperature.

A hybridization oven (HybEZ™ Oven, Advanced Cell Diagnostics, Newark, CA, USA) was preheated to 40°C. A humidifying pad soaked in water was placed in the hybridization chamber 30 minutes before use, which was then inserted into the preheated oven. Concurrently, 700 ml of 1× Target Retrieval solution was prepared in a covered boiling flask.

Deparaffinized sections were treated with 5–8 drops of RNAscope hydrogen peroxide solution, incubated for 10 minutes at room temperature, and washed twice with distilled water. The slides were then immersed in preheated 1× Target Retrieval solution, boiled for 15 minutes under aluminum foil, washed twice with distilled water, once with 96% ethanol, and left to air-dry at room temperature.

Tissue samples were outlined 2-4× using an Immedge™ hydrophobic marking pen and air-dried for 10 minutes at room temperature. Approximately 5 drops of RNAscope Protease Plus solution were applied to fully cover the tissue samples. These sections were incubated in the hybridization oven at 40°C for 25 minutes and subsequently washed twice with distilled water.

RNAscope Oligonucleotide probes (Advanced Cell Diagnostics, Newark, CA, USA) complementary to the target regions were used, specifically **830431 RNAscope® Probe-Cl-TNF** for detecting the canine TNF-alpha messenger-ribonucleic acid (mRNA) and **484101 RNAscope® Probe-Cl-IL1B** for detecting canine IL-1beta mRNA. These probes were designed with 20 pairs of double-Z probes, a multiple signal amplification system, and alkaline phosphatase labeling. With this method, cells expressing the target RNA appeared marked in red.

After pretreatment, 4–5 drops of the specific probe mixture were added to each section. The sections were incubated in the hybridization oven at 40°C for 2 hours and then washed twice for 2 minutes in a prepared wash buffer (Advanced Cell Diagnostics, catalog number: 310091).

Subsequent amplification involved the sequential application of reagents (Amp 1– 6), with each step followed by a wash in the buffer for 2 × 2 minutes (see Table 2). Excess liquid was removed between steps to ensure consistent reagent coverage.

Table 2 *Application of amplification reagents to the slides*

Reagent	Incubation time (minutes)	Incubation temperature (°C)
Amp 1	30	40
Amp 2	15	40
Amp 3	30	40
Amp 4	15	40
Amp 5	30	20
Amp 6	15	20

After completing the final amplification step, 120 µl of a 1:60 mixture of Fast RED-B and Fast RED-A reagents were carefully pipetted onto each section. The sections were incubated in a humidification chamber at room temperature for 10 minutes, followed by a rinse with distilled water. Next, the sections were immersed in 50% hematoxylin solution for 2 minutes, rinsed again with distilled water, briefly dipped in 0.02% ammonia solution for 10 seconds, and washed a final time with distilled water.

After excess liquid was removed, the samples were dried at 60°C for 15 minutes, then immersed in xylene, mounted with BioCare EcoMount medium (Advanced Cell Diagnostics, catalog number: 320409), and covered with a coverslip. The slides were left to

air-dry before being examined under a light microscope after 5 minutes. Finally, the sections were digitized using a slide scanner (Pannoramic Midi Slide Scanner, 3D Histech, Hungary).

6.2.2 Digital analysis of the sample slides

The sample slides were digitized using the software QuPath v0.4.4. Expression of **TNF-alpha mRNA** (marked in Figure 1) and **IL-1beta mRNA** (Figure 2) is visualized as red-stained spots on the digitized slides. Individual spots indicate the expression of single mRNA molecules, whereas clusters represent areas with multiple overlapping spots, making it difficult to distinguish individual molecules. Below, digitized slides from patients with GC and the negative sample slide (Figure 3) are displayed.

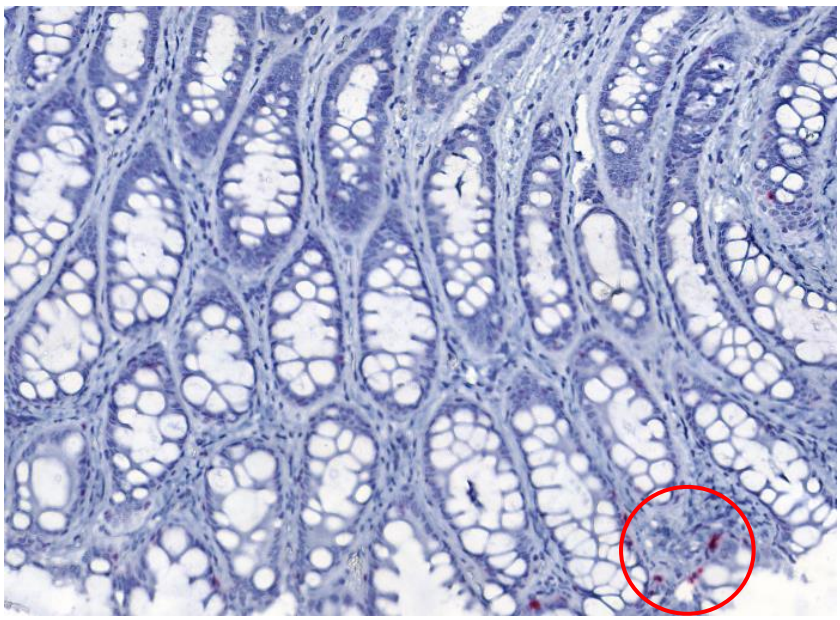

Figure 1 *Digitalized GC sample slide with TNF-alpha mRNA*

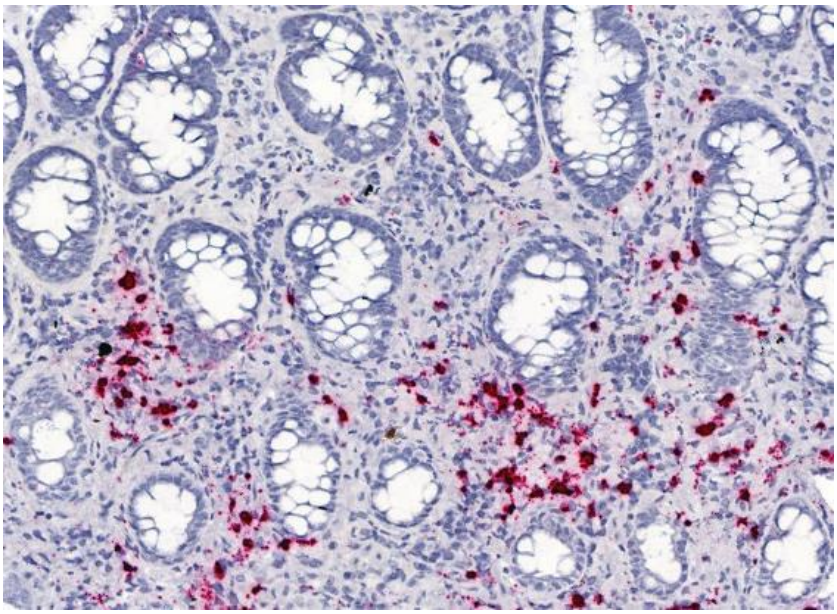

Figure 2 *Digitalized GC sample slide with IL-1beta mRNA*

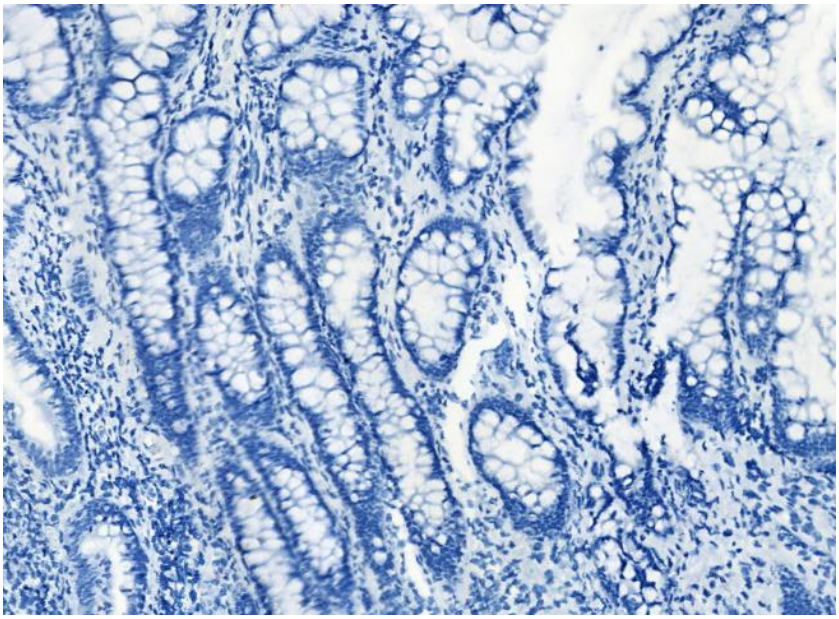

Figure 3 *Digitalized negative sample slide*

6.3 Statistics

In the selected biopsy samples, TNF-alpha and IL-1beta were expressed both in spots and clusters. Spots represent individual points that can be distinguished from each other. However, if several spots are so close to each other that the computer can no longer recognize them as separate units, they are grouped together as a cluster. Therefore, we had to introduce clusters as an additional value to adequately describe and analyze these overlaps.

To evaluate them, we calculated the spot-to-area ratio and cluster-to-area ratio for each sample in the colon. Our aim was to analyze if there is any correlation between the TNF-alpha or IL-1beta expression and the disease.

7 Results

To enhance visual interpretability, the cytokine expression values in the boxplots were scaled by a factor of 1000. This adjustment does not affect the underlying data but improves clarity in graphical representation.

7.1 Cluster/area colon of the TNF-alpha expression

We compared the TNF-alpha expression clusters per area in colon samples from healthy dogs (Neg) and those with GC (Figure 4).

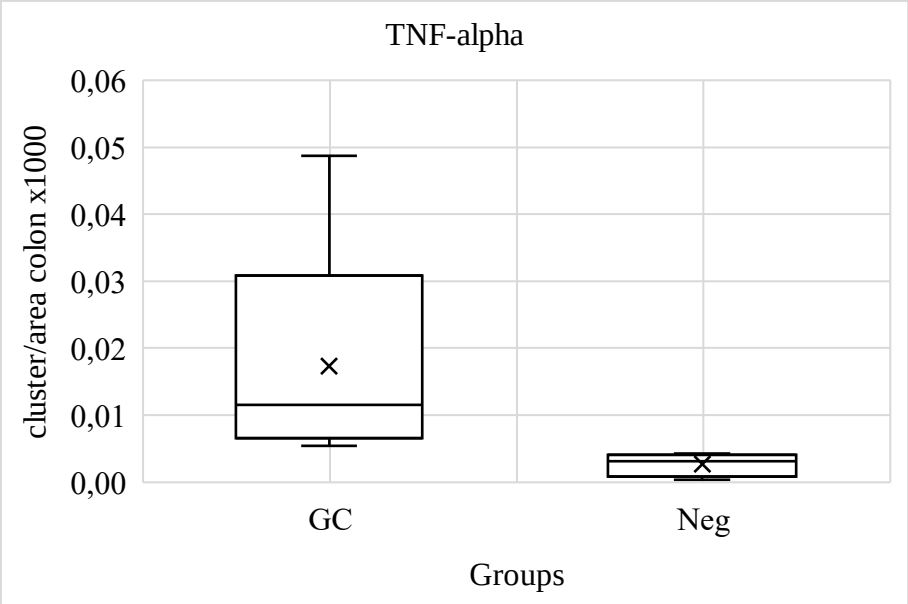

Figure 4 *Cluster per area colon of TNF-alpha expression*

The results show that the median TNF-alpha expression is relevantly higher in the GC group (1.15×10^{-2}) compared to the negative controls (3.12×10^{-3}). The interquartile range (IQR) is broad (6.60×10^{-3} to 3.08×10^{-2}), indicating variability in the expression levels among individuals within the GC group. Some individuals show very high TNF-alpha expression, as indicated by the upper whisker extending towards elevated values (4.87×10^{-2}), while the lower quartile remains above baseline levels (5.42×10^{-3}).

In contrast, TNF-alpha levels in the negative control group are very low and stable, with a narrow IQR (8.63×10^{-4} to 4.12×10^{-3}). The expression values range from a minimum

of 3.42×10^{-4} to a maximum of 4.22×10^{-3}. Both the median and mean values are relevantly lower compared to the GC group.

7.2 Spot/area colon of the TNF-alpha expression

The expression of TNF-alpha in the spot per area in colon samples was analyzed for both the GC group and the negative control group (Figure 5).

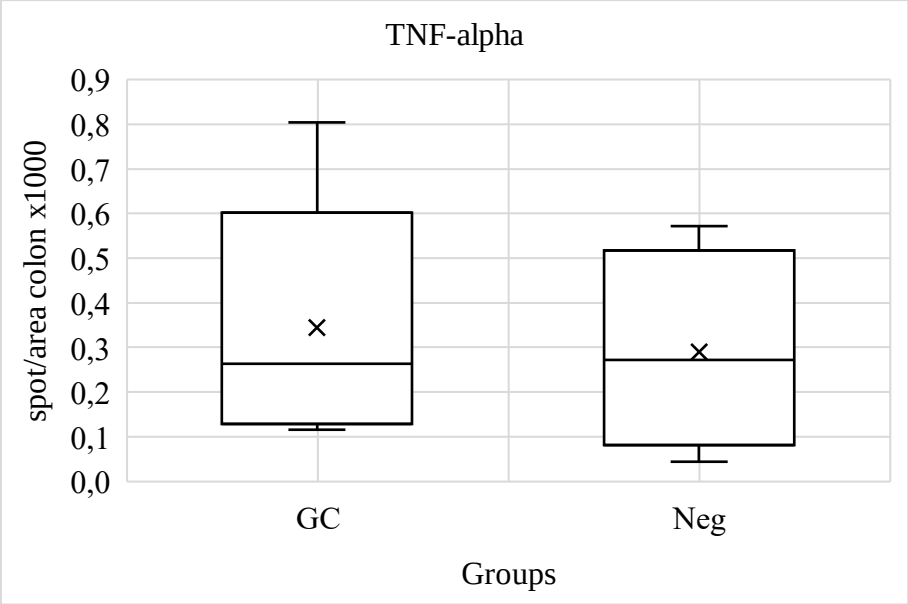

Figure 5 *Spot per area colon of the TNF-alpha expression*

The median TNF-alpha expression in the GC group is slightly lower (2.64×10^{-1}) compared to the negative control group (2.72×10^{-1}). The IQR is broad (1.29×10^{-1} to 6.02×10^{-1}), indicating considerable variability in TNF-alpha levels among the individuals in this group. A few individuals show very high TNF-alpha levels, as indicated by the upper whisker extending towards elevated values (8.04×10^{-1}), while the lower whisker approach baseline levels (1.16×10^{-1}).

The TNF-alpha expression in the negative control group shows a moderately narrow IQR (8.13×10^{-2} to 5.17×10^{-1}), suggesting less variability compared to the GC group. The expression values range from a minimum of 4.37×10^{-2} to a maximum of 5.72×10^{-1}. The median TNF-alpha expression is comparable to that of the GC group.

7.3 Cluster/area colon of the IL-1beta expression

We also compared the IL-1beta expression clusters per area in colon samples from healthy dogs and those with GC (Figure 6).

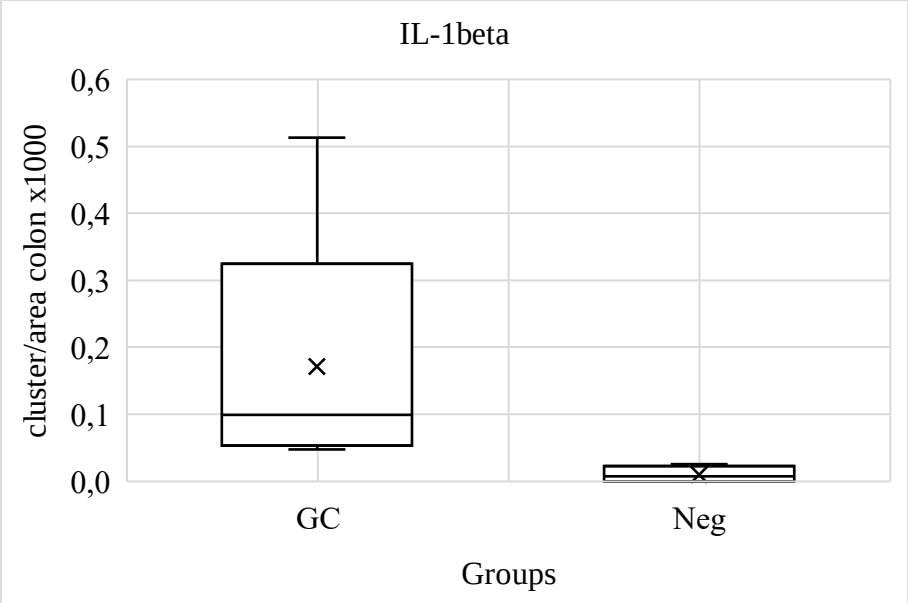

Figure 6 *Cluster per area colon of IL-1beta expression*

The median IL-1beta expression is considerably higher in the GC group (9.88×10^{-2}) compared to the negative control group (7.15×10^{-3}). The IQR in the GC group is wide (5.31×10^{-2} to 3.25×10^{-1}). Some individuals in the GC group exhibit exceptionally high expression levels, as reflected by the upper whisker extending to elevated values (5.13×10^{-1}), while the lower quartile remains above baseline (4.75×10^{-2}).

In contrast, IL-1beta levels in the negative control group are markedly lower and more consistent, with a much narrower IQR (5.53×10^{-5} to 2.24×10^{-2}). The expression values in this group range from a minimum of 0 to a maximum of 2.51×10^{-2}, and both the median and mean values are relevantly lower compared to the GC group.

7.4 Spot/area colon of the IL-1beta expression

We compared the spots of IL-1beta expression per area of the colon samples from healthy dogs and those with GC (Figure 7).

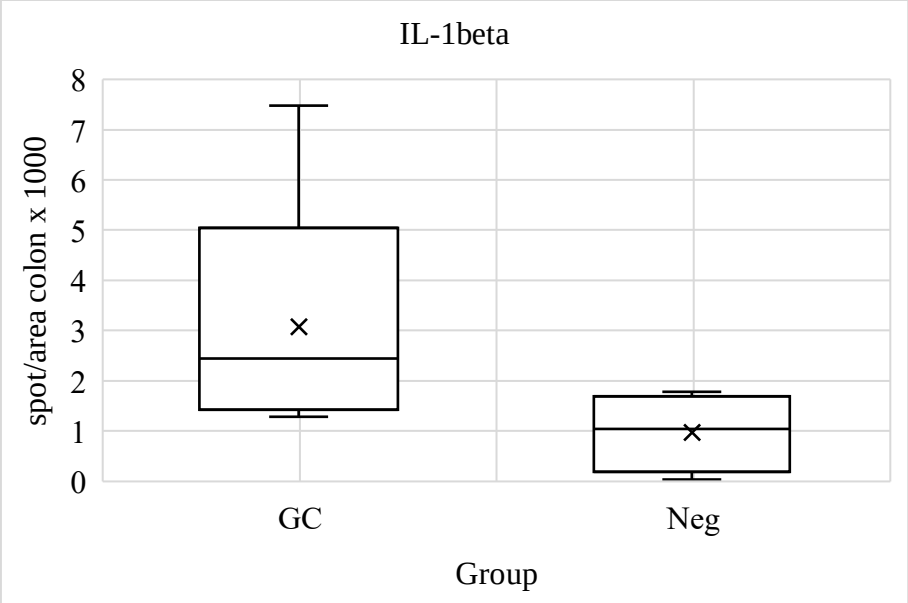

Figure 7 *Spot per area colon of the IL-1beta expression*

The median IL-1beta expression in the GC group is higher (2.44) compared to the negative control group (1.04). The IQR in the GC group is wide (1.43 to 5.04), reflecting relevant variability in IL-1beta levels among individuals. Some individuals exhibit very high IL-1beta expression (7.48) while the lower quartile remains above baseline (1.28).

In the negative control group, IL-1beta expression is lower and more consistent, with a narrower IQR (0.18 to 1.69) compared to the GC group. The expression values range from a minimum of 0.04 to a maximum of 1.78, indicating less variability within this group. Both the median and IQR confirm the lower IL-1beta expression in the negative control group relative to the GC group.

7.5 Cell infiltration in granulomatous colitis

On histopathological examination, the cell infiltration in the lamina propria of the five dogs with GC was also analyzed. The results are shown in Table 3.

Table 3 *Cell infiltration of the lamina propria in the colon in granulomatous colitis*

ID NUMBER	LAMINA PROPRIA LYMPHOCYTES	LAMINA PROPRIA EOSINOPHIL GRANULOCYTES	LAMINA PROPRIA MACROPHAGES
275330	mild	normal	moderate
287936	normal	normal	severe
289332	moderate	normal	severe
281408	normal	normal	severe
271448	moderate	normal	mild

Lymphoplasmacytic infiltration varies from mild to moderate. The number of eosinophil granulocytes remains normal in all samples. Severe macrophage infiltration is clearly visible in three out of five cases.

8 Discussion

The aim of this study was to gain deeper insights into the inflammatory processes underlying GC and to evaluate whether the cytokine expression of TNF-alpha and IL-1beta could serve as diagnostic markers. Treatment often focuses on symptom management or involves the use of expensive antibiotics, raising concerns about antimicrobial resistance. In Germany, the future use of antibiotics in dogs and cats will face increasing restrictions due to the Antibiotic Minimization Concept introduced by the Federal Office of Consumer Protection and Food Safety (dt.: BVL).

This underscores the need for diagnostic, prognostic, and monitoring biomarkers that could enable diagnosis without the necessity of therapeutic trials, allow for the timely initiation of appropriate therapies, and provide more precise prognostic information. Drawing on established knowledge of the role of cytokines in human IBD, and the similarities between IBD and GC in dogs, we consider it essential to explore the potential of TNF-alpha and IL-1beta as biomarkers for GC.

The data presented in this study show relevant differences in the expression of TNF-alpha and IL-1beta between healthy control dogs and those with GC. These results provide insights into potential pathophysiological mechanisms of GC and highlight the role of these cytokines in the disease process.

TNF-alpha expression shows a marked increase in the colon samples when analyzed as cluster per area in GC dogs compared to the negative controls. While TNF-alpha is also expressed in healthy mucosa, its levels are substantially lower than in the diseased dogs. However, the results for TNF-alpha expression at spot per area in the colon are less distinct, with comparable levels observed in both GC dogs and negative controls. These findings suggest that TNF-alpha may play a role in the pathogenesis of granulomatous colitis, particularly by amplifying the inflammatory response. This aligns with the established role of TNF-alpha as a central proinflammatory cytokine in various inflammatory diseases [37, 42].

The expression of IL-1beta shows a marked increase in the colon samples analyzed as cluster per area in GC dogs compared to negative controls. This finding suggests that IL-1beta plays a crucial role in driving the inflammatory processes in GC. While IL-1beta is also expressed in healthy mucosa, its levels are lower than in the mucosa of diseased dogs. Similarly, in the spot per area analysis of the colon, IL-1beta expression is higher in dogs with GC compared to the negative controls, though the difference is less pronounced than in

the cluster per area analysis. This pronounced increase in IL-1beta expression may account for the extensive tissue damage and chronic inflammation that are hallmarks of GC. IL-1beta is known to stimulate the production of additional proinflammatory mediators, potentially exacerbating colonic tissue destruction and sustaining the inflammatory cycle [34, 35].

Our histological findings support the cytokine expression data by demonstrating a partly severe infiltration of macrophages in 3 out of 5 dogs with GC. These results correlate with the known fact that macrophages are a major source of production of both TNF-alpha and IL-1beta [35, 37]. IL-1beta is synthesized almost exclusively by macrophages [35], while TNF-alpha can also be produced by activated T lymphocytes and natural killer cells [37].

This study has several limitations. As a pilot study, the sample size was small, which limits the ability to perform significant statistical analyses. Nonetheless, the results are promising and warrant further investigation.

Moreover, as this is a clinical study, practical and ethical considerations constrained sample collection. Owners are usually unwilling to subject their dogs to additional invasive procedures unless clinically necessary. For the negative control samples, biopsies were not collected endoscopically from living healthy dogs, as clients declined sedation for their pets in the absence of clinical indications. Instead, control samples were obtained from euthanized healthy dogs that were not required for other research purposes. This approach, while ethical, may introduce variability in sample handling.

Despite these limitations, our findings provide valuable preliminary insights into the potential use of TNF-alpha and IL-1beta as biomarkers for GC and highlight the need for further research in this area.

9 Summary

This study explored the inflammatory mechanisms of GC in dogs and assessed the potential of TNF-alpha and IL-1beta as biomarkers. TNF-alpha expression was elevated in GC samples, particularly in cluster-per-area analysis, suggesting a role in amplifying inflammation. IL-1beta expression was consistently higher in GC dogs, indicating its central role in driving chronic inflammation and tissue damage. Histological findings revealed significant macrophage infiltration, supporting their contribution to cytokine production.

While limited by a small sample size and ethical constraints in sample collection, the study provides promising preliminary evidence for using TNF-alpha and IL-1beta as diagnostic biomarkers, emphasizing the need for further research.

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11 Acknowledgments

I would like to thank my thesis supervisor, Dr. Borbála Mózes, for her unwavering support, guidance, and encouragement throughout the entire process of writing this thesis. Her expertise, insightful suggestions, and continuous feedback were invaluable in shaping this work.

I am also grateful to Dr. Péter Fehérvári for his time and expertise in statistical analysis, which was essential in producing the detailed graphs and statistics that contributed to the study´s results.

My thanks also go to the staff of the Department of Pathology for their indispensable resources and support during the research. A special thank you is due to Dr. Lilla Dénes and Renáta Pop for their work with the RNAscope technique and for providing the necessary documentation.

I would like to express my gratitude to Dr. Dávid Géza Horváth for his efforts in conducting the digital analysis of the sample slides and for supplying the images used in this thesis.

I also appreciate Dr. Linda Müller for granting us access to the tissue samples for the control group, which were crucial to the success of this study.

This thesis is the result of many collaborative efforts, and I am truly thankful to everyone who contributed in any way to this research. Your support has been invaluable, and I am deeply appreciative of all your help.