ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

What potential biomarkers should be considered in diagnosing and managing canine chronic inflammatory enteropathies?

Carina Sacoor

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3-BrY - Bromotyrosine
APC - Antigen presenting-cell
ARE - Antibiotic-responsive enteropathy
CCECAI - Canine chronic enteropathy clinical activity index
CIBDAI - Canine inflammatory bowel disease activity index
CIE - Chronic inflammatory enteropathies
CRP - C-reactive protein
CUPRAC - Cupric reducing antioxidant capacity
DAMP - Damage-associated molecular pattern
DC - Dendritic cell
DI - Dysbiosis index
EPI - Exocrine pancreatic insufficiency
FOX - Ferrous oxidation-xylenol orange
FRAP - Ferric reducing ability of the plasma
FRE - Food-responsive enteropathy
GALT - Gut-associated lymphoid tissue
GI - Gastrointestinal
IAP - Intestinal alkaline phosphatase
IBD - Inflammatory bowel disease
IEC - Intestinal epithelial cell
IF - Intrinsic factor
IgA - Immunoglobulin A
IL - Interleukin
IRE - Immunosuppressive-responsive enteropathy
LTE4 - Leukotriene E4
NF-kb - Nuclear factor-kappa B
NMH - N-methylhistamine
NOD - Nucleotide-binding oligomerization domain
pANCA - Perinuclear anti-neutrophilic cytoplasmic antibodies
PLE - Protein-losing enteropathy
PON1 - Paraoxonase 1
PRR - Pattern recognition receptor
RAGE - Receptor for advanced glycation end products
ROS - Reactive oxygen species
SCFA - Short-chain fatty acids
sRAGE - Soluble receptor for advanced glycation end products
TBARS - Thiobarbituric acid reactive substances
TEAC - Trolox equivalent antioxidant capacity
Th cell - T helper cell
TLR - Toll-like receptors
Treg - Regulatory T cell
WSAVA - World Small Animal Veterinary Association
α1PI - Alpha1-proteinase inhibitor

What potential biomarkers should be considered in diagnosing and managing canine chronic inflammatory enteropathies?

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RESUMO

As enteropatias inflamatórias crónicas em cães caracterizam-se pela manifestação de sinais clínicos gastrointestinais persistentes ou recorrentes com uma duração superior a três semanas.

Apesar da falta de clareza na etiopatogenia, considera-se que a predisposição genética, associada a fatores ambientais, como os antigénios alimentares e a microbiota intestinal, poderá induzir uma resposta imunitária anormal no hospedeiro.

O diagnóstico deste quadro clínico requer uma investigação completa, de forma a excluir todas as outras possíveis causas. Atualmente, a observação de sinais clínicos, associada à avaliação histopatológica e a ensaios terapêuticos sistemáticos, constitui o método de eleição para o diagnóstico das enteropatias crónicas. Para além disso, o diagnóstico, a monitorização da progressão da doença e a avaliação da resposta ao tratamento, podem ser exaustivos, visto que todo este processo é extenso, dispendioso e parcialmente invasivo.

Assim, os biomarcadores surgem como ferramentas não invasivas, que podem ser úteis na avaliação da função gastrointestinal, na identificação da presença da doença e na avaliação da sua progressão natural, bem como na deteção de mudanças temporais na atividade clínica. Adicionalmente, os biomarcadores podem ser vantajosos na monitorização da inflamação gastrointestinal, na previsão da resposta ao tratamento e dos desfechos clínicos.

Na última década, vários estudos foram realizados com o intuíto de explorar a utilidade clínica dos biomarcadores. Assim, o objetivo desta dissertação é fornecer uma visão geral dos biomarcadores considerados relevantes para o diagnóstico e gestão de cães com enteropatias inflamatórias crónicas. Os biomarcadores abordados neste estudo poderão ser serológicos, estar presentes nas fezes e urina, ou ainda derivados de tecidos.

Este estudo argumenta que os biomarcadores, em particular a calprotectina e a calgranulina c, têm um grande potencial para serem utilizados na prática clínica, no diagnóstico e gestão de cães doentes. Contudo, um único biomarcador não pode, com certeza, predizer a severidade da doença, a progressão, a resposta ao tratamento e o desfecho clínico. Deste modo, com o intuito de alcançar uma maior precisão, será benéfico se estas ferramentas forem utilizadas em conjunto com as ferramentas contemporâneas. Futuras investigações são necessárias, com o objetivo de melhor determinar a utilidade destas ferramentas no diagnóstico e gestão de cães com enteropatias crónicas inflamatórias.

Palavras-chave: Biomarcadores; Canino; Doença Intestinal Inflamatória; Enteropatias inflamatórias crónicas; Inflamação gastrointestinal

ABSTRACT

Chronic inflammatory enteropathies in dogs are characterized by persistent or recurrent gastrointestinal signs that last for more than three weeks.

Despite unclarity in the etiopathogenesis, it is considered that a genetic predisposition associated with environmental factors, such as dietary antigens and the intestinal microbiota, might induce an abnormal immune response in the host.

The diagnosis of this condition requires full investigation in order to exclude all other possible causes. Currently, observation of clinical signs associated with histopathologic evaluation and systematic therapeutic trials is the gold standard for the diagnosis of chronic enteropathies. Furthermore, diagnosis, monitoring the disease progression and treatment response evaluation can be exhausting, since this whole process is time-consuming, costly and partially invasive.

Therefore, biomarkers appear as non-invasive tools, which can be useful in evaluating gastrointestinal function, identifying the presence of disease, and assessing its natural progression, as well as detecting temporal changes in clinical activity. Moreover, it can be advantageous in monitoring gastrointestinal inflammation, predicting response to treatment and clinical outcomes.

Over the past decade, several studies were conducted in order to explore the clinical utility of biomarkers. Thus, the aim of this dissertation is to provide an overview of the biomarkers considered relevant in the diagnosis and management of dogs with chronic inflammatory enteropathies. The biomarkers addressed in this study may be serological, present in urine and feces or even tissue-derived.

This study argues that biomarkers, in particular calprotectin and calgranulin c, have great potential to be used in clinical practice in the diagnosis and management of dogs affected dogs. However, a single biomarker cannot assuredly predict disease severity, progression, response to treatment and clinical outcome. Therefore, in order to achieve greater accuracy, it would be beneficial if these tools are used in conjunction with the c ontemporary ones. Future research is needed with the aim of better determine the usefulness of these tools in diagnosing and managing chronic inflammatory enteropathies in dogs.

Key-words: Biomarkers; Canine; Chronic inflammatory enteropathies; Inflammatory bowel disease; Gastrointestinal inflammation.

1. INTRODUCTION

Chronic inflammatory enteropathies (CIE) are a group of gastrointestinal (GI) disorders characterized by persistent or recurrent GI signs (Heilmann & Steiner, 2018; Moser, Mitze, Teske, von Bomhard, & Stockhaus, 2018), lasting for more than three weeks (AlShawaqfeh *et al*., 2017) with histologic evidence of primary intestinal mucosal inflammation (Heilmann *et al*., 2018). There were inconsistencies in the articles pertaining to this research, as some authors used the term "chronic enteropathies" and others used the term "CIE". For the purpose of this study, the term "CIE" will be used.

The main cause of GI disease in dogs are considered to be CIE (Volkmann *et al*., 2017) which have a cyclical remission-relapse nature (Heilmann, Volkmann, *et al.,* 2016). They can be further classified in different forms, based on a clinical responsiveness to different therapeutic interventions (Heilmann, Volkmann, *et al*., 2016), including food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (ARE), corticosteroid or immunosuppressive-responsive enteropathy (IRE), and non-responsive enteropathy (Dandrieux, 2016; Volkmann *et al*., 2017) if patients do not respond to immunomodulatory treatment (Heilmann & Steiner, 2018). In the cases of CIE that do not respond to food trials nor antimicrobial treatments, therefore requiring immunosuppressive-responsive treatment, are also known as idiopathic inflammatory bowel disease (IBD) (Dandrieux, 2016; Volkmann *et al*., 2017). In addition to this classification, the term protein-losing enteropathy (PLE) is used when there is evidence of intestinal protein loss, revealing a worse prognosis and a poor clinical outcome (Dandrieux, 2016; Heilmann & Steiner, 2018; Volkmann *et al*., 2017).

While their exact etiologies and pathogenesis mechanisms remain partially uncertain, an abnormal immune response against dietary and bacterial antigens, associated with genetic predisposition appears to play a central role (AlShawaqfeh *et al*., 2017; Hanifeh *et al*., 2018; Heilmann *et al*., 2018). Some breeds have been reported as predisposed for developing CIE, such as Weimaraner, Rottweiler, German sharped dog, Border collie, Boxer (Dandrieux, 2016), Basenjis and French bulldogs (Jergens *et al*., 2009)

Observation of clinical signs associated with histopathologic evaluation and systematic therapeutic trials is currently the gold standard for the diagnosis of CIE (Gerou-Ferriani *et al*., 2018). Histopathology allows the evaluation of the intestinal inflammatory infiltrate in the lamina propria. According to its nature, the inflammatory infiltrate can be divided into neutrophilic, eosinophilic and lymphocytic-plasmacytic, the latter described as the most frequent form of enteritis (AlShawaqfeh *et al*., 2017; Moser *et al*., 2018). Nevertheless, histopathology methods do not differentiate the various forms of CIE (Heilmann & Steiner, 2018). Hence, at the present date, the most accurate treatment is one of trial-and-error. Moreover, clinical outcomes for individuals vary widely and are difficult to predict (Gerou-Ferriani *et al*., 2018). As a result, novel biomarkers have been investigated in efforts to provide a more objective method to assess the natural progression of the disease, help in diagnostic evaluation, assess the temporal changes in clinical activity, patient monitorization, treatment evaluation, response and outcome

prediction (Gerou-Ferriani *et al*., 2018; Heilmann & Steiner, 2018; Hof *et al*., 2012; Jergens & Simpson, 20212; Otoni *et al*., 2018).

The aim of this study is to provide an overview of the current status of biomarkers and their usefulness in diagnosing and managing CIE in dogs. Another goal of this dissertation is to evaluate their potential clinical advantages, as well as possible limitations, based on the results of studies conducted mostly over the last decade.

2. ETIOPATHOGENESIS

Predisposed animals can develop CIE as a result of a dysregulation of mucosal immunity. The exact etiologies remain unknown and the underlying mechanisms of the pathogenesis have not been elucidated (Somu *et al*., 2017), however, one mechanism that could justify the development of chronic inflammation is the loss of immunologic tolerance against antigens, such as harmless dietary components and commensal microorganisms. The articles pertaining to this research believe that this failure of immunological tolerance occurs as a consequence of intestinal barrier integrity dysfunction (Eissa, Kittana, Gomes-Neto, & Hussein, 2019), dysregulation of gut-associated lymphoid tissue (GALT), disturbances in the bacterial flora, or a combination of these factors (Ogawa *et al*., 2018), resulting in pathological inflammations (Somu *et al*., 2017).

Intestinal epithelial cells (IECs) form a biochemical and physical barrier that separates luminal bacteria, dietary elements, toxins and antigens from the host, preventing mucosal inflammation and tissue damage (Celi, Verlhac, Pérez Calvo, Schmeisser, & Kluenter, 2019; Gram, Milner, & Lobetti, 2018; Ogawa *et al*., 2018; Osada *et al*., 2016). Furthermore, IECs can secrete mucus and antimicrobial peptides, in response to a stimulus (Eissa *et al*., 2019), contributing to epithelial repair and defending against bacterial invasion (Abraham & Cho, 2009). In CIE, the intestinal barrier has increased permeability as a result of a defective regulation of tight junctions and adherent junctions (Abraham & Cho, 2009; Ohta *et al*., 2014). A primary defect in barrier function can cause the abovementioned abnormalities; however, those same abnormalities can be an outcome of inflammation (Abraham & Cho, 2009). Also, an impaired mucosal barrier function can result in increased exposure of immune cells to bacteria and intestinal luminal antigens, thus contributing to an unsuppressed immune response (Ohta *et al*., 2014). As such, IECs are essential in maintaining intestinal homeostasis (Osada *et al*., 2016) through the balance between physiological and pathological inflammation (Eissa *et al*., 2019).

A complex immunological network constitutes GALT, (Junginger, Schwittlick, Lemensieck, Nolte, & Hewicker-Trautwein, 2012) which is composed by secondary lymphoid organs, including Peyer patches in the small intestine, isolated lymphoid follicles throughout the GI tract, and the mesenteric lymph nodes; and effector sites, such as the lamina propria mucosa (Karin Allenspach, 2011). The point of a framework like GALT is to promote tolerance towards environmental antigens, such as commensals and

food antigens, while at the same time having a protective immune response against pathogens. Consequently, a failure in maintaining this tolerance is the main factor leading to chronic intestinal inflammation (Karin Allenspach, 2011; Gram *et al*., 2018). A complex population of innate and adaptive immune cells participate in the pathogenesis of CIE in dogs (Figure 1) (Abraham & Cho, 2009; Karin Allenspach, 2011; Eissa et al., 2019).

The innate immunity pathways provide an initial and rapid response (Abraham & Cho, 2009; Heilmann & Allenspach, 2017) and consist of IECs, neutrophils, dendritic cells (DCs), macrophages and eosinophils interaction, as well as their secreted products (Eissa *et al*., 2019).

Enterocyte pattern recognition receptors (PRRs) are responsible for the recognition of microbe-associated molecular patterns (Schnyder *et al*., 2018), which are conserved molecules found on bacteria or other infectious agents (Karin Allenspach, 2011). Based on PRRs, one can determine whether the antigens are tolerated or reacted against (Cerquetella *et al*., 2010). PRRs include nucleotide-binding oligomerization domain (NOD) 2 and toll-like receptors (TLRs), which are located on the surface or in the cytoplasm of IEC, DCs (Karin Allenspach, 2011) and macrophages (Schnyder *et al*., 2018). TLRs, namely TLR-2, TLR-4, TLR-5, TLR-9, recognize specific bacterial products. To begin with, TLR-2 recognizes lipopeptides from Gram-positive bacteria; Secondly, TLR-4 identifies lipopolysaccharides from Gram-negative bacteria; Moreover, TLR-5 recognizes the main protein of bacteria flagella, flagellin (Karin Allenspach, 2011; Hall, 2009; Heilmann & Allenspach, 2017); and finally, TLR-9 identifies bacterial and viral unmethylated CpG oligonucleotides (Schnyder *et al*., 2018). Also, NOD-2 detects the muramyl dipeptide molecule, a peptidoglycan component of gram-positive and gram-negative bacteria, and possibly viral constituents as well (Heilmann & Allenspach, 2017). Canine CIE have been linked with genetic polymorphisms in genes encoding TLR2, TLR4, TLR5, TLR9, that may contribute to individual predisposition (Heilmann & Allenspach, 2017; Maeda *et al*., 2012; Schnyder *et al*., 2018). These PRRs play an important role in the homeostasis and host defense. However, an abnormal activation of these PRRs can potentially lead to a loss of controlled homeostatic tolerance, causing chronic inflammations (Heilmann & Allenspach, 2017). After binding to TLRs, a complex intracellular signaling pathway is initiated (Karin Allenspach, 2011), with up-regulation of pro-inflammatory cytokines, chemokines, costimulatory molecules, inflammatory mediators, such as prostaglandins and leukotrienes, reactive oxygen species and nitrogen intermediates (Kołodziejska-Sawerska *et al*., 2013; Schnyder *et al*., 2018), culminating in the activation of nuclear factor-kappa B (NF-kB) (Karin Allenspach, 2011; Heilmann & Allenspach, 2017). In this changed environment, the immune system loses its tolerance, triggering an active immune response (Hall, 2009). Clinical studies have demonstrated that several innate immunity receptors, including TLR-2, TLR-4, TLR-5, TLR-9 and NOD-2, are dysregulated within the intestines of dogs suffering from CIE (Aono et al., 2019; Okanishi et al., 2013; Schnyder et al., 2018). Thus, representing consistent evidence that the innate immunity is hyperactive in this disease (Karin Allenspach, 2011; Heilmann & Allenspach, 2017).

After innate immunologic mechanisms, activated antigen presenting-cells (APCs) trigger an adaptive immune response by presenting peptide antigens to naïve CD4+ T helper cells (Th cells) in secondary lymphoid organs. Based on their cytokine profile, Helper T cells can be differentiated into Th1 cells, that mediate cytotoxicity and cell-mediated immunity; Th2 cells, that mediate humoral immunity; Th17 cells or regulatory T cells (Tregs) (Heilmann & Suchodolski, 2015). On top of that, memory lymphocytes are also developed (Abraham & Cho, 2009). In canine CIE, intestinal inflammation can be marked by Th1 responses, which are mediated mainly by the secretion of Interferon gamma, tumor necrosis factor alpha. Th1 cells can be antagonized by Th2 cells that primarily produce interleukin (IL)-4, IL-5 and IL-13, and are typically associated with responses to allergens and parasites (Eissa *et al*., 2019). Other studies have not been able to demonstrate a clear Th1 or Th2 cytokine expression in dogs with CIE (Dumusc *et al*., 2014; Eissa *et al*., 2019; Heilmann & Suchodolski, 2015; Jergens *et al*., 2009; Kołodziejska-Sawerska *et al*., 2013). Experts in the field observed that IL-23p19 is increased in the inflamed intestinal mucosa of dogs. This cytokine is produced by macrophages and plays an important role in the promotion of Th17 cell differentiation (Tamura *et al*., 2014). Th17 cells can produce proinflammatory cytokines, such as IL-17 and IL-22, but most importantly can demonstrate anti-inflammatory properties due to their ability to transdifferentiating into Tregs. These cells play an important role in maintaining immunotolerance. They are in charge of suppressing effector T cells and APCs through the secretion of immunosuppressive cytokines, such as IL-10 and transforming growth factor beta (Eissa *et al*., 2019; Heilmann & Steiner, 2018; Maeda, Ohno, Fujiwara-Igarashi, Uchida, & Tsujimoto, 2016).

In dogs with CIE, this imbalance between proinflammatory and anti-inflammatory cytokines results in disrupted intestinal immunity (Kołodziejska-Sawerska *et al*., 2013). Although Th1, Th2 and Th17 are crucial for the defense against pathogens and elevated intake of luminal bacteria, their extension and overactivity can result into intestinal inflammation (Abraham & Cho, 2009).

In addition, intestinal B lymphocytes have the ability to turn into plasma cells and produce immunoglobulin (Ig) A antibodies, which contribute to immune protection without causing inflammation (Abraham & Cho, 2009). IgA antibodies not only contribute to the prevention of bacteria crossing the epithelial barrier, but can also shape the intestinal microbiota composition (Maeda *et al*., 2013), keeping them from triggering an immune response in the intestine (Lee *et al*., 2015).

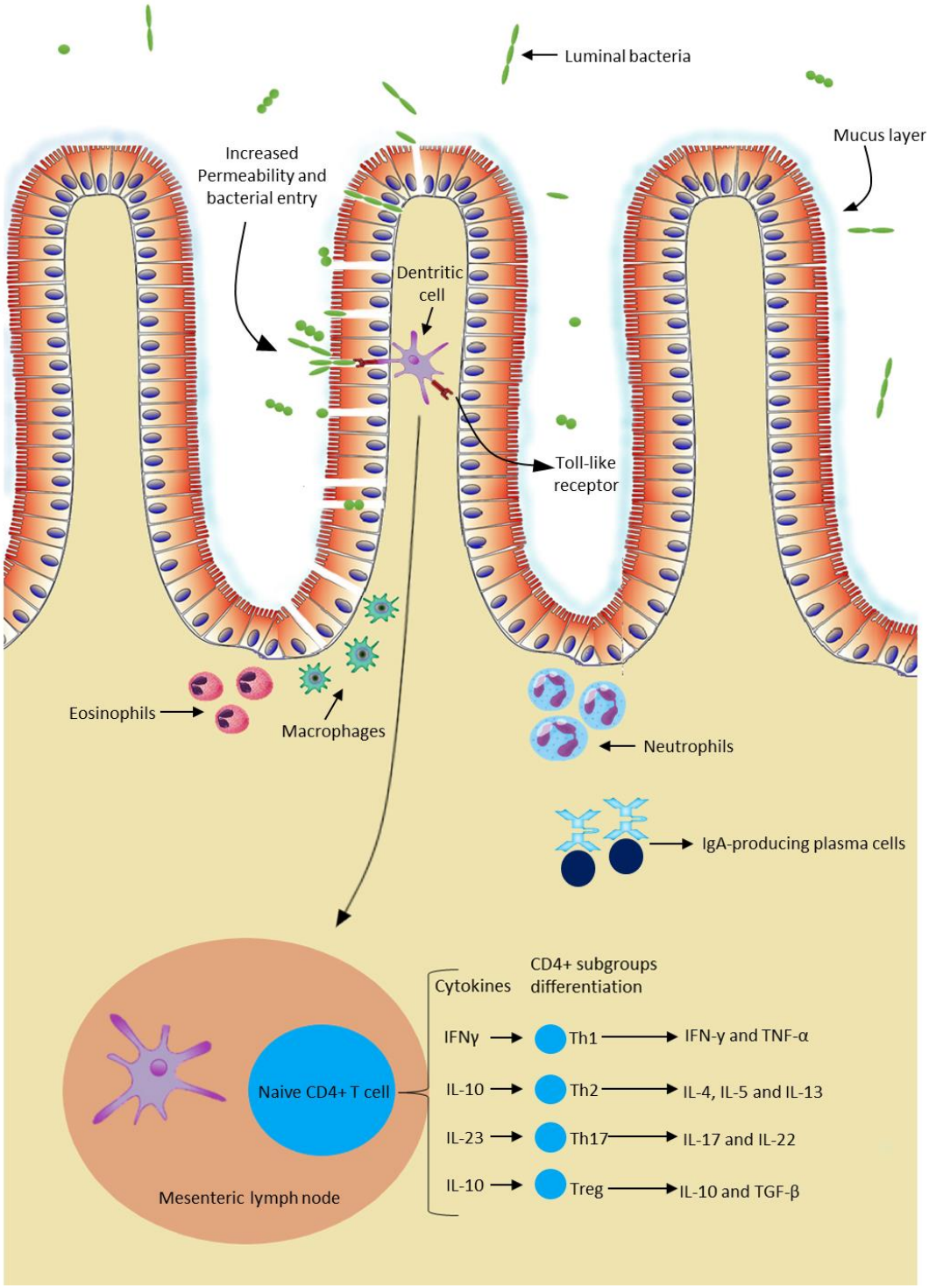

Figure 1 – The intestinal immune system in canine chronic inflammatory enteropathies. The intestinal barrier has increased permeability in dogs with CIE. An impaired barrier function can increase the exposure of immune cells to luminal antigens. These can be tolerated or reacted against, based on the PRRs, such as TLRs. Innate response consists in the interaction of IECs, neutrophils, macrophages, DCs and eosinophils, as well as their secreted products. After innate immunologic mechanisms, activated APCs trigger an adaptive immune response by presenting peptide antigens to naïve CD4+ T helper cells in secondary lymphoid organs, such as the mesenteric lymph node. Based on their cytokine profile, Th cells can be differentiated into Th1, Th2, Th17 cells and Tregs and consequently produce their respective cytokines. In addition, intestinal B lymphocytes have the ability to turn into plasma cells and produce IgA antibodies, which contribute to immune protection. (Original illustration based on Abraham *et al.*, 2009; Eissa *et al.*, 2019; Karin Allenspach, 2011).

3. CURRENT DIAGNOSTIC CHALLENGES

The diagnosis of CIE requires a complete investigation in order to exclude other possible causes of GI signs (Heilmann & Steiner, 2018; Moser et al., 2018), including infectious, neoplastic, metabolic or endocrine diseases (Moser *et al*., 2018). This includes a detailed medical history, clinical examination, complete blood cell count, serum biochemical analyses, parasitological and bacteriologic fecal analyses, pancreatic function tests, medical imaging, including radiography and abdominal ultrasonography, which give information about intestinal layering and wall thickness. Furthermore, to acquire more specific information on the intestinal inflammation, an endoscopic evaluation with intestinal biopsies and histopathological evaluation, which distinguishes the various subtypes of mucosal infiltration, should be performed (Karin Allenspach, 2015; Cerquetella *et al*., 2010; Moser *et al*., 2018; Wdowiak, Rychlik, & Kołodziejska-Sawerska, 2013). The interpretation of the obtained biopsies contributes to the assessment of the severity and distribution of the disease (Moser et al., 2018).

Clinical signs result from uncontrolled inflammation (Dandrieux, 2016) and generally include abdominal pain, vomiting, diarrhea, anorexia, weight loss, flatulence, bloating (Eissa *et al*., 2019), inappetence and borborygmi (Kalenyak, Isaiah, Heilmann, Suchodolski, & Burgener, 2018). Important tools for clinical evaluation are the canine IBD activity index (CIBDAI) and the canine chronic enteropathy clinical activity index (CCECAI) (Cerquetella *et al*., 2010). The former evaluates six parameters including attitude/activity, appetite, vomiting, stool consistency, stool frequency and weight loss. Each variable is scored from 0 (normal) to 3 (severe change). Based on a cumulative score, it classifies the disease as insignificant (0-3), mild (4-5), moderate (6-8) or severe (≥ 9) (Jergens et al., 2003). The abovementioned clinical signs are also considered in CCECAI, together with albumin concentration, the presence of ascites, peripheral edema and pruritus. Based on a similar scoring pattern, CCECAI classifies the disease as insignificant (0-3), mild (4-5), moderate (6-8), severe (9-11) and very severe (≥12) (Karin Allenspach, Wieland, Grone, & Gaschen, 2007). However, these scoring systems only allow a semi-objective assessment of clinical disease activity (Heilmann *et al*., 2018). Typically, in clinical practice, veterinarians rely mainly on the severity of the clinical signs to estimate the disease severity and the response to treatment (Collins, 2013; Grellet *et al*., 2013). This evaluation is based on partially subjective assessments (Grellet et al., 2013; Hof et al., 2012), and additionally the severity of clinical signs has no proven correlation to the severity of histologic lesions (Heilmann *et al*., 2018; Heilmann, Grellet, *et al*., 2014), hence not reflecting intestinal inflammation (Collins, 2013).

It is worth noting that the endoscopic evaluation of the intestinal mucosa and histopathologic findings are usually not sufficient in differentiating the various forms of CIE (Heilmann & Steiner, 2018). However, these tools remain the golden standard for detecting and quantifying intestinal inflammation (Collins, 2013). Endoscopy procedures are costly, time-consuming (Heilmann, Grellet, *et al*., 2014), influenced by operator experience. Additionally, these are relatively invasive procedures (Otoni *et al*., 2018) that requires general anesthesia, and the preparation of the colon. Histopathological examination of GI inflammation might be considered limited, as clinical outcomes can be influenced by several factors.

Amongst those are the biopsy procedure, number of tissues samples, biopsy sample quality, and the diverse interpretations of GI histopathological findings among pathologists (Karin Allenspach et al., 2018; Day et al., 2008; Simpson & Jergens, 2011; Wdowiak et al., 2013). The World Small Animal Veterinary Association (WSAVA) GI Standardization Group developed a grading scheme about histopathological standards for the characterization of inflammatory and associated morphological abnormalities in the canine and feline GI tract, in order to reduce fluctuations among interpretations (Day *et al*., 2008). However, even with the use of the WSAVA standardization grading scheme, significant interobserver variability in the diagnostic interpretation of endoscopic mucosal specimens still exists (Karin Allenspach, 2015; Karin Allenspach et al., 2018), as well as lack of consensus (Simpson & Jergens, 2011). Another limitation to consider is the fact that improvement of the histopathologic lesions does not always correspond to response to therapy and clinical improvement (Collins, 2013; Heilmann, Volkmann, *et al*., 2016). These procedures are unlikely to be frequently performed (Heilmann, Grellet, *et al*., 2014). Furthermore, there are no currently available systems to accurately assess the degree of active inflammation.

Considering all these constraints , there is a need for a simple, minimally or non-invasive and objective method that evaluates intestinal inflammation (Collins, 2013). Thus, biological markers for clinical indices, that objectively reflect mucosal disease severity, might be useful in clinical practice in diagnosing and managing GI inflammation (Heilmann, Grellet, *et al*., 2014; Otoni *et al*., 2018). It could be considered an attractive option for estimating a diagnosis, prognosis and defining disease severity (Otoni *et al*., 2018; Wdowiak *et al*., 2013).

4. CLINICAL RELEVANCE

According to the European Commission Health Research Directorate (2010), “A biomarker is a biological characteristic, which can be molecular, anatomic, physiologic, or biochemical. These characteristics can be measured and evaluated objectively. They act as indicators of a normal or a pathogenic biological process. A biomarker shows a specific physical trait or a measurable biologically produced change in the body that is linked to a disease or a particular health condition”. In CIE, biomarkers can be very useful tools in identifying the presence of disease, site of origin, evaluating the GI function, determining the progression of the disease, as well as the current response to treatment, and monitoring the severity of GI inflammation.

In the past decade, diverse biomarkers have been evaluated in dogs with CIE. In clinical practice a useful biomarker should have characteristics that make it valuable (Heilmann & Steiner, 2018). In order to have an added value, a biomarker should aim to be measurable without temporal delay in expression or secretion, specific to the disease process (Heilmann, Grellet, *et al*., 2014), easy to perform, have the ability to accurately identify individuals at risk (Jergens & Simpson, 2012), affordable, and minimally

invasive, as well as being stable in routine biological samples (Heilmann & Steiner, 2018). In clinical practice, when using a single biomarker, it is essential to understand that it is improbable to meet all the criteria. Hence, clinical information about a specific biomarker should be taken into account for a better understanding of the data in a specific clinical situation (Heilmann & Steiner, 2018). The biomarkers addressed in this study may be serological, present in urine and feces or even tissue-derived (Wdowiak et al., 2013).

5. BIOMARKERS IN CHRONIC INFLAMMATORY ENTEROPAHIES

5.1. Serological biomarkers

5.1.1. Cobalamin and folate concentrations

Cobalamin (vitamin B12) and folate (vitamin B9) are water-soluble vitamins (Heilmann & Steiner, 2018) of diagnostic and therapeutic importance (Collins, 2013). Most commercial pet foods usually contain cobalamin and folate, thus a dietary deficiency is very uncommon (Berghoff & Steiner, 2011). Some breeds, such as Chinese Shar Peis, Giant Schnanuzers, Border Collies and Beagles, have cobalamin deficiency. Besides genetic predisposition, other causes for cobalamin deficiency in dogs are CIE and EPI (Toresson, Steiner, Suchodolski, & Spillmann, 2016).

Hypocobalaminemia is more likely to occur due to disturbances in its absorptive mechanism. In the diet, cobalamin is bond to animal protein preventing it from being absorbed. In the stomach, protein is partly digested. As a result, cobalamin is released and immediately binds to R binding protein. When entering the small intestine, pancreatic proteases breakdown the R binding protein and the liberated cobalamin has high affinity for the intrinsic factor (IF), which is mainly secreted by the pancreas. After binding to IF, this complex is later absorbed into the ileum, the distal part of the small intestine by specific receptors (Berghoff & Steiner, 2011; Toresson *et al*., 2016).

Several factors can disturb this mechanism, resulting in cobalamin malabsorption. Chronic inflammation of the ileal mucosa can cause reduced expression of the cobalamin-IF receptors in enterocytes (Berghoff & Steiner, 2011). Furthermore, as the main source of IF in dogs is the exocrine pancreas, its condition can also influence the binding nature with IF, and consequently affect cobalamin absorption (Toresson *et al*., 2016). Also, exocrine pancreatic insufficiency (EPI) may inhibit cobalamin dissociation from R-binding proteins (Berghoff & Steiner, 2011), disturbing the absorptive mechanism of cobalamin. Besides distal small intestinal malabsorption, hypocobalaminemia can also occur due to small intestine bacterial overgrowth, as cobalamin coupled with IF can be highly consumed by anaerobic intestinal bacteria (Berghoff & Steiner, 2011; Moser *et al*., 2018; Toresson *et al*., 2016). In accordance, Volkmann *et al*., (2017) identified the most severe decrease in serum cobalamin concentrations in dogs with IBD and EPI. A recent study showed hypocobalaminemia in 30% of dogs diagnosed with CIE (Heilmann *et al*., 2018). This condition is a negative prognostic factor in dogs with CIE, and can result in severe metabolic consequences (Toresson *et al*., 2016) and increased risk of euthanasia (Karin Allenspach,

2015).

Hypocobalaminemia is not specific for CIE (Heilmann & Steiner, 2018). In accordance, no significant differences were observed in serum cobalamin levels between dogs diagnosed with ARE, and those not responding to antibiotic treatment, or with other causes of chronic GI signs (German *et al*., 2003). Therefore, the measurement of serum cobalamin concentrations is not sufficient to differentiate the various forms of CIE, as demonstrated by Allenspach, Culverwell & Chan (2016). However, a normal serum cobalamin concentration does not exclude a CIE diagnosis (Heilmann & Steiner, 2018), since the patient's body stores of cobalamin might still be sufficient to maintain a normal serum cobalamin concentration, despite the malabsorption. Concentrations under the reference range require supplementation that should only be dropped if the underlying condition is fully resolved and when the patient's cobalamin concentrations is within the normal range values (Berghoff & Steiner, 2011).

Similar to cobalamin, alterations in serum folate concentrations are more likely to occur due to a reduced absorption, or alterations in intestinal microbiota (Berghoff & Steiner, 2011). Contrarily to cobalamin, folate is principally absorbed in the proximal part of the small intestine, the duodenum and proximal jejune (Heilmann & Steiner, 2018). Dietary folate, present in the form of folate polyglutamate is hydrolyzed by folate conjugase, an enzyme produced by the jejunal brush border (Berghoff & Steiner, 2011). Folate is then absorbed into the proximal part of the small intestine in the form of folate monoglutamate, by specific folate carriers (Heilmann & Steiner, 2018). When the proximal small intestinal mucosa is damaged, malabsorption of folate can occur due to an impaired folate conjugase activity, making folate unabsorbable, or even due to damaged folate carriers. Thus, hypofolatemia can occur if the condition has become chronic and the folate body stores have become depleted. Furthermore, due to dysbiosis in the small intestine, some intestinal bacteria can increase its folate production, becoming available for absorption by the host, resulting into a false, normal or higher serum concentrations (Berghoff & Steiner, 2011).

In a study performed by Heilmann *et al*., (2018), hypofolatemia was shown in 14% and hyperfolatemia in 5% of dogs diagnosed with CIE. Yet, even though hypofolatemia can result from chronic malabsorption in dogs with CIE, it is not specific for this condition. Researchers observed no significant differences in serum folate concentrations in dogs with CIE responsive to diet, antibiotic or immunosuppressive treatment (Allenspach *et al*., 2016). Moreover, German *et al*., (2003) observed insignificant differences in serum folate concentrations between dogs diagnosed with ARE, and those not responding to antibiotic treatment or with other causes of chronic GI signs. Moreover, a normal serum folate concentration does not rule out a CIE diagnosis (Heilmann & Steiner, 2018).

Folate and cobalamin serum concentrations have been reported as nonspecific findings, nevertheless, its supplementation is important during treatment (Cerquetella *et al*., 2010).

5.1.2. C-reactive protein

C-reactive protein (CRP) is a positive type II acute phase protein of the pentraxin family. It is produced in the liver as a response to IL-6, IL-1β and tumor necrosis factor alpha (Jergens *et al*., 2009) during periods of infection, inflammation, or cancer (Heilmann *et al*., 2018).

Researchers showed increased CRP concentrations in dogs with idiopathic IBD, when compared to healthy dogs, and diseased dogs after treatment (Otoni *et al*., 2018). Authors also noted a lack of correlation with clinical severity, as determined by CIBDAI, as well as with histopathologic lesions. Interestingly, in a larger study, it was reported a significant correlation between serum CRP concentrations and clinical severity, as determined by CIBDAI (Jergens *et al*., 2010).

Despite being considered a sensitive indicator of inflammation (Otoni *et al*., 2018), increased levels should be interpreted with caution (Karin Allenspach, 2015) since CRP is not specific for the intestinal tract (Berghoff & Steiner, 2011) and can be increased in other diseases (Karin Allenspach, 2015). Thus, this biomarker has a limited utility as a diagnostic biomarker for CIE in dogs due to its high biological variability (Heilmann *et al*., 2018); Notwithstanding, it is valuable in other aspects such as monitoring treatment response and disease progression (Collins, 2013; Heilmann & Steiner, 2018).

5.1.3. Perinuclear anti-neutrophilic cytoplasmic antibodies

Perinuclear anti-neutrophilic cytoplasmic antibodies (pANCA) are serum autoantibodies directed towards neutrophil granule components (Heilmann & Steiner, 2018; Mancho *et al*., 2010), including nuclear histone, proteinase 3, myeloperoxidase (Heilmann & Steiner, 2018), lactoferrin, elastase and lysozyme (Wdowiak *et al*., 2013). These anti-neutrophilic cytoplasmic antibodies can be detected by indirect immunofluorescence methods (Heilmann & Steiner, 2018; Mancho *et al*., 2010), through the visualization of a typical perinuclear staining pattern (Karin Allenspach, 2015).

In general, canine antibodies might cross-react with diverse antigens, possibly resulting in the development of an autoimmune reaction that could be on the basis of chronic inflammation in dogs with IBD (Mancho, Sainz, García-sancho, Villaescusa, & Rodríguez-franco, 2011). Thus, the detection of this biomarker has been proposed as a complementary tool to help in differentiating dogs with IBD from dogs with other chronic GI diseases (Mancho *et al*., 2011).The seropositivity of pANCA can also be detected in other infectious, inflammatory, autoimmune or oncologic disorders, as it is non-CIE specific (Karin Allenspach, 2015; Heilmann & Steiner, 2018)**.** In accordance, (Mancho *et al*., 2011) found no significant differences in pANCA expression between dogs with IBD and dogs with intestinal lymphoma.

With regards to intestinal inflammation, pANCA has not shown significant utility (Otoni *et al*., 2018). Furthermore, researchers failed to correlate pANCA seropositivity with CIBDAI and histopathologic scores (Otoni *et al*., 2018). Other authors notified the importance of pANCA in differentiating dogs with

FRE and IBD (Karin Allenspach, 2015). Dogs responding to food trials, yielded more positive results, as cited in Otoni *et al*., (2018).

Although pANCA's utility has been considered limited, it might be useful in differentiating various forms of CIE, as cited in Otoni *et al*., (2018). Future studies should be performed in order to evaluate the potential of pANCA as a useful biomarker in canine CIE.

5.1.4. Citrulline

Citrulline is a non-dietary amino acid produced and released by the enterocytes of the small intestinal mucosa (Gerou-Ferriani *et al*., 2018; Xu *et al*., 2016). Citrulline is not available in food, therefore its serum concentration depends exclusively on the production by small intestine mucosa enterocytes. In concurrence, a reduced serum concentration corresponds to a reduced enterocyte mass and absorptive function (Gerou-Ferriani *et al*., 2018). The authors suggested that citrulline has the potential to be an effective biomarker in chronic intestinal diseases in dogs. However, this study failed to show differences in serum citrulline concentrations between dogs with CIE and healthy dogs, as well as among dogs with different forms of CIE (Gerou-Ferriani et al., 2018). Furthermore, researchers observed failure not only in predicting treatment response, but also in correlating citrulline concentrations with disease severity, as determined by CIBDAI (Gerou-Ferriani et al., 2018). These unexpected findings could be explained by an insufficiently damaged enterocyte, which in turn would make a reduction in serum citrulline concentration undetectable (Gerou-Ferriani *et al*., 2018). Conversely, others observed, in a smaller study, a lower concentration of plasma citrulline in dogs diagnosed with IBD (Xu *et al*., 2016). Moreover, Rossi *et al*., (2014) reported a significant increase in plasma citrulline concentrations in dogs treated with a multi-strain probiotic, suggesting the restoration of mucosal barrier. Thus, more studies are required in order to accurately evaluate the potential use of citrulline as a biomarker in CIE in dogs.

5.1.5. Soluble receptor for advanced glycation end products

The receptor of advanced glycation end products (RAGE) is a multi-ligand PRR (Heilmann, Otoni, et al., 2014), which is implicated in dogs with CIE (Heilmann & Allenspach, 2017). Signaling pathways of RAGE, leads to the activation of several kinases, including the activation and nuclear translocation of NF-Kb. As a consequence of this activation, inflammatory cells are recruited and a proinflammatory microenvironment is installed. Soluble RAGE (sRAGE) is a truncated variant of RAGE. sRAGE functions as an anti-inflammatory decoy receptor, that can sequester RAGE ligands, preventing their interaction with cell surface RAGE. Thus, sRAGE has the capacity to modulate and abolish cell signaling, nullifying the proinflammatory effect of ligands for this receptor (Heilmann & Allenspach, 2017; Heilmann & Steiner, 2018).

Dogs with CIE have significantly decreased serum sRAGE concentrations (Heilmann & Steiner, 2018), as specifically demonstrated in dogs with IBD (Heilmann, Otoni, *et al*., 2014). However, in this study, researchers could not determine whether this decrease in sRAGE concentrations was due to its consumption or decreased production. The lower concentrations of circulating sRAGE, functioning as a decoy receptor, might permit the ligand-RAGE binding, leading to the activation of RAGE pathways and consequently, potentiating the inflammatory response. In addition, sRAGE concentrations were not correlated with CIBDAI nor histopathologic disease scores. However, only esophagogastroduodenoscopies were performed and lesions in the ileum or colon could have been unintentionally disregarded, which could be considered as a limitation of this study (Heilmann, Otoni, *et al*., 2014).

(Heilmann & Steiner, 2018) have reported the potential of serum sRAGE concentration to assess response to treatment in dogs with CIE, since serum sRAGE concentrations only stabilized in dogs after achieving complete clinical remission (Heilmann, Otoni, *et al*., 2014).

Assuming that sRAGE has the capacity to downregulate the proinflammatory response mediated by RAGE, a possible therapeutic strategy for dogs with CIE using this anti-inflammatory receptor should be further investigated (Heilmann, Otoni, *et al*., 2014).

5.1.6. Metabolite profile

Oxidative stress is hypothesized to play a role in the pathogenesis of IBD, resulting from a significant disproportion between the production of reactive oxygen species (ROS) and their elimination by antioxidants (Rubio *et al*., 2017).

Rubio and collaborators have analyzed a profile of several serum biomarkers of oxidative stress in dogs with idiopathic IBD and compared it to healthy dogs (Rubio *et al*., 2017). In order to determine the antioxidant response, biomarkers of total antioxidant status, such as Trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC) and ferric reducing ability of the plasma (FRAP) were evaluated. In addition, individual antioxidant biomarkers were analyzed, including total thiol concentrations and paraoxonase 1 (PON1) activity. Moreover, biomarkers of oxidant status, such as ferrous oxidation-xylenol orange (FOX), thiobarbituric acid reactive substances (TBARS), and ROS production concentrations were measured in order to evaluate oxidative damage. Results showed a significant reduction in TEAC, CUPRAC, thiol and PON1 in dogs with IBD, in comparison with healthy dogs, revealing a decrease in the antioxidant response. Interestingly, no alteration was detected in serum FRAP. This finding might be explained as FRAP may vary according to its individual antioxidants contributors, unlike the other total antioxidant capacity assays. Hence, it is recommended the use of different methods to determine the total antioxidant status. Moreover, TBARS, FOX and ROS levels were increased, suggesting the presence of an elevated oxidative stress status in canine IBD. The

authors referred that this intense and permanent oxidative stress, could lead to the decrease of the antioxidant resources, surpassing the body production capacity. Therefore, the decreased antioxidant response observed could be justified. In addition, the authors suggested that the lymphocytes and plasma cells present in the inflamed intestinal mucosa of dogs with IBD, might be a source of systemic ROS production (Rubio *et al*., 2017).

Future studies should be performed, in order to assess the potential of this metabolite profile in evaluating the oxidative stress response in dogs with IBD.

Table 1 – Summary of the results obtained in clinical trials evaluating serum biomarkers in dogs with chronic inflammatory enteropathies.

SERUM BIOMARKERS					
Biomarkers	**Group comparison**	**Biological Sample**	**Results**	**Correlations**	**Reference**
Cobalamin	132 dogs with CD	Serum	Severe hypocobalaminemia in dogs with IBD and EPI	Significant correlation with poor clinical outcome	Volkmann *et al*., 2017
	29 dogs with chronic GI signs vs. 38 healthy dogs	Serum	No significant differences in dogs diagnosed with ARE and those not responding to antibiotics	-	German *et al*., 2003
	203 dogs with CIE	Serum	No significant differences in dogs diagnosed with ARE, FRE and IRE	-	Allenspach *et al*., 2016
Folate	29 dogs with chronic GI signs vs. 38 healthy dogs	Serum	No significant differences in dogs diagnosed with ARE and those not responding to antibiotics	-	German *et al*., 2003
	203 dogs with CIE	Serum	No significant differences in dogs diagnosed with ARE, FRE and IRE	-	Allenspach *et al*., 2016
CRP	16 dogs with IBD vs. 13 healthy dogs	Serum	Higher concentrations in dogs with IBD	No correlation with CIBDAI or histopathological severity	Otoni *et al*. , 2018
	54 dogs with IBD	Serum	Lower concentrations after treatment	Significant correlation with CIBDAI	Jergens *et al*., 2010
p-ANCA	124 dogs with IBD vs. 23 dogs with intestinal lymphoma	Serum	No significant change		Mancho *et al*., 2011
	16 dogs with IBD vs. 13 healthy dogs	Serum	No significant change	No correlation with CIBDAI or histopathological severity	Otoni *et al*. , 2018
Citrulline	74 dogs with CIE vs. 83 dogs healthy dogs	Serum	No significant change	No correlation with CIBDAI	Gerou-Ferriani *et al*., 2018
	23 dogs with IBD vs. 10 healthy dogs	Plasma	Lower concentrations in dogs with IBD		Xu *et al*., 2016
	20 dogs with IBD vs. 10 healthy dogs	Plasma	Higher concentrations in dogs treated with a multistrain probiotic		Rossi *et al*., 2014
sRAGE	20 dogs with IBD vs. 15 healthy dogs	Serum	Lower concentrations in dogs with IBD	No correlation with CIBDAI or histopathologic scores	Heilmann, Otoni *et al*., 2014

TEAC	18 dogs with IBD vs. 20 healthy dogs	Serum	Lower concentrations in dogs with IBD		Rubio *et al*., 2017
CUPRAC			Lower concentrations in dogs with IBD		
Thiol			Lower concentrations in dogs with IBD		
PON1			Lower concentrations in dogs with IBD		
FRAP			No change		
FOX			Higher concentrations in dogs with IBD		
TBARS			Higher concentrations in dogs with IBD		
ROS			Higher concentrations in dogs with IBD		

ABBREVIATIONS:

ARE – Antibiotic-responsive enteropathy

CD – Chronic diarrhea

CIBDAI – Canine inflammatory bowel disease activity index

CIE – Chronic inflammatory enteropathies

CRP – C reactive protein

CUPRAC – Cupric reducing antioxidant capacity

FOX – Ferrous oxidation-xylenol orange

FRAP – Ferric reducing ability of the plasma

FRE – Food-responsive enteropathy

GI – Gastrointestinal

IBD – Inflammatory bowel disease

pANCA – Perinuclear anti-neutrophilic cytoplasmic antibodies

PON1 – Paraoxonase 1

ROS – Reactive oxygen species

sRAGE – Soluble receptor for advanced glycation end products

TBARS – Thiobarbituric acid reactive substances

TEAC – Trolox equivalent antioxidant capacity

2

5.2. Fecal and urinary biomarkers

5.2.1 Alpha 1-proteinase inhibitor

Alpha1-proteinase inhibitor (α1PI) is a major proteinase inhibitor that is synthesized in the liver (Heilmann & Steiner, 2018), macrophages and in the intestinal mucosa (Wdowiak *et al*., 2013). This plasma protein has a similar weight to albumin and both can be lost from the intersticium to the GI lumen at the same rate in PLE. However, unlike albumin, α1PI is not affected by proteolysis (Heilmann & Steiner, 2018), and it is able to persist unaltered throughout the intestinal tract, allowing its extraction and measurement from fecal samples (Cerquetella *et al*., 2010; Heilmann & Steiner, 2018; Wdowiak *et al*., 2013). Elevated fecal canine α1PI concentrations are clinically useful as a marker of GI protein loss, at the same time being a risk factor for negative outcome in CIE (Berghoff & Steiner, 2011).

This biomarker has shown to be correlated with histopathologic lesions seen in dogs with PLE, such as lacteal dilatation and/or crypt abscesses (Heilmann, Parnell, *et al*., 2016). In accordance, Murphy *et al*., (2003) observed higher concentrations of fecal α1PI in dogs with GI disorders showing histologic abnormalities, such as IBD and lymphangiectasia. Furthermore, the authors did not correlate fecal α1PI concentrations with serum albumin concentrations. This finding could be explained based on the assumption that hypoalbuminemia would only develop if protein loss were severe enough to exceed hepatic production capacity. Hence, the assessment of fecal α1PI concentrations may identify patients that have ongoing intestinal protein loss, before the occurrence of clinical signs (Berghoff & Steiner, 2011), making this biomarker principally useful in detecting protein loss during early stages of disease (Murphy *et al*., 2003). Furthermore, it might also be useful to differentiate hepatic causes from GI protein loss (Heilmann & Steiner, 2018).

Fecal α1PI concentrations may vary significantly from one day to another. Ideally, fecal samples should be collected on three consecutive days and a three-day mean should be taken in order to improve test accuracy (Karin Allenspach, 2015; Heilmann & Steiner, 2018). Despite its high sensitivity, measurement of fecal α1PI is not considered a specific biomarker for this condition, as GI protein loss can be associated with several other GI systemic disorders, however, it may have a role in monitoring disease progression and treatment response (Collins, 2013).

5.2.2. Immunoglobulin A

In mucosal lymphoid tissues, IgA is largely produced. This antibody, (Maeda *et al*., 2013) is secreted in the intestinal mucosa, mainly in its dimeric form (Karin Allenspach, 2011) and plays a crucial role in maintaining intestinal homeostasis (Maeda *et al*., 2013). IgA provides a first line of immune protection at mucosal surfaces (Maeda *et al*., 2013), by preventing commensal and dietary antigens from triggering an immune response (Lee *et al*., 2015). The interaction between commensals and the cells of the intestinal immune system is critical to the secretion of IgA, as it promotes B lymphocytes to switch its

class to IgA-producing plasma cells (Lee *et al*., 2015), via T cell dependent and independent mechanisms (Eissa *et al*., 2019). Even though IgA may be detected in different biological samples, this biomarker will be addressed in this section, since its fecal concentrations reflect more accurately the inflammation degree, in comparison with serum concentrations (Heilmann & Steiner, 2018; Maeda et al., 2013).

German shepherd dogs are known for their predisposition to polymorphisms in the gene encoding TLR-5. In addition, this breed is known for its abnormal IgA production (Lee *et al*., 2015; Maeda *et al*., 2013), being highly susceptible to CIE (Jergens & Simpson, 2012). However, Lee *et al*., (2015) failed to correlate IgA-positive plasma cells in the mucosa of dogs with CIE with single nucleotide polymorphisms in the gene encoding TLR-5.

The involvement of IgA in the pathogenesis mechanism remains unclear (Maeda *et al*., 2013), however it has been considered whether an impaired function of IgA-producing plasma cells and decreased fecal IgA concentrations could be a cause or a consequence of the disease (Heilmann & Steiner, 2018). Studies have observed decreased concentrations of IgA in the duodenum and feces of dogs with IBD, without observing any change in serum IgA concentrations (Maeda *et al*., 2013). In addition, researchers failed to correlate fecal IgA concentration with clinical severity.

Despite detecting decreased fecal IgA concentrations in dogs with CIE, available data is inconclusive about its clinical utility as biomarkers (Heilmann & Steiner, 2018).

5.2.3. 3-Bromotyrosine

Eosinophils are typically present in low numbers in the intestinal mucosa of healthy dogs. Eosinophilic enteritis, the second most commonly diagnosed form of IBD in dogs, is characterized by a mixed infiltration of inflammatory cells, predominantly eosinophils. During inflammatory processes, eosinophils are activated and migrate to the villi, where degranulation occurs. Eosinophils release various highly cytotoxic granule proteins, such as eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin, and major basic protein, resulting in tissue damage and dysfunction (Bastan *et al*., 2017). Bromotyrosine (3-BrY) is the stable metabolite of eosinophil peroxidase, a potent granular cytotoxic heme-protein (Panpicha Sattasathuchana *et al*., 2015). 3-BrY concentrations reflect eosinophilic inflammation, according to the eosinophilic component that can be present in the cellular infiltrate of the lamina propria (Heilmann & Steiner, 2018). This biomarker can be detected in different biological samples. Nevertheless, the focus of this analysis is on the feces, as reports have evidenced that the assessment of 3-BrY concentrations in fecal samples reflect the level of eosinophil activation in the GI tract more accurately than serum biomarkers (Sattasathuchana et al., 2019).

A recent study revealed higher concentrations of fecal 3-BrY concentrations in dogs with CIE, however,

sensitivity and specificity to differentiate between the different forms of CIE are yet to be determined (Sattasathuchana, Thengchaisri, Suchodolski, Lidbury, & Steiner, 2019). Sattasathuchana and colleagues assessed 3-BrY concentrations in the serum of dogs with CIE (Sattasathuchana, Allenspach, Lopes, Suchodolski, & Steiner, 2017; Panpicha Sattasathuchana et al., 2015). Researchers showed increased serum 3-BrY concentrations in dogs with eosinophilic gastroenteritis, as well as in dogs with lymphocytic-plasmacytic enteritis (Sattasathuchana *et al*., 2015). These finding highlights the pathophysiological role of eosinophil activation in dogs with CIE that have these types of cellular infiltrates. Later, the same authors observed increased serum 3-BrY concentrations in dogs with FRE and IRE in comparison with healthy dogs. Additionally, higher concentrations were noted in IRE dogs, suggesting an increased severity of inflammation in dogs with this form of CIE. No significant correlation was established with clinical severity, as determined by CCECAI (Sattasathuchana, Allenspach, Lopes, Suchodolski, & Steiner, 2017). Researchers proposed that the simultaneous use of these two independent predictors might improve diagnosis specificity and prediction of the most likely outcome.

The clinical utility of 3-BrY should be further investigated before the use of this biomarker can be recommended to the clinical practice (Heilmann & Steiner, 2018).

5.2.4. N-methylhistamine

In canine CIE, mast cells participate in inflammatory processes through the release of multiple inflammatory mediators in the intestinal mucosa, such as histamine (Wdowiak *et al*., 2013). Serum histamine concentrations may directly reflect the degree of mast cell activation, however it is not usually measured due to its short half-life (Anfinsen *et al*., 2014). A stable metabolite of histamine, N-methylhistamine (NMH) has been suggested as a proinflammatory marker of mast cell degranulation and GI inflammation (Berghoff & Steiner, 2011; Heilmann & Steiner, 2018), as it can more accurately reflect the overall mast cell activity (Anfinsen *et al*., 2014). NMH is generated via the histamine N-methyltransferase enzyme system (Berghoff *et al*., 2014) and can be readily measured from urine and fecal specimens (Anfinsen *et al*., 2014; Berghoff *et al*., 2014).

Studies failed to establish a correlation between fecal and urinary NMH concentrations and clinical severity, as well as with the degree of mast cell infiltration (Anfinsen *et al*., 2014; Berghoff *et al*., 2014). However, higher quantities of intestinal mast cells were observed in dogs responding to dietary trials, when compared to those requiring immunosuppressive treatment, suggesting that mast cell activation might be implicated in the pathogenesis of FRE (Anfinsen *et al*., 2014). Unlike the previous study, (Berghoff *et al*., 2014) observed a significant correlation between urinary NMH concentrations and the histological grade of inflammation, implying that urinary NMH concentrations might be a more accurate indicator of disease severity when compared to fecal concentrations.

NMH has been reported as a potentially useful biomarker in clinical practice, however, more studies are

needed in order to determine its sensitivity and specificity (Heilmann & Steiner, 2018).

5.2.5. Leukotriene E4

Leukotriene E4 (LTE4) is a proinflammatory product derived from 5-lipoxygenase that contributes to the inflammatory response by increasing vessel permeability, chemotaxis and mucous secretion in the colonic mucosa (Dumusc *et al*., 2014; Hof *et al*., 2012; Wdowiak *et al*., 2013). This metabolite of cysteinyl leukotriene enzymatic pathway is obtainable in urine samples. The collection of multiple samples in short periods is recommended, in order to more accurately estimate de LTE4 production (Hof *et al*., 2012).

Researchers showed no significant difference in urinary LTE4 concentrations in dogs with IBD, compared to dogs diagnosed with FRE. Yet, both groups presented significantly higher concentrations in comparison with healthy dogs. These findings might indicate how important the contribution of cysteinyl leukotriene pathway activation can be to the intestinal inflammation. Hence, the potential use of 5-lipoxygenase inhibitors or leukotriene receptor antagonists for therapeutic interventions should be further investigated. Moreover, LTE4 concentrations were not correlated with clinical severity, as determined by CIBDAI (Hof *et al*., 2012).

The levels of LTE4 may have potential to be used as biomarkers in canine CIE. Yet, future studies with a larger number of dogs are required in order to support the existing data (Hof *et al*., 2012).

5.2.6. Calprotectin

Calprotectin, also referred as S100A8/A9, is a heterodimeric protein complex (Collins, 2013) that belongs to the S100/ calgranulin family of damage-associated molecular pattern (DAMP) molecules (Heilmann *et al*., 2018; Heilmann & Steiner, 2018). Activated macrophages and neutrophils express and release calprotectin in the extracellular space (Heilmann & Steiner, 2018). However, this calcium- and zinc-binding protein (Celi *et al*., 2019) can also be induced in epithelial cells (Heilmann & Steiner, 2018). Calprotectin is a ligand for TLR-4 (Heilmann *et al*., 2019), which is upregulated in dogs with idiopathic IBD, as reported by Heilmann *et al*., (2012). Thus, it is speculated that calprotectin might be involved in the expression of proinflammatory cytokines and chemokines (Heilmann & Steiner, 2018), playing a role in acute and chronic inflammation (Heilmann *et al*., 2019). Even though calprotectin can be measured in different biological samples, it will be addressed in this section since fecal concentrations of this biomarker are reported as more specific for detecting gastrointestinal inflammatory processes (Heilmann & Steiner, 2018).

Calprotectin is considered stable in natural feces, thus allowing a simple collection in the dog's home environment (Otoni *et al*., 2018). One study revealed increased concentrations of fecal calprotectin in

dogs with IBD, at the time of diagnosis, which decreased significantly after treatment (Otoni *et al*., 2018). Accordingly, Grellet *et al*., (2013) observed higher fecal calprotectin concentrations in dogs with chronic diarrhea. Furthermore, fecal calprotectin concentrations have been positively correlated with clinical and histopathologic severity (Grellet *et al*., 2013; Heilmann *et al*., 2018; Otoni *et al*., 2018). When evaluating the histopathologic lesions, researchers noted a correlation between fecal calprotectin concentration and lymphocytes in the ileal lamina propria, rather than a correlation with intestinal lamina propria neutrophils and macrophages. This finding could be explained, as calprotectin expression might reflect the activity of these cells, instead of their quantity (Heilmann *et al*., 2018). Moreover, this biomarker appears to have potential in differentiating the various forms of CIE, with dogs responsive to immunosuppressive treatment, showing higher concentrations (Heilmann *et al*., 2018). However, assessing fecal calprotectin in combination with serum CRP and CCECAI score, was demonstrated to increase the ability to differentiate between these conditions (Heilmann *et al*., 2018). In addition, this biomarker seems to be clinically useful in predicting response to treatment in dogs with CIE (Heilmann & Steiner, 2018) as it is specific for the GI tract (Heilmann *et al*., 2018). Furthermore, the authors also verified that fecal calprotectin concentrations did not correlate with serum CRP concentrations, suggesting that the intestinal inflammation in dogs with CIE is not related to the systemic inflammatory response (Heilmann *et al*., 2018). This biomarker appears to be useful for the noninvasive evaluation of intestinal inflammation, because of its potential to monitor disease intensity and detect both active and inactive periods of the disease (Otoni *et al*., 2018).

Calprotectin can also be detected in the serum. Calprotectin concentrations are reported be increased in the serum of dogs with CIE, however it is not specific for the GI tract (Heilmann & Steiner, 2018). Researchers documented an increase in serum calprotectin concentrations in dogs with idiopathic IBD, compared with healthy dogs (Heilmann *et al*., 2012). Contrarily, Otoni *et al*., (2018) showed no differences in serum calprotectin concentrations between healthy dogs and those with idiopathic IBD, nor in dogs before and after treatment. Additionally, both studies failed to correlate serum calprotectin concentrations with CIBDAI scores, as well as with histopathologic severity. Serum calprotectin appears to be useful in detecting inflammation, however the inability to identify the exact inflamed organ limits its clinical utility (Heilmann *et al*., 2012).

Calprotectin has been considered a biomarker with a great potential to be used in dogs with CIE, particularly in monitoring GI inflammation (Celi et al., 2019; Heilmann et al., 2018).

5.2.7 Calgranulin C

Calgranulin C, also referred as S100A12, is an endogenous DAMP, involved in the phagocyte activation (Heilmann, Otoni, *et al*., 2014). This calcium binding protein (Wdowiak *et al*., 2013) is principally expressed and secreted by activated neutrophils, macrophages and monocytes (Hanifeh *et al*., 2018) and has a significant role in inflammatory immune responses (Heilmann & Steiner, 2018). After being

released in the extracellular space, (Hanifeh *et al*., 2018) it works as a ligand for RAGE (Heilmann & Steiner, 2018; Heilmann, Volkmann, *et al*., 2016). Binding to this PRR can trigger signaling pathways that lead to the activation of NF-kb, resulting in the production of proinflammatory cytokines and chemokines. Also, a positive feedback on the expression of transmembrane RAGE itself, leads to the perpetuation and amplification of the inflammatory response and consequently to tissue damage (Hanifeh *et al*., 2018; Heilmann, Grellet, *et al*., 2014).

Calgranulin C is a quite sensitive and specific biomarker for localized inflammatory disorders, such as GI inflammation. This biomarker might be detected in different biological samples. However, concentrations in serum might also be increased in other inflammatory disorders. Thus, this biomarker will be addressed in this section, since its fecal concentrations are more specific for detecting gastrointestinal inflammatory processes (Heilmann & Steiner, 2018).

Calgranulin C can be detected in fecal samples. Higher concentrations of fecal calgranulin C have been detected in dogs with IBD (Heilmann, Grellet, *et al*., 2014), as well as in dogs with CIE in general (Hanifeh *et al*., 2018). This finding indicates an increased infiltration of phagocytes, supporting the idea that phagocyte activation plays a role in the pathogenesis of the disease (Heilmann, Grellet, *et al*., 2014). Furthermore, studies have been demonstrating a significant correlation between fecal calgranulin C concentrations and clinical severity (Heilmann, Grellet, *et al*., 2014), endoscopic lesions (Heilmann *et al*., 2018; Heilmann, Grellet, *et al*., 2014), as well as histopathologic alterations in the colon, but not with the severity of histopathologic lesions overall (Heilmann, Grellet, *et al*., 2014). Additionally, fecal calgranulin C concentrations have been correlated with a negative outcome (Hanifeh *et al*., 2018). Moreover, researchers showed the potential of fecal calgranulin C concentrations in distinguishing dogs with CIE that are more likely to respond to dietary trials, antibiotic treatment or immunosuppressive therapy (Heilmann, Volkmann, *et al*., 2016). Results also indicated the utility of this biomarker in predicting the lack of response to treatment in dogs with CIE, suggesting its prognostic value.

A recent study reported increased levels of calgranulin C in the intestinal mucosa of dogs with CIE. (Hanifeh *et al*., 2018). The authors established a significant correlation between colonic mucosal calgranulin C concentrations and the severity of epithelial injury. Furthermore, they also associated increased mucosal calgranulin C concentrations with the presence of macrophages or neutrophil inflammatory infiltrate components.

Calprotectin has been considered a highly attractive biomarker to be used in canine CIE (Heilmann *et al*., 2018). The measurement of calgranulin C in fecal samples, as a non-invasive test is reported to be particularly advantageous for monitoring GI inflammation (Celi *et al*., 2019).

5.2.8. Intestinal alkaline phosphatase

Intestinal alkaline phosphatase (IAP) is an isoenzyme of alkaline phosphatase that contributes positively to the maintenance of homeostatic conditions of the intestinal flora (Ide *et al*., 2016). It is expressed in high quantities in the duodenum, mainly in villus enterocytes, having a gradual decline throughout the rest of the intestinal tract (Celi et al., 2019). During digestion IAP is not dissolved, thus fecal concentrations reflect the original expression in epithelial cells (Ide *et al*., 2016). This biomarker has been reported as an indicator of mature enterocytes (Celi et al., 2019).

Important functions carried out by IAP includes pH modulation, assimilation of organophosphorus acid and fat absorption into the intestinal tract. In the intestinal mucosa, IAP has an important role of protection, as it is capable of lipopolysaccharide dephosphorylation. This is a component of the outer cell membrane of gram-negative bacteria, which are overrepresented in dogs with CIE. In this way, dogs with CIE have, simultaneously, a significant number of intestinal lipopolysaccharides and a defective capacity to neutralize them. IAP neutralizes bacteria endotoxic properties and protects the intestinal mucosa from the detrimental effects of endotoxins; abnormalities in both IAP expression and function may alter the lipopolysaccharide, and result in endotoxin-induced inflammation or in an abnormal response against the intestinal flora (Ide *et al*., 2016).

A significant decrease in IAP's expression have been documented in the duodenal mucosa of dogs with CIE, particularly in those with moderate and severe disease (Ide *et al*., 2016). Researchers hypothesized that a decrease in IAP production might be either a cause or a consequence of the intestinal inflammation by increasing the intestinal mucosa exposure to active endotoxins. Additionally, researchers noted an increased expression of IAP in the duodenum, compared with the colon in affected dogs (Ide *et al*., 2016).

Future studies need to be carried out in order to investigate the role of IAP in canine CIE pathogenesis (Ide *et al*., 2016), as well as to assess its potential as a biomarker (Heilmann & Steiner, 2018).

233 **Table 2 – Summary of the results obtained in clinical trials evaluating fecal and urinary biomarkers in dogs with chronic inflammatory enteropathies.**

URINARY AND FECAL BIOMARKERS					
Biomarkers	**Group comparison**	**Biological sample**	**Results**	**Correlations**	**Reference**
α1-PI	21 healthy dogs vs. 16 dogs with GI disorders	Feces	Higher concentrations in dogs with histologic abnormalities	No significant correlation with serum albumin concentration	Murphy *et al*., 2003
	120 dogs undergoing GI tissue biopsies		Higher concentrations in dogs with crypt abscesses and/or lacteal dilation	Moderate correlation with albumin	Heilmann *et al*., 2016
IgA	37 dogs with chronic GI signs vs. 20 healthy dogs	Feces	Lower concentrations in dogs with IBD	-	Meda *et al*., 2013
3-BrY	40 dogs with CIE vs. 40 healthy dogs	Feces	Higher concentrations in dogs with CIE	-	Sattasathuchana *et al*., 2019
	27 dogs with EGE, 25 dogs with LPE, 26 dogs with EPI, 27 dogs with pancreatitis vs. 52 healthy dogs	Serum	Higher concentrations in dogs with EGE and LPE	-	Sattasathuchana et al., 2015
	38 dogs with FRE, 14 dogs with IRE and 46 healthy dogs	Serum	Higher concentrations in dogs with IRE, followed by dogs with FRE	No correlation with peripheral eosinophil counts or CCECAI	Sattasathuchana *et al*., 2017
NMH	28 dogs with CIE vs. 55 healthy dogs	Urine	No significant change	No correlation with CCECAI, histopathologic severity, or degree of mast cell infiltration	Ansifen *et al*., 2014
	28 dogs with CIE vs. 55 healthy dogs	Feces	No significant change	No correlation with CCECAI, histopathologic severity, or degree of mast cell infiltration	Ansifen *et al*., 2014
	16 dogs with CIE vs 49 healthy dogs	Feces	Higher 3-day maximum concentrations in dogs with CIE	Correlated with histopathologic severity; No correlation with CCECAI or mast cell infiltration degree	Berghoff *et al*., 2014
		Urine	No significant change	Correlated with histopathologic severity; No correlation with CCECAI or mast cell infiltration degree	
LTE4	37 dogs with CIE vs. 23 healthy dogs	Urine	Higher concentrations in dogs with IBD, followed by dogs with FRE	No correlation with CIBDAI	Hof *et al*., 2012

Calprotectin	27 dogs with CD vs. 69 healthy dogs	Feces	Higher concentrations in dogs with CD	Correlated with CCECAI and histopathologic severity	Grellet *et al*., 2013
	16 dogs with IBD vs. 13 healthy dogs	Feces	Higher concentrations in dogs with IBD	Correlation with CIBDAI and residually correlated with histopathological severity	Otoni *et al*., 2018
	127 dogs with CIE	Feces	Higher concentrations in dogs with IRE	Correlated with CCECAI and histopathologic inflammatory lesions	Heilmann *et al*., 2018
	34 dogs with idiopathic IBD vs. 139 healthy dogs	Serum	Higher concentrations in dogs with IBD	No correlation with CIBDAI,CRP or histopathological severity	Heilmann *et al*., 2012
	16 dogs with idiopathic IBD vs. 13 healthy dogs	Serum	No significant change	No correlation with CIBDAI or histopathological severity	Otoni *et al*. , 2018
Calgranulin C	26 dogs with IBD vs. 90 healthy	Feces	Higher concentrations in dogs with IBD	Correlated with CCECAI, endoscopic severity in the duodenum and colon, and histopathologic lesions in the colon	Heilmann, Grellet, *et al*., 2014
	40 dogs with CIE vs. 18 healthy dogs	Duodenum, ileum, colon and cecum mucosas	Higher concentrations in the duodenum and colon	Correlated with histopathologic severity, but not with CIBDAI	Hanifeh *et al*., 2018
	64 dogs with chronic GI signs	Fecal	Higher concentrations in dogs with IBD	-	Heilman *et al*., 2016
IAP	28 dogs with CIE vs. 118 healthy dogs	Fecal	Lower expression and activity in dogs with CIE, especially in those with moderate or severe diseases.	-	Ide *et al*., 2016
	28 dogs with CIE vs. 9 healthy dogs	Duodenal and colonic mucosa	Higher expression and activity in the luminal side of epithelial cells in the mucosa and intestinal crypts in the duodenum of dogs with CIE	-	

ABBREVIATIONS:

3-BrY – Bromotyrosine

CCECAI – Canine chronic enteropathy clinical activity index

CD – Chronic diarrhea

CIBDAI – Canine inflammatory bowel disease activity index

CIE – Chronic Inflammatory enteropathies

CRP – C reactive protein

EGE – Eosinophilic gastroenteritis

EPI – Exocrine pancreatic insufficiency

FRE – Food-responsive enteropathy

GI – Gastrointestinal

IAP – Intestinal alkaline phosphatase

IBD – Inflammatory Bowel Disease

IgA – Immunoglobulin A

IRE – Immunosuppressive-responsive enteropathy

LPE – Lymphocytic plasmacytic-enteritis

LTE4 – Leukotriene E4

NMH – N-methylhistamine

α1-PI – Alpha1-proteinase inhibitor

234

5.2.9. Intestinal microbiota

The GI microbiota is a complex population of living microorganisms, comprising bacteria, archaea, fungi, protozoa, and viruses (Eissa *et al*., 2019; Honneffer, Minamoto, & Suchodolski, 2014; Redfern, Suchodolski, & Jergens, 2017). This highly complex ecosystem plays an essential role in GI health (Honneffer *et al*., 2014; Omori *et al*., 2017), mainly in digestion, absorption, energy metabolism, immune system development and in the prevention of infections (Celi *et al*., 2019). A significant proportion of this system are bacterial species comprised in the phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*.

Intestinal microbiota and the host immune system have a complex mutual relationship (Eissa *et al*., 2019). In a balanced environment, resident microbiota compete against pathogens for available resources and space in the GI tract, preventing pathogen colonization (Omori *et al*., 2017). In addition, commensals produce short-chain fatty acids (SCFAs) through the fermentation of substrates, such as nondigested dietary residues, endogenous mucus and sloughed epithelial cells (Eissa *et al*., 2019; Xu *et al*., 2016). As a result, commensals obtain energy for their metabolism and allow epithelial cell growth (Omori *et al*., 2017). In this mutualistic interaction, the host contributes with nutrients and niches that are crucial for microbiota colonization (Eissa *et al*., 2019).

Gut microbiota also contributes to the homeostasis of systemic immunity (Omori *et al*., 2017) by promoting self-tolerance (Redfern *et al*., 2017). Hence, an imbalance in bacteria populations within the GI tract, defined as dysbiosis, can significantly affect their functions (Redfern *et al*., 2017). Alterations of intestinal microbiota have been associated with CIE, thus an inappropriate activation of immune responses against GI microbiota is thought to contribute to the mechanisms of the disease (Omori *et al*., 2017).

Dogs with CIE have been associated with a lower microbiota diversity (Eissa *et al*., 2019), characterized by an overrepresentation of the phylum *Proteobacteria* (Honneffer *et al*., 2014), particularly in the *Enterobacteriaceae* family (Simpson & Jergens, 2011) and in the *Delftia* genus (Kalenyak *et al*., 2018).

Increases in the phylum *Actinobacteria* were also reported (Honneffer *et al*., 2014), particularly in the genus *Corynebacterium* (Kalenyak *et al*., 2018).

Affected dogs have been characterized by a decrease in the phylum *Firmicutes* (Honneffer *et al*., 2014), specifically in the *Clostridiales* order (Eissa *et al*., 2019). With regards to this phylum, Xu *et al.,* (2016) observed that dogs demonstrating higher CCECAI scores showed a gradual decrease in *Lactobacillus* strains. These commensal organisms carry out important functions such as the production of SCFAs (Redfern *et al*., 2017) and the downregulation of pro-inflammatory cytokines, as demonstrated in murine models (Xu *et al*., 2016). Interestingly, Kalenyak *et al.,* (2018) showed there was a decrease in

the *Enterococcus* genus, from the same phylum.

Furthermore, lower populations of the phylum *Bacteroidetes* were shown (Honneffer *et al*., 2014), particularly in the *Bacteroidales* order, as reported by Eissa *et al* (2019). Yet, Kalenyak *et al.,* (2018) observed increased populations of the *Bacteroides* genus in dogs with FRE and IBD after treatment. *Bacteroides* are considered valuable for their ability to reduce carbohydrates and breakdown of bile acid. Therefore, the potential use of these strains as a marker to assess response to treatment has been suggested.

Discrepancies amongst studies analyzing microbiota composition, could be justified by the different sampling methods to evaluate microbiota, differences in study population, diet variability within the individuals (Kalenyak *et al*., 2018), the use of medications, such as antibiotics, and the different washout periods applied (Omori *et al*., 2017).

Recently, researchers developed a mathematical algorithm to evaluate alterations in the intestinal microbiota in fecal samples (AlShawaqfeh *et al*., 2017). The fecal dysbiosis index (DI) consists of a quantitative polymerase chain reaction panel to assess eight bacterial groups that are normally changed in dogs with CIE, including *Blautia, Clostridium hiranonis, Escherichia coli, Faecalibacterium, Fusobacterium, Streptococcus, Turicibacter*, and total bacteria. This tool evaluates the occurrence of dysbiosis and may also be useful to track whether the microbiota normalizes in response to treatment.

In order to estimate the clinical utility of DI as a tool to analyze microbiota dysbiosis in dogs with CIE and their response to treatment, future studies have to be carried out (AlShawaqfeh et al., 2017).

6. FINAL CONSIDERATIONS

Canine CIE comprehend a group of idiopathic GI disorders with a chronic cyclical remission-relapse nature, which are considered immunologically-mediated (Heilmann & Steiner, 2018; Jergens & Simpson, 2012). This condition has been recognized, so far, as the biggest cause for chronic GI signs in dogs. However, it is currently overdiagnosed in clinical practice, possibly due to the difficulties inherent to the diagnosis process (Somu *et al*., 2017). This evidence, associated to the fact that their etiologies and pathogenesis mechanisms remains partially unclear, emphasizes the need to further investigate this subject (Wdowiak et al., 2013).

Currently, the diagnosis and monitoring of CIE rely predominantly on clinical, laboratory, endoscopic and histologic parameters (Karin Allenspach, 2015; Cerquetella et al., 2010; Moser et al., 2018; Wdowiak et al., 2013). However, these methods have several limitations. Besides the subjective nature of clinical score systems, clinical signs usually do not reflect intestinal inflammation (Heilmann et al., 2018). Furthermore, endoscopic and histopathologic procedures are semi-invasive, expensive and unlikely to be frequently performed (Heilmann, Grellet, et al., 2014). Additionally, the interpretation of

histopathologic tissue specimens depends significantly on interobserver variability. Added together, all these limitations constitute a big challenge to the approach and management of patients with CIE. To provide a solution for all of these concerns, biomarkers appear as a more objective and non-invasive tool that can have great advantages in estimating diagnosis, defining disease severity and predicting the most likely outcome (Otoni et al., 2018; Wdowiak et al., 2013).

Biomarkers have a great potential in helping in the diagnosis and management of dogs with CIE. Based on the literature used for this study, one may argue that fecal calprotectin and fecal calgranulin C are promising biomarkers of intestinal inflammation (Celi et al., 2019). When compared to other biomarkers, the concentrations of these two DAMP molecules have been positively correlated with clinical and histopathological severity (Grellet et al., 2013; Heilmann et al., 2018; Heilmann, Grellet, et al., 2014; Otoni et al., 2018). Furthermore, their usefulness in predicting clinical outcomes (Hanifeh et al., 2018; Heilmann & Steiner, 2018) as well as in differentiating the various forms of CIE, has been proven (Heilmann et al., 2018; Heilmann, Volkmann, et al., 2016). Moreover, calprotectin and calgranulin C have been analyzed the most through clinical trials and articles substantiating its value, over the other biomarkers. Even though the remaining biomarkers discussed in this article have not presented results as solid as calprotectin and calgranulin C, future investigations should take place with the goal of contributing for current knowledge advancement of biomarkers clinical utility in canine CIE. It is also important to flag that, even though the investigations in the intestinal microbiome are recent, it is a promising area with great potential to be used in clinical practice. In addition, a single biomarker cannot assuredly predict disease severity, progression, response to treatment and clinical outcome. Therefore, in order to achieve greater accuracy, it would be beneficial if these tools are used in conjunction with the contemporary ones (Collins, 2013).

In the present study, it is important to point out its limitations. Firstly, when reporting clinical trials, the different methods for the detection of biomarkers expression, as well as their sensitivity, specificity and cut-off values were not taken into consideration. This limitation can be justified, since the majority of the studies analyzed in this article did not have this information available. Moreover, there were not enough clinical trials behind some of the reported biomarkers that could support the conclusions about their clinical utility. In order to address these limitations, it is of upmost urgency to raise awareness about biomarkers' usefulness in this field. Furthermore, although recent investigations on biomarkers' utility in dogs with CIE have been performed, none of them are routinely used in clinical practice (Heilmann *et al*., 2018). Hence, one can draw the conclusion that future research is needed in order to better determine the usefulness of these tools in diagnosing and managing CIE in dogs.

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