From the
Department for Small Animals
Faculty of Veterinary Medicine, Leipzig University

Evaluation of RAGE (receptor for advanced glycation end products) in dogs with chronic enteropathy

Inaugural-Dissertation
To obtain the degree of a
Doctor medicinae veterinariae (Dr. med. vet.)
From the Faculty of Veterinary Medicine
Leipzig University

Submitted by
Angela Isabel Cabrera Garcia
From Córdoba, Spain

Leipzig, 2021

Mit Genehmigung der Veterinärmedizinischen Fakultät der Universität Leipzig

Dekan: Prof. Dr. Dr. Thomas W. Vahlenkamp

Betreuerin: Prof. Dr. Romy M. Heilmann

Gutachter: Prof. Dr. Romy M. Heilmann, Klinik für Kleintiere, Veterinärmedizinische Fakultät, Universität Leipzig

Prof. Dr. Karin Allenspach-Jorn, Lloyd Veterinary Medical Center, College of Veterinary Medicine, Iowa State University, Ames, IA, USA

Tag der Verteidigung: 07.09.2021

Dedicated to Sara C., Mocca M., Pepe, and Egon

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
APCs	Antigen-presenting cells
ARE	Antibiotic responsive enteropathy
α_1PI	Alpha$_1$-proteinase inhibitor
BUN	Blood urea nitrogen
cAMP	Cyclic adenosine monophosphate
CBC	Complete blood cell count
CCK	Cholecystokinine
cGMP	Cyclic guanosinmonophosphate
CIBDAI	Canine inflammatory bowel disease activity index
CFU/g	Colony-forming units per gram
Cl^-	Chloride
CCECAI	Canine chronic enteropathy clinical activity index
CIE	Chronic inflammatory enteropathies
cPLI	Canine pancreatic lipase immunoreactivity
cTLI	Canine trypsin-like immunoreactivity
CRP	C-reactive protein
DAMP	Damage-associated molecular pattern
HCO_3^-	Hydrogencarbonate
HMGB1	High-mobility group box-1
IF	Intrinsic factor
FLL	Focal lipogranulomatous lymphangitis
FPS-ZM1	N-benzyl-N-cyclohexyl-4-chlorobenzamide
FIG	Figure

FRE	Food-responsive enteropathy
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
IBD	Inflammatory bowel disease
MDB	Minimum database
NaCl	Sodium chloride
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular pattern
PLE	Protein-losing enteropathy
PRR	Pattern-recognition receptor
RAGE	Receptor for advanced glycation end products
ROI	Region of interest
sRAGE	Soluble receptor for advanced glycation end products
SRE/IRE	Idiopathic steroid- and/or immunosuppressant-responsive (or -refractory) enteropathy
Spec cPL	Specific canine pancreatic lipase
TLR	Toll-like receptor
WSAVA	World Small Animal Veterinary Association
WK	Week

1. INTRODUCTION

Chronic inflammatory enteropathies (CIE) occur frequently and are an important group of conditions in dogs. Similar to people with inflammatory bowel disease (IBD), dogs with CIE typically present with chronic gastrointestinal signs, including diarrhea, vomiting, weight loss, and sometimes abdominal pain. Distinguishing small bowel diarrhea (characterized by an increased fecal volume, melena, weight loss, and sometimes steatorrhea [HALL AND GERMAN 2010]), from large bowel diarrhea (mucus in the feces, tenesmus, and fecal urgency [ALLENSPACH 2010]) can aid in the clinical decision-making process (e.g., planning the optimal diagnostic steps). In a dog suspected of vomiting, it is also important to differentiate vomiting from regurgitation because the differential diagnoses for those two clinical problems differ profoundly.

Gastrointestinal signs in dogs can be acute, chronic, or acute-on-chronic in nature. Infections (viral, parasitic, or bacterial), dietary indiscretion, and toxin ingestion are the most common causes of acute gastrointestinal signs in canine patients (ALLENSPACH and GASCHEN 2011). Chronic enteropathies are characterized by the presence of persistent or intermittent clinical signs for at least 3 weeks (WASHABAU ET AL. 2010, ALLENSPACH 2016, DANDRIEUX 2016). Beyond the common signs of an acute gastroenteropathy (vomiting, diarrhea, and abdominal pain), dogs with CIE may show borborygmus, flatulence, hyporexia, lip-smacking, and/or licking fits. A definitive diagnosis requires laboratory and fecal examinations as well as diagnostic imaging to rule out infectious or mechanical-obstructive gastrointestinal or extra-gastrointestinal causes (ERDMANN and HEILMANN 2017). Histopathological evaluation of gastrointestinal tissue biopsies is then needed to document inflammation, rule out a neoplastic condition, and characterize the inflammatory infiltrate. Dogs with CIE are further subclassified based on the response to treatment as either food-responsive enteropathy (FRE; if the clinical signs significantly improve or resolve following an elimination diet trial) or idiopathic steroid- and/or immunosuppressant-responsive (or -refractory) enteropathy (SRE/IRE; if treatment with a corticosteroid [e.g., prednisolone or budesonide] and/or other immunosuppressant medication [e.g., cyclosporine, azathioprine, or chlorambucil] is needed) (ALLENSPACH 2016, DANDRIEUX 2016). Protein-losing enteropathy is characterized by an intestinal protein loss due to primary or secondary lymphangiectasia, which generally carries a worse prognosis, and affected dogs can develop hypoalbuminemia.

Presenting a primary (idiopathic) inflammatory condition, IRE/SRE is a diagnosis of exclusion. The pathogenesis of this disease entity appears to be complex, and current knowledge suggests a dysregulated immune response to play a key role in this disease. There are two arms of the

immune system: the innate immune response (also called natural or non-specific immunity) and the acquired immune response (also referred to as adaptive or specific immunity). An innate immune system exists in virtually all living creatures, even simple single-celled organisms such as bacteria have enzymatic systems that protect against viral infections. In higher-order organisms, the innate immune system can detect various damage-associated molecular pattern (DAMP) molecules released from cells (also called alarmins) as well as signals associated with the presence of pathogens called pathogen-associated molecular pattern (PAMP) molecules, thus allowing for the identification of damaged or infected cells (LOZANO SOTO 2012). The acquired immune system has equipped vertebrates, including humans and dogs, with more sophisticated defense mechanisms interconnected with the innate immune system in a dynamic but more longitudinal fashion. With lymphocytes as the functional basis of the adaptive immune system, this immune system arm can adapt to recognize specific pathogens more efficiently (immune memory) and provides the basis for immune tolerance towards certain antigens encountered at mucosal surfaces (BERKOW 2008, LOZANO SOTO 2017).

Pattern-recognition receptors (PRRs) belong to the innate immune system and can recognize DAMP and PAMP molecules (HEILMANN and ALLENSPACH 2017). One of these innate immune receptors is the receptor for advanced glycation end products (RAGE), which has a central role in the pathogenesis of chronic inflammatory diseases in humans (BIERHAUS et al. 2005). RAGE as a non-specific PRR binds to several DAMP molecules, including S100/cal-granulin proteins S100A8/A9 and S100A12 (BIERHAUS et al. 2005, HEILMANN and ALLENSPACH 2017). Transmembrane (full-length) RAGE consists of 5 different domains, of which the cytoplasmic tail presents the distal portion of the receptor and can activate the intracellular inflammatory cascade (HEILMANN and ALLENSPACH 2017). Soluble RAGE (sRAGE) is a decoy receptor that can bind circulating ligands of RAGE but lacks the cytoplasmic part to induce pro-inflammatory post-receptor signaling. Because sRAGE competes with transmembrane (full-length) RAGE for ligand (e.g., DAMP) binding, the intracellular inflammatory cascade is not activated (HEILMANN and ALLENSPACH 2017), potentially leading to an abrogation of inflammation.

There are 24 variants of RAGE in dogs (STERENCZAK et al. 2009). This PRR has been evaluated in canine patients with malignant neoplasms (histiocytic sarcoma, lymphoma) (STERENCZAK et al. 2010, STERENCZAK et al. 2011) but has not been studied or reported in dogs with chronic gastrointestinal inflammation (i.e., CIE). Decreases in serum sRAGE concentrations were detected in canine CIE. However, the RAGE/sRAGE axis and its role in the inflammatory response have not been further investigated in dogs with this condition.

2. REVIEW OF LITERATURE

2.1 Functional Anatomy and Physiology of the Intestines

2.1.1 Anatomy of the Intestines

The intestine (Latin *intestinum*, Greek ἔντερον), or gut, is the longest part of the digestive tract of higher multicellular animals, including dogs. Anatomically, the intestine is divided into two main portions: the small intestine and the large intestine or colon (HELANDER 2014).

The small intestine (*intestinum tenue*) is the section of the digestive tract where most of the hydrolytic cleavage of the ingesta by endogenous pancreatic and intestinal brush border enzymes occurs (HELANDER 2014). The small intestine constitutes of three segments: the duodenum, jejunum, and ileum. The duodenum emerges right from the gastric pylorus. The proximal portion of the duodenum (*pars cranialis duodeni*) directly faces the intestinal surface of the liver, which it connects to by the hepatoduodenal ligament. The duodenum then follows a caudal direction with the cranial duodenal flexure (*flexura duodenalis cranialis*). The subsequent *pars descendens duodeni* has attached a comparatively long mesentery in the dog, in which the right lobe of the pancreas is embedded (SCHUMMER 1987). The *pars ascendens duodeni* is attached to a shorter mesentery and is located between the cecum and descending colon. The *pars ascendens duodeni* and *colon descendens* are connected through the *plica duodenocolica*, which continues to the rectum. With the *flexura duodenojejunalis,* the ascending duodenum enters into the jejunum, the longest segment of the small intestine comprised of six to eight jejunal loops located between the stomach and pelvic entrance (SCHUMMER 1987). The ileum, as the last segment of the small intestine, connects to the colon. It opens into the large intestine, between the cecum and ascending colon, with the *ostium ileale* (WISSDORF 1976).

The intestinal wall has a similar structure in all three small intestine segments and comprises the mucosa, submucosa, muscularis propria, subserosa, and serosa (Fig. 1). Blood is supplied to the small intestine from the *arteria coeliaca* and *arteria mesenterica cranialis*, and nutrient-rich blood from the intestine is transported to the liver via the portal vein (WISSDORF 1976). The lymphatic drainage system of the small intestine originates from chylous capillaries of the small intestinal villi (Fig. 1). Regional lymph nodes of the small intestine include the jejunal, pancreaticoduodenal, and colic lymph nodes.

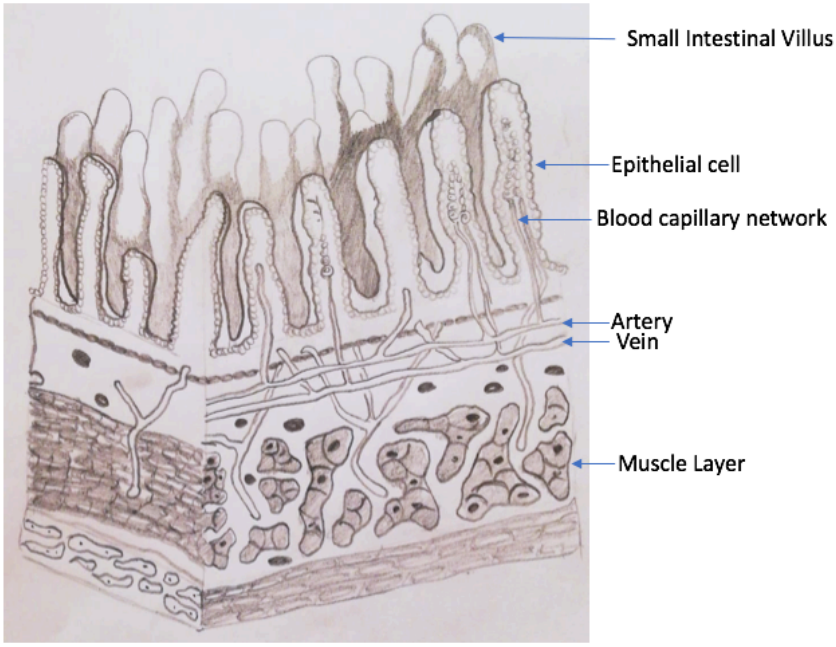

Figure 1: Detailed cross-section showing the organization of blood and lymph vessels of the small intestinal wall.

The large intestine (*intestinum crassum*) is essentially the main segment to absorb water and electrolytes from the intestinal lumen. The large intestine consists of the cecum, colon, and rectum. The cecum in dogs has a corkscrew-like appearance and measures between 40 and 150 mm; the length doubles when folds elapse. Through the mesocecum of the mesenteric root and the *plica ileocecalis*, the location of the cecum remains constant, mainly within the right quadrant of the abdomen (SCHUMMER 1987). The colon describes a U-shaped loop that rises to the right, then crosses over to the left, cranial to the mesenteric root, and then descends to the pelvic entrance. Accordingly, the colon is comprised of the *colon ascendens*, *colon transversum*, and *colon descendens*. The rectum begins at the level of the 7th lumbar vertebra. Within the peritoneal cavity, before entering the pelvic canal, the rectum is attached to a short mesentery, the mesorectum (SCHUMMER 1987).

The histomorphology of the large intestinal wall follows in principle that of the small intestine, with the exception that the large intestinal mucosa lacks any villi (Fig. 2). The lymphatic structures in the mucosa are referred to as *noduli lymphatici solitarii*.

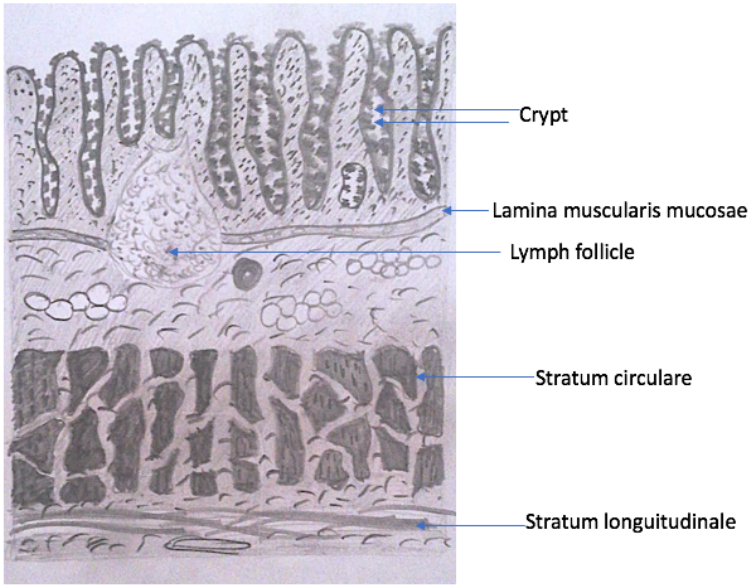

Figure 2: Schematic of the large intestinal wall.

2.1.2 Gastrointestinal Physiology

Several aspects of gastrointestinal function are important for the physiologic mechanisms of the gastrointestinal tract and to understand and address gastrointestinal pathologies. These include gastrointestinal motility, secretion, digestion and absorption, and mucosal barrier function.

Following the ingestion of a meal, the stomach contents are released into the small intestine by rhythmic contractions of the pyloric muscles, which occur every 15–20 seconds. In the small intestine, the ingesta are gradually mixed with intestinal secretions and are transported aborally by periodic segmentation and pendulous contractions of the intestinal wall. The chyme then reaches the large intestine approximately 60–90 minutes after passing into the duodenum (ZENTEK 2016). During passage through the small intestine, the crucial steps of digestion occur. These include the breakdown of nutrient complexes into smaller, soluble substances and the absorption of these small molecules through the intestinal wall and into the circulation. At the distalmost portion of the ileum, the digestion of proteins and fats is nearly complete with highly digestible rations, but not with complex rations that are more difficult to digest (ZENTEK 2016).

Pancreatic secretions are essential in gastrointestinal physiology. The secretion of electrolytes and water occurs in the acinar cells of the pancreatic acini and the duct cells of the pancreatic

duct system, although the duct cells generally play a dominant role (ENGELHARDT and BREVES 2000). Digestive enzymes such as peptidases, nucleases, amylases, and lipases are secreted by the acinar cells of the pancreas via exocytosis, partly as inactive pro-enzymes and partly as activated enzymes (ENGELHARDT and BREVES 2000). The inactive pro-enzyme forms of pancreatic enzymes are activated in the small intestine. Enteropeptidase (also referred to as enterokinase) is an intestinal brush border enzyme that activates trypsinogen to trypsin by enzymatic removal of a hexapeptide. Other pancreatic pro-enzymes secreted into the small intestinal lumen are also activated by trypsin via peptide cleavage (ENGELHARDT and BREVES 2000).

2.1.3 Small Intestinal Physiology

The small intestine is primarily responsible for nutrient digestion and absorption (CAMPBELL and REECE 2003). The acidic chyme from the stomach is released into the duodenum, where it mixes with digestive juices from the pancreas, liver, and gallbladder.

The small intestinal mucosa has a very large surface area. Broad folds and finger-like extrusions (intestinal villi) of the mucosa contribute to the enlarged surface area. Further, each epithelial cell of the intestinal villi carries microscopic microvilli, which protrude from the luminal cell surface into the intestinal lumen (CAMPBELL and REECE 2003). The center of each intestinal villus harbors a network of blood capillaries and a central lymphatic vessel. Nutrient products absorbed via the intestinal epithelium are transported through the endothelium of the capillaries or lymphatic vessels (CAMPBELL and REECE 2003).

Fluid secretion in the small intestine plays a central role in absorptive processes. Chloride is primarily released into the intestinal lumen through a chloride channel in the apical membrane of the epithelium. Sodium and potassium follow paracellularly through the *zonulae occludentes*, driven by an electrical gradient (ENGELHARDT and BREVES 2000). By activating chloride and sodium channels, the electrolyte- and osmotically-driven intestinal secretion of water can be stimulated (ENGELHARDT and BREVES 2000). Three intracellular second-messenger systems regulate the secretion of chloride from the intestinal epithelial cells: cAMP-, cGMP-, and calcium-signaling pathways. These, in turn, offer different pathomechanisms for chloride hypersecretion. cAMP directly promotes the secretion of chloride by triggering the opening of chloride channels in the brush border membrane (ENGELHARDT and BREVES 2000). All three signaling pathways also inhibit the absorption of sodium chloride (NaCl). However, this mechanism is limited to NaCl transport via Na^+/H^+ and Cl^-/HCO_3^- exchangers and does not affect sodium transport via Na^+ channels (ENGELHARDT and BREVES 2000).

2.1.4 Large Intestinal Physiology

Chyme from the small intestine ultimately enters the large intestine. At the junction between these two intestinal portions is a valve-like sphincter that controls the aborad transport of intestinal contents (CAMPBELL and REECE 2003). Major functions of the large intestine include the reabsorption of water, including the portion of water presenting the basic substance of digestive secretions (CAMPBELL and REECE 2003). A large part of water absorption occurs with the absorption of nutrients in the small intestine, and the colon reabsorbs the remainder of the fluid that has remained in the lumen. Together, both small and large intestines reabsorb about 99% of the water that enters the alimentary tract (CAMPBELL and REECE 2003).

Microorganisms colonize the gastrointestinal tract of all mammals immediately after birth, and the large intestine is the main site of microbial colonization in monogastric animals (ENGELHARDT and BREVES 2000). The physiologic microbiome includes various species of the genera *Bacteroides*, *Fusobacterium*, *Streptococcus*, *Eubacterium*, *Ruminococcus*, *Lactobacillus*, and *Treponema,* as well as coliforms such as *Escherichia coli*. Several factors are important to maintain a physiologic composition and function of the gastrointestinal (and particularly colonic) microbiome and its metabolic functions (ENGELHARDT and BREVES 2000). These include the neutralization of acidic products of microbial metabolism, sufficient residence time of the ingesta in the large intestine, dilution of metabolic end-products by the liquid phase of colonic content, and resorption of the metabolites from microbial fermentation. Also, the gastrointestinal barrier function is important to prevent microbial translocation.

Goblet cells are mostly located in the large intestine and differ from cells in the small intestine or colonic crypts by having numerous mucus-containing secretory granules (ENGELHARDT and BREVES 2000). Mucous secretion from goblet cells occurs through a process called collective exocytosis, which is induced by acetylcholine and prostaglandin E (ENGELHARDT and BREVES 2000). Mucous secretion is essential for defection. Feces are stored in the distal large intestine (rectum) until defecation. This reflex involves two sphincters, an internal (involuntary) and an external (voluntary) sphincter located between the rectum and anus (CAMPBELL and REECE 2003). Activation of stretch receptors within the rectal wall causes the inner sphincter to relax, creating an urge to defecate. Defecation then requires the external sphincter to be voluntarily relaxed and increased abdominal pressure (CAMPBELL and REECE 2003).

2.1.5 Gastrointestinal Neuronal and Endocrine System

The intestinal mucosa has three types of detectors: neuronal, endocrine, and immunological (FURGESS et al. 1999). The intrinsic nervous system allows the intestine to autonomously regulate its functions depending on the microenvironment, including the amount and type of food ingested. The secondary control exerted by the central nervous system on the intestine influences the intrinsic systems (nervous and endocrine) and directly regulates intestinal functions (FURNESS et al. 1999).

The neurons found in the myenteric and submucosal plexus are primary afferent neurons. They comprise three different types (FURNESS et al. 1999): (1) intrinsic primary afferent neurons comprised of cell bodies and fibers within the gut wall, (2) extrinsic primary afferent neurons, with cell bodies in the vagal and dorsal root, and (3) intestinofugal neurons, with cell bodies in the muscularis propria and projections to neurons outside the gut wall.

The intestine produces more than 20 different hormones that are secreted by the enteroendocrine cells. One of the most important endocrine mediators is cholecystokinin (CCK). This hormone is released after the ingestion of a meal. CCK mediates the release of digestive enzymes from the pancreas and causes bile salts to empty into the duodenum (FURNESS et al. 1999).

2.1.6 Gastrointestinal Immune System

The gastrointestinal tract has an intrinsic immune system, referred to as the gut-associated lymphoid tissue (GALT). The GALT is the most significant accumulation of immune cells within the body. Approximately 70% of all immunologically active cells are localized (resident cell population) or recruited to the intestinal mucosa. The GALT plays a major role in maintaining a delicate balance between mounting an immune response to fight pathogenic microorganisms and remaining in an anergic state (immunotolerance) while recognizing a large number of different dietary antigens as well as beneficial organisms of the gut microbiome. The presentation of the antigens by antigen-presenting cells (APCs) as structural components of the GALT is a fundamental process for the decision between inflammation and immunotolerance (SPAHN and KUCHARZIK 2004). Antigen presentation occurs in the intestinal tract, where lymphoid follicles of the mucosa are accumulated. The strong presence of these follicles distinguishes the GALT from the immune system or immune structures in other parts of the body. The GALT functions as the intestinal frontline of the systemic immune response (SPAHN and KUCHARZIK 2004). The GALT is comprised of:

- the tonsils and lymphoid follicles of the palate,
- the solitary lymph follicles of the intestinal segments,

- the Peyer's patches, and
- the lamina propria of the intestinal segments.

The mesenteric lymph nodes connect the GALT to the immune system of the body. As part of the immune system, tonsils provide an immunity as a first line of defense against ingested or inhaled pathogens. The GALT surface contains antigen-capture cells, the so-called M cells, that can capture pathogens by detecting specific surface antigens. These M cells function to signal the presence of a pathogen to underlying B and T lymphocytes, resulting in the initiation of an immune response (KATO et al. 2013).

Peyer's patches are also an essential structural component of the intestinal immune system. These structures play a central role in the surveillance of intestinal bacterial populations and preventing intestinal colonization and infection with pathogenic bacteria. The lamina propria of the intestinal segments has several immune functions and has a significant role in protecting the host from pathogenic microorganisms that can enter from the gastrointestinal tract (VAROL et al. 2009).

2.1.7 Innate Immunity and Acquired Immunity

Mammals have an immune system that includes two main branches: innate immunity and acquired immunity.

The innate immune system presents the host's first line of defense against pathogens. Phagocytes, including macrophages and dendritic cells, mediate the innate immune response. The innate immune system can recognize microorganisms through pattern recognition receptors (PRRs), unlike the acquired immune system that contains a vast repertoire of rearranged receptors (AKIRA et al. 2006).

Acquired immunity plays an essential role in the late phase of the immune response, eliminating pathogens at this stage and generating the humoral and cellular immune response as well as their cross-talk. This is accomplished through the individual development and separation of lymphocytes that carry specific antigen receptors generated by gene rearrangement (AKIRA et al. 2006).

2.1.8 Intestinal Microbiome

The intestinal microbiome consists of bacteria, fungi, viruses, and protozoa (SUCHODOLSKI 2016). Longitudinal differences are known to exist in the intestinal microbiome, with the large intestine harboring a higher number of bacteria (up to 10^{11} colony-forming units per gram

[CFU/g]) compared to the small intestine, where the number reaches about 10^5 CFU/g (SUCHODOLSKI 2016). Shotgun deep-sequencing and fluorescence *in situ* hybridization (FISH) analysis allow for the specific identification of most bacterial species. The intestinal microbiota is of vital importance to the host. Not only does the physiological microbiome help with intestinal epithelial development and maintenance (homeostasis), but it also constitutes an important barrier (or competitor) against pathogenic organisms (SUCHODOLSKI 2016).

Another important role of the intestinal microbiota is providing nutritional factors to the host, including the synthesis of essential vitamins (such as folic acid) and the degradation of polysaccharide complexes (NEISH 2009, SWANSON et al. 2011). The latter function also has an anti-inflammatory effect by inducing regulatory T lymphocytes (ARPAIA et al. 2013). The Firmicutes, Bacteroidetes, Proteobacteria, and Fusobacteria are the most prevalent bacterial phyla of the intestinal microbiome in healthy dogs (SUCHODOLSKI et al. 2008; XENOULIS et al. 2008, SUCHODOLSKI et al. 2009, HANDL et al. 2011).

Previous studies demonstrated that the host susceptibility (ALLENSPACH et al. 2010, KATHRANI et al. 2012, and KATHRANI et al. 2014) and the intestinal microbiome (XENOULIS et al. 2008, ALLENSPACH et al. 2010, SUCHODOLSKI et al. 2012a, SUCHODOLSKI et al. 2012b, HONNEFFER et al. 2014, and MINAMOTO et al. 2015) are of central importance in the pathogenesis of chronic inflammatory enteropathies (CIE) in dogs.

Several recent investigations into the intestinal microbiota of dogs with CIE point to the presence of an intestinal dysbiosis (SUCHODOLSKI 2016).

Not only alterations in the interaction of the mucosa and the host's enteric microbiota but also the intestinal microbiota contribute to the pathogenesis of inflammatory bowel disease (IBD) in dogs and cats (SUCHODOLSKI 2016). However, it remains to be determined whether these changes are a definitive cause or a consequence of IBD (or even a combination of both).

2.2 Enteropathies in Dogs

2.2.1 Definition

Enteropathy refers to any pathology of the intestines. Clinical signs such as vomiting, nausea, diarrhea, decreased appetite, or weight loss, amongst others, are described in patients with these conditions. Enteropathies can be acute or chronic.

2.2.2. Acute Enteropathy

Acute diarrhea in dogs is mostly related to dietary or infectious causes. In this species, the ingestion of spoiled food, garbage (e.g., containing mold), or substances or things that are not

suitable for consumption are common causes of acute gastrointestinal signs. Too rapid of a change in diet or an intolerance to the type of food offered can also lead to acute diarrhea. Toxic reactions (e.g., medications, other toxins) are another common cause of acute diarrhea in dogs (LANGLOIS et al. 2019).

Hypoadrenocorticism (Addison's disease), especially in its atypical form, can be primarily associated with waxing and waning acute or acute-on-chronic gastrointestinal signs. Thus, this differential also needs to be considered an extra-gastrointestinal cause of gastrointestinal signs mimicking an enteropathy. The exclusion or diagnosis of this condition is by determining a serum baseline cortisol concentration and/or an ACTH stimulation test.

2.2.3. Chronic Enteropathies

Chronic inflammatory enteropathies (CIE) are an important group of gastrointestinal diseases in dogs, the diagnosis of which requires (WASHABAU et al. 2010, ALLENSPACH 2016, DANDRIEUX 2016):

- the presence of chronic gastrointestinal signs (≥3 weeks),
- histopathologic evidence of intestinal mucosal inflammation, and
- the exclusion of other underlying causes.

The diagnostic evaluation and patient management can be challenging in dogs with CIE. Canine CIE are further subclassified (usually retrospectively) based on the response treatment (Fig. 3).

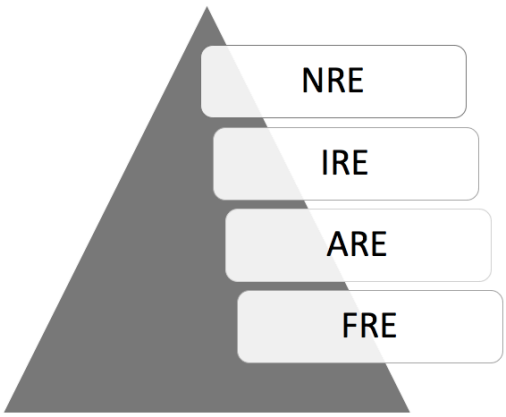

Figure 3: Frequencies of the different subgroups of chronic inflammatory enteropathies (CIE) in dogs. Food-responsive enteropathy (FRE); antibiotic-responsive enteropathy (ARE); immunosuppressant-responsive enteropathy (IRE); and non-responsive enteropathy (NRE).

2.2.3.1 Food-Responsive Enteropathy (FRE)

Dogs showing a significant improvement or resolution of their clinical signs after being placed on an elimination diet are classified as FRE. Typically, these dogs are younger and have less severe clinical disease than dogs with other CIE forms. FRE is also the most prevalent group (approximately 64%; Fig. 3) of CIE in dogs (ALLENSPACH et al. 2016, DANDRIEUX 2016, VOLKMANN et al. 2017).

Treatment of FRE consists of a dietary change to either an elimination diet with a novel protein and carbohydrate source (hitherto unknown to the dog) or a hydrolyzed protein diet (that is supposed to be less immunogenic). Novel protein/carbohydrate diets can be home-cooked (and supplemented/ balanced if considered for long-term feeding) or commercial diets. An excellent long-term response in dogs with FRE has been shown with a commercial hydrolyzed protein diet (MANDIGERS et al. 2010, ALLENSPACH et al. 2016). It is essential that the owners are instructed not to feed any other food source (e.g., table scraps, treats containing other protein sources) during an elimination diet trial.

2.2.3.2 Antibiotic-Responsive Enteropathy (ARE)

Dogs that show a marked and long-lasting improvement or resolution of their clinical signs after administering an antibiotic (metronidazole and/or tylosin) for two weeks are classified as ARE (DANDRIEUX 2016). Because most dogs that respond to an antibiotic trial will relapse after discontinuation of antimicrobial treatment, the true existence of this subgroup of canine CIE is currently a matter of debate (ERDMANN and HEILMANN 2017, CERQUETELLA 2020).

2.2.3.3. Steroid- or Immunosuppressant-responsive enteropathy (SRE/IRE)

Dogs that require treatment with glucocorticosteroids or other immunosuppressive drugs are diagnosed as steroid-responsive (SRE) or immunosuppressant-responsive enteropathy (IRE) (ALLENSPACH 2016, DANDRIEUX 2016, ERDMANN and HEILMANN 2017). The diagnosis of SRE/IRE is a diagnosis of exclusion. Thus, the diagnostic evaluation of dogs suspected to have SRE/IRE can be very complex and present a challenge for both the owners or caretakers and the veterinarian. Despite a predisposition in some dog breeds (e.g., German shepherd dog), many different breeds of dogs can develop SRE/IRE, which supports the hypothesis of a multifactorial etiopathogenesis for the development of SRE/IRE in dogs, involving genetic effects and environmental factors (Fig. 4).

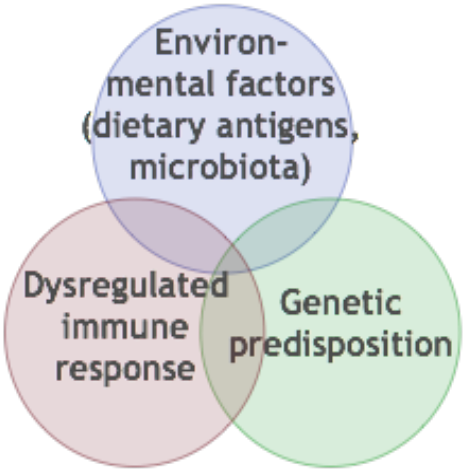

Figure 4: The pathogenesis of chronic inflammatory enteropathies, particularly SRE/IRE, in dogs is multifactorial. Environmental and genetic factors and a dysregulation of the immune system are involved in SRE/IRE E pathogenesis. Heilmann RM, PhD Thesis. Texas A&M University, 2015.

Like with inflammatory bowel disease (IBD) in human patients, medical treatment of dogs with SRE/IRE usually involves the administration of glucocorticosteroids and/or other immunosuppressive drugs such as cyclosporine or azathioprine. These drugs can have significant side effects (ALLENSPACH 2016, DANDRIEUX 2016, ERDMANN and HEILMANN 2017). More targeted treatment strategies available in human medicine (e.g., tumor necrosis factor-α blockers) would be desirable as a glucocorticosteroid-sparing treatment option for dogs with SRE/IRE but are currently lacking in veterinary medicine.

2.2.3.4 Non-Responsive Enteropathy (NRE)

Studies suggest that approximately 15–40% of dogs with CIE (initially classified as SRE/IRE) do not adequately respond to medical therapy, thus worsening the individual prognosis (CRAVEN 2004, ALLENSPACH et al. 2007). Whether these patients could potentially benefit from additional or alternative immunomodulatory treatment options (including prebiotics, probiotics, or synbiotics; fecal microbiota transplantation [PEREIRA et al. 2017]; stem cell therapy [PÉREZ-MERINO et al. 2015a, 2015b]; or a combination of these modalities) requires further study.

2.2.3.5 Protein-Losing Enteropathy (PLE)

Canine PLE is a special form of chronic enteropathies in dogs. This condition can occur secondary to an infiltrative intestinal disease process (e.g., lymphoplasmacytic enteritis with

SRE/IRE, or diffuse intestinal neoplasia such as lymphoma) or present a primary intestinal lymphangiectasia (with a breed predisposition in Yorkshire Terriers, Rottweilers, Maltese, Basenjis, and Irish Soft Coated Wheaten Terriers).

As a significant component of the immune system, the lymphatic system is a network of organs, lymph nodes, ducts, and lymphatic vessels that produce and transport lymph from the tissues to the bloodstream. This system contributes to the extracellular fluid hemostasis, fat absorption and transport, and immune system function (CRAVEN and WASHABAU 2018). Mechanisms of PLE in dogs involve structural lesions and/or dysfunctions of these lymphatic structures (Fig. 5). The most common clinical signs shown by affected dogs are chronic vomiting, diarrhea, weight loss, loss of lean muscle mass, lethargy, ascites, and/or pleural effusion (DOSSIN and LAVOUÉ 2011). Clinicopathological abnormalities can include lymphopenia, hypoalbuminemia or panhypoproteinemia, hypocholesterolemia, hypocalcemia/ hypomagnesemia, and an increased blood urea nitrogen (BUN) concentration (CRAVEN and WASHABAU 2018).

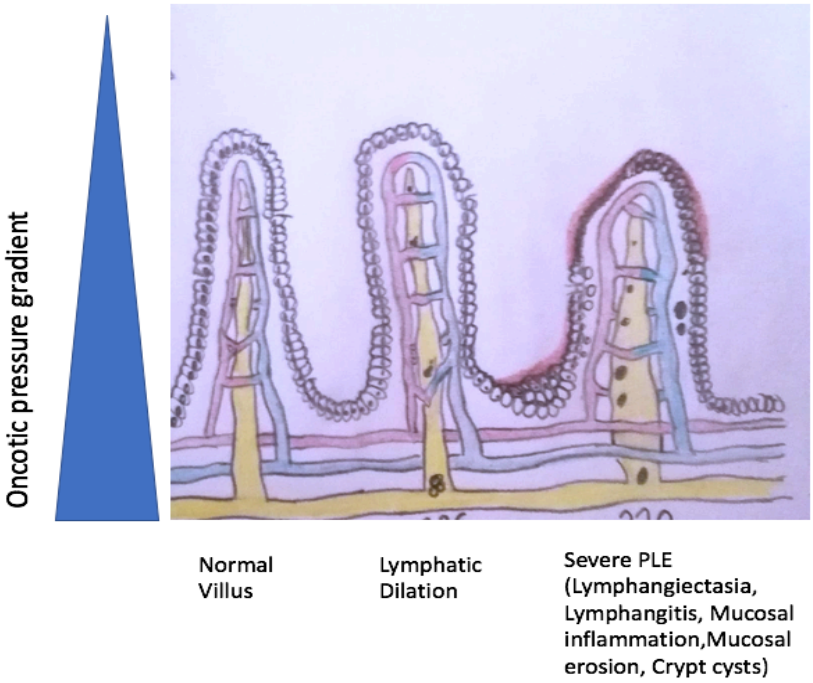

Figure 5: Schematic of the different mechanisms of gastrointestinal protein loss in dogs with protein-losing enteropathy (PLE).

A rapid diagnostic work-up is desirable and important in dogs with marked hypoalbuminemia or panhypoproteinemia, where the risk of complications (e.g., ascites/pleural effusion resulting from a marked reduction in plasma colloid-osmotic pressure or thromboembolism associated

with a hypercoagulability) is also high (DOSSIN and LAVOUÉ 2011, GOODWIN et al. 2011). Hypoalbuminemia/panhypoproteinemia and hypovitaminosis D are linked to poor outcomes in dogs with PLE (ALLENSPACH et al. 2008, TITMARSH et al. 2015). Diets that are ultra-low in fat (<10–15%) and contain highly digestible proteins are recommended for these patients (RUDINSKY et al. 2018, NAGATA et al. 2020), and some dogs diagnosed with PLE will require additional treatment options to prevent complications of the disease (e.g., anticoagulant drugs) or to address the underlying problem (e.g., immunosuppressive drugs).

Another subtype of PLE in dogs is known as focal lipogranulomatous lymphangitis (FLL). The clinical signs of affected dogs are those typically seen with CIE and PLE (vomiting and diarrhea for more than three weeks' duration, and in some cases also weight loss and a profound loss of lean muscle mass), and these patients can also be hypoalbuminemic, hypocobalaminemic, or both. There may be mesenteric lymphadenopathy, and lymphangiectasia is also common. A characteristic finding on abdominal ultrasonography with FLL is an increased intestinal wall thickness of the jejunum, ileum, or both with focal or solitary mass-like lesions (Fig. 6) (LECOINDRE et al. 2016). The underlying etiology of FLL is unknown, but the current consensus is that the pathogenesis – similar to SRE/IRE – involves environmental and genetic factors leading to a dysregulated immune response (WATSON et al. 2014, LECOINDRE et al. 2016).

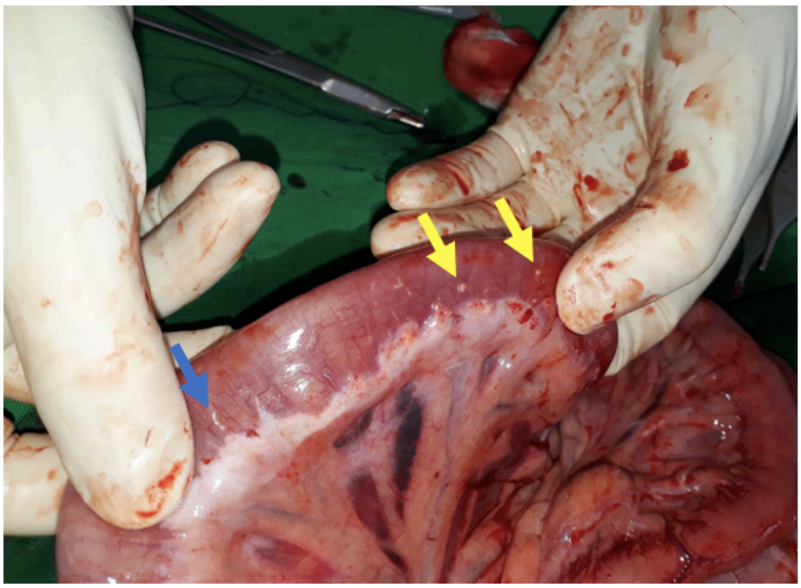

Figure 6: Lymphangiectasia (yellows arrows) and small multifocal mass (blue arrow) like lesions at the serosal aspect of the duodenum in a dog (4- year old male intact Maltese) affected with focal lipogranulomatous lymphangitis (FLL). Courtesy of Dr. Cora Siebenaller, Small Animal Clinic Marienberg, Germany).

2.3 Diagnostic evaluation of dogs with suspected CIE

A thorough patient medical history is essential in dogs suspected to have a CIE. It should focus on information about general clinical signs (diarrhea and vomiting) but also other characteristic clinical signs of CIE (e.g., weight loss, smacking while eating or gulping after food intake, eating grass, flatulence, salivation, or pica) (ERDMANN and HEILMANN 2017). A complete physical examination, including a rectal palpation, should follow, with particular emphasis on the presence of abdominal pain, nausea, or ballottement during abdominal palpation (WASHABAU et al. 2010, ERDMANN and HEILMANN 2017).

Given that the clinical signs in dogs with CIE are common to many other diseases, a sequential diagnostic evaluation should be initiated, with non-invasive diagnostic tests (including routine clinicopathological parameters, fecal parasitology, and diagnostic imaging of the abdomen) performed first before more invasive diagnostic tests are being considered. The owners need to be educated that the entire process of sequential steps might take some time and require patience, increasing owner compliance along that path (WASHABAU et al. 2010, DANDRIEUX 2016, ERDMANN and HEILMANN 2017).

2.3.1. Clinical and Clinicopathologic Approach

A semi-objective method to assess the severity of clinical disease in dogs with suspected or confirmed CIE is the use of a clinical disease activity index: the canine inflammatory bowel disease activity index (CIBDAI) (JERGENS et al. 2003) or the canine chronic enteropathy clinical activity index (CCECAI) (ALLENSPACH et al. 2007). These indices are quantitative and repeatable measures that can help assess the clinical disease severity at the time of first evaluation and determine the clinical response to treatment (JERGENS et al. 2003).

The CIBDAI scoring system consists of six clinical parameters (attitude/activity, appetite, vomiting, stool consistency, defecation frequency, and weight loss) that are evaluated on a scale from 0 (normal) to 3 (severely abnormal). The sum of the results for the individual criteria are reported as the cumulative CIBDAI score, where a score of 0–3 reflects clinically insignificant disease, a score of 4–5 mild clinical disease, a score of 6–8 moderate clinical disease, a score of 9–11 severe clinical disease, and a score of ≥12 very severe clinical disease (ALLENSPACH et al. 2007). The CCECAI scoring system is similar to the CIBDAI system. However, it includes assessing three additional criteria: serum albumin level, the presence of ascites and/or peripheral edema, and pruritus (Table 1). The CCECAI score has predictive value for the success of treatment, and a cut-off value of 12 was shown to be the best predictor for a negative outcome (ALLENSPACH et al. 2007).

CCECAI (Canine Chronic Enteropathy Clinical Activity Index)	
Criteria	**Assessment**
Attitude/Activity	0 = normal
	1 = slightly decreased
	2 = moderately decreased
	3 = severely decreased
Appetite	0 = normal
	1 = slightly decreased
	2 = moderately decreased
	3 = severely decreased
Vomiting	0 = normal
	1 = mild (1×/wk)
	2 = moderate (2-3×/wk)
	3 = severe (>3×/wk)
Stool consistency	0 = normal
	1 = slighty soft feces
	2 = very soft feces
	3 = watery diarrhea
Stool frequency	0 = normal
	1 = slightly increased (2-3×/d) or fecal blood, mucus or both
	2 = moderately increased (4-5×/d)
	3 = severely (>5×/d)
Weight loss	0 = none
	1 = mild (<5%)
	2 = moderate (5-10%)
	3 = severe (10%)
Serum albumin concentration	0 = serum albumin concentration >20 g/L
	1 = serum albumin concentraion 15-19 g/L
	2 = serum albumin concentration 12-14 g/L
	3 = serum albumin concentration <12 g/L
Ascites and/or Peripheral edema	0 = none
	1 = mild ascites or peripheral edema
	2 = moderate amount of ascites/peripheral edema
	3 = severe ascites/pleural effusion/peripheral edema
Pruritus	0 = none
	1 = occasional
	2 = regular episodes of itching, but stop when dog asleep
	3 = dog regularly wakes up because of itching

Table 1: Individual parameters of the CCECAI scoring system (ALLENSPACH et al. 2007).

Routine clinicopathological parameters are the next diagnostic step, and the minimum database (MDB) should consist of a complete blood cell count (CBC), serum biochemistry profile (including BUN and creatinine; alanine aminotransferase, alkaline phosphatase, and γ-glutamyltransferase activity; total bilirubin, total protein, albumin, total calcium, cholesterol, and triglyceride concentrations; sodium, potassium, and chloride concentrations), urinalysis (with a urine protein-to-creatinine ratio and/or bacterial culture of urine with antimicrobial susceptibility testing, if indicated), and a fecal parasitology (Fig. 7). While there usually will be no specific findings pointing to a diagnosis of CIE, the MDB is essential to rule out extra-gastrointestinal etiologies and assess the overall condition of the patient (ERDMANN and HEILMANN 2017).

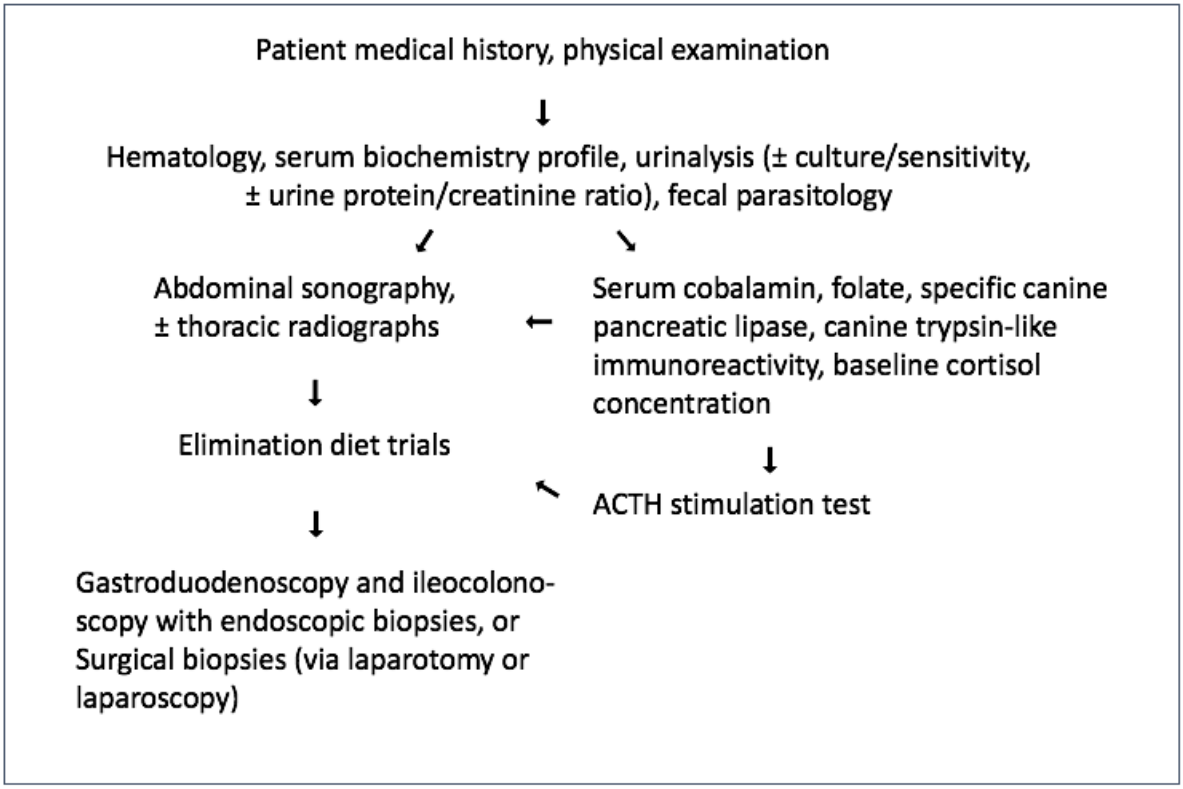

Figure 7: Diagnosis scheme for a complete clarification of a chronic enteropathy.

A gastrointestinal profile can be performed to further evaluate the dog for pancreatic and chronic small intestinal disease. Serum canine trypsin-like immunoreactivity (cTLI) can rule out a diagnosis of exocrine pancreatic insufficiency (EPI) (ERDMANN and HEILMANN 2017). Serum specific canine pancreatic lipase (Spec cPL) concentration is a marker for pan-

creatitis (ERDMANN and HEILMANN 2017). The water-soluble B vitamins cobalamin (vitamin B_{12}) and folic acid (vitamin B_9) are markers of gastrointestinal absorptive function (see 2.3.3.1. and 2.3.3.2.) (ERDMANN and HEILMANN 2017, KATHER et al. 2020). An increased serum Spec cPL concentration is a negative prognostic factor in dogs with CIE, specifically SRE/IRE (KATHRANI et al. 2009). A serum baseline cortisol concentration (and if indicated, an ACTH stimulation test) can help exclude atypical hypoadrenocorticism (atypical Addison's disease) as a differential diagnosis (LATHAN and THOMPSON 2014), and a bile acid stimulation test can be used to further evaluate the patient for a possible hepatopathy (Fig. 7).

2.3.2. Diagnostic Imaging of the Abdomen

Another critical part of the diagnostic work-up of dogs with suspected CIE is abdominal imaging. Abdominal radiographs can be useful to rule out other disease processes (e.g., chronic gastrointestinal foreign bodies, large intraabdominal masses, or decreased serosal detail with the presence of ascites). Abdominal ultrasonography is a more sensitive technique to evaluate the gastrointestinal tract, mesenteric lymph nodes, liver, and pancreas. Structural changes of organs (e.g., intestinal wall thickness or loss of layering, intussusceptions, or solitary neoplasia) can also be detected (Fig. 8–9) or ruled out as possible causes of chronic gastrointestinal signs.

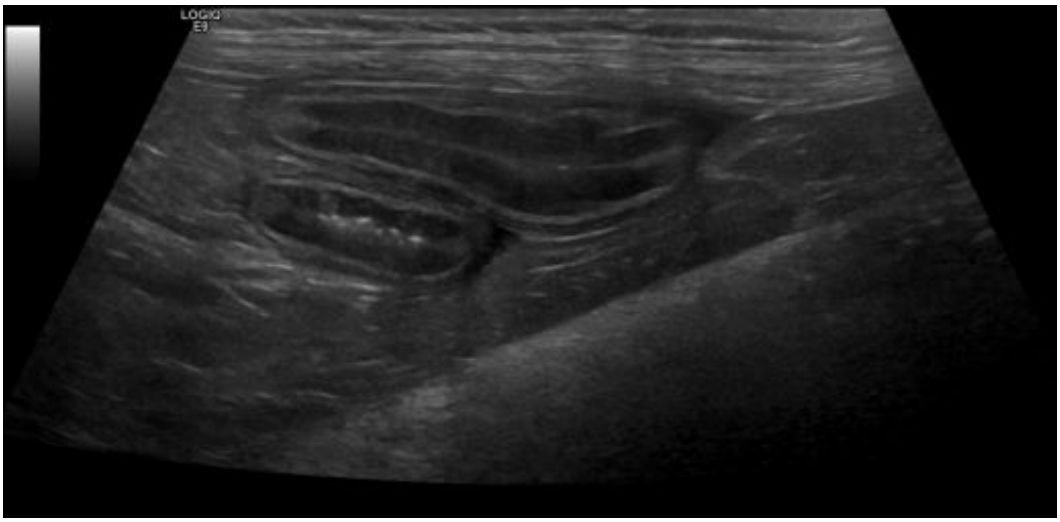

Figure 8: Abdominal ultrasonography in a dog with PLE due to SRE/IRE. Increased intestinal wall thickness (characterized predominantly by an enlarged mucosal layer) and a scant amount of free peritoneal fluid surrounding the intestinal loops are seen. Hyperechogenic striations oriented perpendicular to the intestinal lumen are also detected within the mucosa. © Small Animal Clinic, Veterinary Teaching Hospital, College of Veterinary Medicine, University of Leipzig.

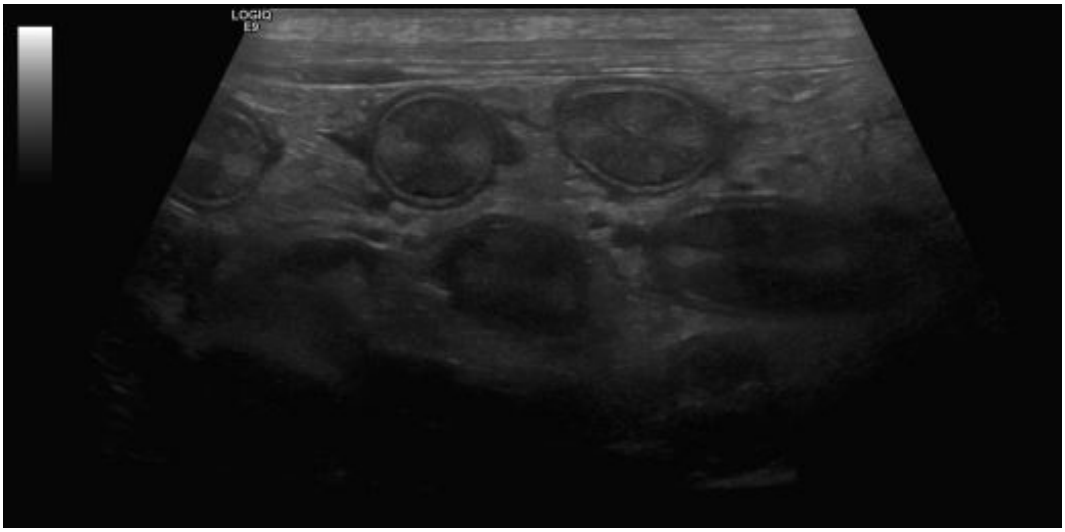

Figure 9: Abdominal ultrasonography in a dog with PLE. A generalized hyperechogenicity is noted throughout the abdominal cavity. Anechoic free peritoneal fluid surrounding the intestinal loops is also detected. The mucosa is diffusely thickened (with the intestinal wall layering being preserved) and has a heterogeneous appearance with multifocal hyperechogenic areas (stipples). © Small Animal Clinic, Veterinary Teaching Hospital, College of Veterinary Medicine, University of Leipzig.

2.3.3 Laboratory Tests for Gastrointestinal Disease

Several biomarkers of gastrointestinal function, inflammation, or protein loss can be useful surrogate tests in the clinical evaluation or monitoring of dogs with suspected or confirmed CIE (HEILMANN and STEINER 2018). These biomarkers include serum cobalamin (vitamin B_{12}), folate (vitamin B_9), and C-reactive protein (CRP) concentrations; and serum and fecal calprotectin (S100A8/A9 complex), S100A12 protein, and alpha$_1$-proteinase inhibitor (α_1PI) concentrations (JERGENS et al. 2010, HEILMANN et al. 2014a, HEILMANN et al. 2016a, HEILMANN et al. 2018). Notably, decreased serum concentrations of cobalamin and albumin are negative prognostic indicators in canine CIE (ALLENSPACH et al. 2007).

2.3.3.1 Serum cobalamin (vitamin B_{12})

Vitamin B_{12} exerts its effect on various cobalamin-dependent enzymes. This vitamin plays an essential role as a cofactor of the methylmalonyl-coenzyme A-mutase, necessary for the transformation of propionate to succinate, and tetrahydrofolate-methyltransferase, which is essential in the metabolism of methionine.

Cobalamin is bound to dietary proteins and is digested in the proximal gastrointestinal tract (stomach and duodenum) by gastric and pancreatic proteases. Because cobalamin cannot be

absorbed in its unbound (free) form, it must be complexed to a specific glycoprotein, the intrinsic factor (IF). In dogs, it is predominantly produced by the exocrine pancreas. The cobalamin-IF complex is then absorbed in the distal small intestine (ileum). Dogs, unlike ruminants and other herbivores, are not able to synthesize cobalamin. Thus, cobalamin has to be either ingested with the diet or supplemented in canines (KATHER et al. 2020).

Hypocobalaminemia or cobalamin deficiency can indicate a chronic enteropathy involving at least the distal small intestine (BATT et al. 1982, KATHER et al. 2020). However, no further conclusions regarding the type of chronic enteropathy (e.g., CIE *versus* small-cell intestinal lymphoma), disease extent and severity, or disease location(s) can be drawn from the serum cobalamin concentration alone (KATHER et al. 2020). Also, a normal serum cobalamin status does not exclude the possibility of a chronic intestinal disease process (ERDMANN and HEILMANN 2017). Hypocobalaminemia in dogs with CIE is defined as a serum cobalamin concentration <350–400 ng/L (KATHER et al. 2020). Cobalamin supplementation (parenteral or oral route) is indicated in dogs with a suboptimal serum cobalamin status (KATHER et al. 2020). Hypercobalaminemia (without prior supplementation) is infrequently seen in dogs with CIE, but the clinical or prognostic importance of this finding remains to be elucidated (SIELSKI et al. 2020).

In patients with a serum cobalamin concentration of less than 400 ng/L, cyanocobalamin supplementation is recommended. Two supplementation protocols exist, via parenteral and oral route. The dose of cobalamin does not vary between protocols and is recommended at 50 µg/kg. With the parenteral protocol, cobalamin is administered weekly for at least 6 weeks and another dose one month later (KATHER et al. 2020). Oral administration of cobalamin is daily over at least 12 weeks (KATHER et al. 2020). Serum cobalamin concentration should be checked one month after the last dose.

2.3.3.2. Serum folic acid (vitamin B_9)

Folic acid is also a water-soluble B vitamin involved in tissue growth and cell division (e.g., erythrocyte formation) in the body. This vitamin is abundant in commercial dog foods. Thus, a dietary deficiency is extremely rare in dogs. Hypofolatemia or folic acid deficiency is an indicator of chronic disease affecting the proximal small intestine (BATT et al. 1982, HEILMANN and STEINER 2018).

Because folic acid acts as a cofactor in some cobalamin-dependent enzymatic processes, it can accumulate in cobalamin deficiency states, resulting in falsely higher (or falsely normal) serum concentrations of folic acid (ERDMANN and HEILMANN 2017).

Folic acid supplementation (200–400 µg once daily for 7–28 days) is recommended for dogs with moderate to marked hypofolatemia.

2.3.3.3. Serum C-reactive protein (CRP)

CRP is produced by hepatocytes due to systemic inflammation, and this biomarker has no specificity for the gastrointestinal tract. A previous study (JERGENS et al. 2003) found a correlation between the severity of clinical disease activity and the concentration of CRP in serum and that serum CRP concentrations decrease with a successful response to immunomodulatory treatment. However, other studies (ALLENSPACH et al. 2007, OTONI et al. 2017) contradict these findings because the decrease in serum CRP concentrations was not directly related to the clinical improvement of patients, given that an increase in CRP in serum can be influenced by different causes including infectious, neoplastic, and inflammatory conditions (BUSER et al. 2019).

2.3.3.4. Fecal calprotectin and S100A12 protein

Calprotectin, also known as the S100A8/A9 protein complex, is released from neutrophils and activated macrophages, whereas S100A12 is a proinflammatory protein secreted by granulocytes (FOELL et al. 2008, FOELL et al. 2009). If measured in serum, both biomarkers are not specific for a particular (localized) inflammatory disease. However, both markers (if increased) can indicate an acute or chronic gastrointestinal inflammatory process (HEILMANN and STEINER 2018). Dogs with CIE also have increased mucosal expression levels of calprotectin and S100A12 compared to healthy individuals (HEILMANN et al. 2019). Measurement of calprotectin concentrations in feces can help detect dogs with CIE and predict a suboptimal clinical response to treatment in dogs with SRE/IRE (HEILMANN et al. 2018). Fecal concentrations of S100A12 can also indicate intestinal inflammation and severe endoscopic lesions in the small intestine in dogs and help distinguish FRE cases from SRE/IRE (HEILMANN et al. 2016).

2.3.3.5. Fecal alpha$_1$-proteinase inhibitor (α_1PI)

In contrast to albumin, alpha$_1$-proteinase inhibitor (α_1PI) is not enzymatically degraded during the intestinal passage. This allows for the extraction and quantification of α_1PI in fecal samples (HEILMANN et al. 2011). Fecal α_1PI concentrations reflect histopathological lesions in the intestinal mucosa that are typically seen with a PLE (lacteal dilation, crypt abscesses). Increased

fecal α_1PI concentrations can be used as an early indicator of gastrointestinal protein loss before clinical or clinicopathological signs of a PLE occur (HEILMANN and STEINER 2018).

2.4 Gastrointestinal Histopathology

Obtaining gastrointestinal tissue biopsies is needed to document inflammation and assess the quality and severity of inflammatory infiltration. The quality of tissue samples and the goal to obtain samples representing mucosal lesions in the sampled segments of the gastrointestinal tract is critical to arriving at a definitive and correct diagnosis in any disease of the digestive tract (WILLARD et al. 2008). Gastrointestinal tissue biopsies can be obtained by choosing one of three different methods: endoscopic biopsy, laparoscopic biopsy, or a surgical approach via celiotomy. Each method of biopsy has advantages and disadvantages.

An advantage of endoscopy over surgical biopsy is the visualization of the intestinal mucosa allowing the endoscopist to obtain samples directly from affected and macroscopically normal areas (Fig. 10).

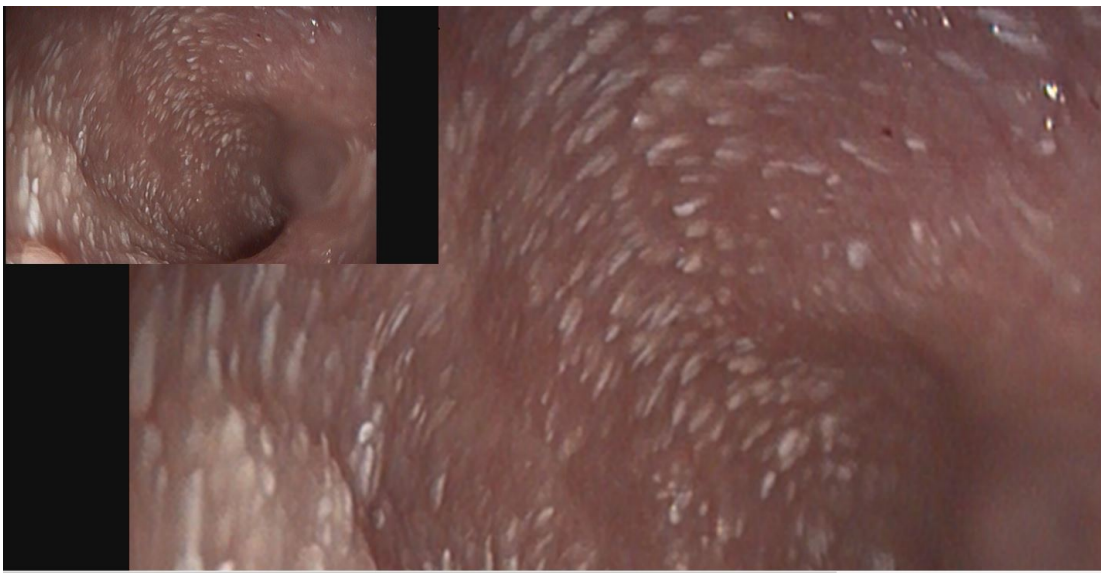

Figure 10: Endoscopic view of the duodenum in a dog with protein-losing enteropathy (PLE). The multifocal white spots represent macroscopically enlarged lacteals (intestinal mucosal lymphangiectasia). © Small Animal Clinic, Veterinary Teaching Hospital, College of Veterinary Medicine, University of Leipzig.

Further, endoscopy allows for the acquisition of multiple (at least 10–15) mucosal biopsies that may be more representative of the lesions in the sampled segment than an individual full-thickness biopsy. Endoscopy also has a minimal risk of perforation (WOOLHEAD et al. 2020). However, the disadvantages of endoscopy are that superficial mucosal samples are obtained

with the potential to miss some pathologies. Laparoscopy or celiotomy allows for full-thickness gastric and small intestinal biopsies to be obtained and other intraabdominal organs (e.g., liver, pancreas, or lymph nodes) to be sampled but is more invasive and carries the risk of suture dehiscence. Also, anti-inflammatory or immunosuppressive treatment may be delayed with this approach (ERDMANN and HEILMANN 2017).

Histopathologic evaluation of gastrointestinal tissue biopsies is performed according to the *Endoscopic, Biopsy, and Histopathologic Guidelines for the Evaluation of Gastrointestinal Inflammation in Companion Animals* established by an initiative of the *World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group* and released by the *American College of Veterinary Internal Medicine* as a consensus statement (WASHABAU et al. 2010). Using this standard histopathologic evaluation scheme (DAY et al. 2008), each of the inflammatory and structural criteria are assessed and assigned a score between 0 (normal) and 3 (severely abnormal) (WASHABAU et al. 2010, DAY et al. 2008):

- score 0 = normal
- score 1= mildly abnormal
- score 2 = moderately abnormal
- score 3 = severely abnormal.

However, the recognition and interpretation of inflammatory and structural changes in endoscopic biopsies can be challenging even for board-certified and experienced veterinary pathologists (WILLARD et al. 2002 and 2010). Also, normal gastrointestinal histology can be affected by various factors (e.g., age, diet, medications) that can vary from patient to patient (BAUM et al. 2007).

2.5 Pattern Recognition Receptors

The innate immune system is equipped with receptors called pattern recognition receptors (PRRs). These receptors recognize exogenous pathogen-associated molecular pattern (PAMP) molecules as well as endogenous damage-associated molecular pattern (DAMP) molecules (HEILMANN and ALLESPACH 2017). There are many different PRRs, for example, Toll-like receptors (TLR) such as TLR1, TRL2, TLR3, TLR4, TLR5, TLR6, TRL7, TLR8, TLR9, TLR10, TLR11, TLR12, and TLR13, nucleotide-binding oligomerization domain (NOD)-like receptors such as NOD2, and the receptor for advanced glycation end products (RAGE). PRRs can be located either on the cell surface (e.g., full-length RAGE, TLR 4, or TLR 5) or within cells (e.g., NOD2) (HEILMANN and ALLENSPACH 2017). PRRs generally consist of several domains but have an individual sequence of different domains and associated functions.

Dysregulations in the signaling cascades of PRRs have been linked to the development of chronic primary inflammatory or autoimmune diseases (BIERHAUS et al. 2005, WALSH et al. 2013). The innate immunity also appears to play a key role in the complex pathogenesis of CIE in dogs (JERGENS and SIMPSON 2012, HEILMANN and SUCHODOLSKI 2015, SCHMITZ et al. 2015, HEILMANN and ALLESPACH 2017). Genomic and also functional dysregulations have been demonstrated for TLR2, TLR4, and TLR9 (genetic polymorphisms and increased mucosal expression), TLR5 (genetic polymorphisms and decreased mucosal expression), NOD2 (single-nucleotide polymorphisms), and soluble RAGE (decreased serum levels) in dogs with CIE (ALLENSPACH et al. 2010, KATHRANI et al. 2012, KATHRANI et al. 2014.) and some of these changes were related to the clinical disease activity (assessed by the CCECAI scoring system). These findings agree with genetic polymorphisms and functional aberrations in human patients with IBD (Crohn's disease and ulcerative colitis) (HEILMANN and ALLENSPACH 2017). Further research in the area of PRR in canine CIE is warranted to better characterize the innate immune arm of CIE pathogenesis and explore potential novel (and more targeted) treatment strategies (HEILMANN and ALLESPACH 2017).

2.5.1 Receptor for Advanced Glycation End Products

The receptor for advanced glycation end products (RAGE) belongs to the group of innate immune PRRs. It can recognize both exogenous PAMPs and endogenous DAMPs released with inflammation and tissue damage (BIERHAUS et al. 2005, HEILMANN and ALLENSPACH 2017).

The complete (full-length) or transmembrane form of RAGE (Fig. 11) consists of five receptor domains (HEILMANN and ALLENSPACH 2017):

- 1 variable (V-type) domain for ligand binding,
- 2 constant (C-type) domains,
- 1 transmembrane domain (anchor), and
- 1 cytoplasmic tail domain (which initiates post-receptor signaling).

Full-length RAGE is a type I transmembrane glycoprotein that is constitutively expressed on the surface of certain cells (e.g., alveolar cells). In contrast, its expression in other cells (e.g., macrophages) is induced by the accumulation of RAGE ligands (i.e., the existence of a proinflammatory microenvironment) or the activation of certain transcription factors (e.g., nuclear factor-κB) (BIERHAUS et al. 2005, MEIJER et al. 2014, HEILMANN and ALLENSPACH 2017). In addition to the complete transmembrane (full-length) receptor, there are soluble forms of RAGE (sRAGE) from which the cytoplasmic portion is missing. Soluble RAGE functions

as a decoy receptor through its ability to bind and capture proinflammatory RAGE ligands (Fig. 12). The canine *RAGE* gene and its gene products have been characterized, and there are 24 currently known variants of this receptor in dogs (STERENCZAK et al. 2009).

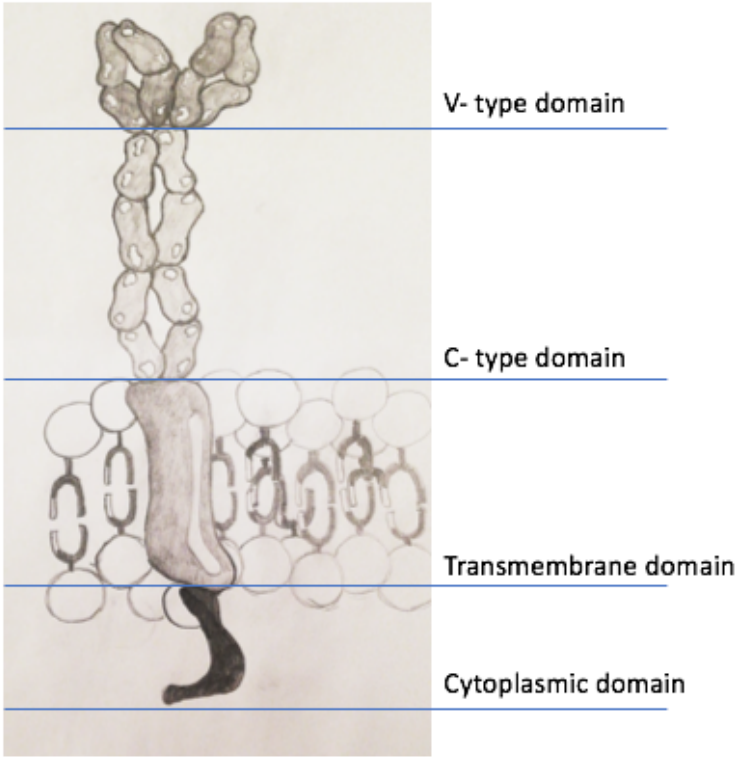

Figure 11: Structure of the transmembrane (full-length) receptor for advanced glycation end products (RAGE).

RAGE plays an important role in the pathogenesis of chronic inflammatory diseases in humans. Sustained RAGE-ligand interaction was suggested to be associated with the perpetuation and amplification of the proinflammatory immune response in autoimmune diseases, such as human IBD (BIERHAUS et al. 2005). Shortened isoforms of RAGE, such as the decoy receptor sRAGE that result from alternative splicing or proteolytic cleavage (MEIJER et al. 2014, HEILMANN and ALLENSPACH 2017) can abrogate the RAGE-ligand interaction (Fig. 12) and thus modulate downstream signaling (BIERHAUS et al. 2005).

A recent study that investigated the intestinal expression of RAGE in human IBD and also the effect of RAGE-blockade by the specific inhibitor FPS-ZM1 (N-Benzyl-N-cyclohexyl-4-chlorobenzamide) in a murine experimental model of human IBD (BODY-MALAPEL et al. 2019) showed RAGE to be expressed in the colon and small intestine in people as well as in mice and to be upregulated with inflammatory conditions (Crohn's disease and ulcerative colitis). RAGE-blockade by FPS-ZM1 administration was associated with less severe enteritis

in the ileum and jejunum (BODY-MALAPEL et al. 2019). FPS-ZM1 binds specifically to domain 5 and blocks RAGE-binding of some DAMPs (e.g., S100B and high-mobility group box 1 [HMGB1] protein). Thus, further investigation of the RAGE/sRAGE axis might be a valuable in the ileum and jejunum (BODY-MALAPEL et al. 2019). FPS-ZM1 binds specifically to domain 5 and blocks RAGE-binding of some DAMPs (e.g., S100B and high-mobility group box 1 [HMGB1] protein). Thus, further investigation of the RAGE/sRAGE axis might be a valuable route to explore potential novel therapeutic strategies (BODY-MALAPEL et al. 2019, BRAMHALL et al. 2020).

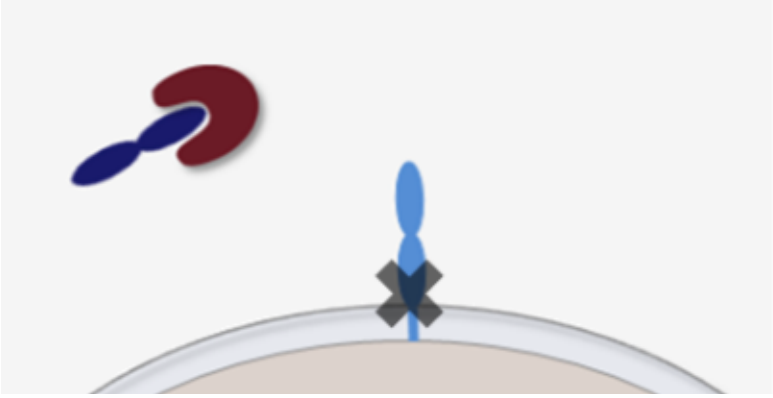

Figure 12: Soluble RAGE (sRAGE) competes with complete (full-length) transmembrane RAGE for ligand binding. Modified from: Heilmann RM, PhD Thesis, Texas A&M University, TX, USA (2015).

The expression of RAGE has also been investigated in canine lymphoma and histiocytic sarcoma (STERENCZAK et al. 2010, STERENCZAK et al. 2011). In the last study, STERENCZAK et al. (2011) showed that the RAGE-HMGB1 complex plays an important role in the maturation, migration, and immune response of dendritic cells. This finding suggests that the dysregulation of RAGE pathways is involved in the progression of histiocytic disorders such as disseminated histiocytic sarcoma (a very aggressive neoplasm of dendritic cells) in dogs. Dysregulations of RAGE-HMGB1 are also associated with other neoplastic conditions in humans (e.g., cholesteatoma and pancreatic, prostate, and colonic neoplasia) (SCZPANSKI et al. 2014 and ZHAO et al. 2014).

Conversely, downregulation of RAGE expression has been demonstrated in non-small cell lung cancer (BARTLING et al. 2005). Less RAGE expression was associated with higher histological stages of cancer and overexpression of RAGE with decreased tumor growth. This

suggests RAGE regulation to differ between different tissues (e.g., lung *versus* hematopoietic or histiocytic cells) and linked to physiological levels of post-natal RAGE expression in these tissues.

A previous pilot study in dogs showed that serum sRAGE concentrations are significantly (and in some cases severely) decreased with CIE, particularly SRE/IRE, compared to serum sRAGE levels in healthy dogs (HEILMANN et al. 2014b). It was hypothesized that this finding results from an increased consumption of this decoy receptor (i.e., an attempt to abrogate intestinal proinflammatory RAGE signaling) in affected dogs. However, it is also possible that serum sRAGE deficiency contributes to the pathogenesis of canine CIE or SRE/IRE. Interestingly, the same study showed that serum sRAGE concentrations increased or returned to normal (i.e., serum sRAGE levels detected in healthy controls) only in dogs that achieved clinical remission during the induction phase of treatment (HEILMANN et al. 2014b). However, intestinal tissue RAGE expression has not been investigated in canine CIE. Research to further investigate the RAGE/sRAGE axis and its potential for developing targeted (pathway-specific) treatment options in canine CIE – a good spontaneous model for human IBD (JERGENS and SIMPSON 2012) – might also benefit human IBD research.

2.6 Aims and Hypotheses

The aim of this study is to determine serum sRAGE concentrations in canine CIE and their association with histological and clinicopathological evidence of CIE, including serum and fecal biomarkers of gastrointestinal inflammation or protein loss. Further, the expression of full-length RAGE along the gastrointestinal tract (stomach, duodenum, ileum, and colon) is evaluated in canine CIE by immunofluorescence analysis. Quantitative expression of RAGE in the gastrointestinal epithelium in dogs with CIE is compared to gastrointestinal RAGE expression levels in healthy control dogs. It is evaluated for the possibility of an association with serum sRAGE concentrations and with clinical, clinicopathological, and histological markers of gastrointestinal disease.

The study evaluates the hypothesis that (1) gastrointestinal epithelial RAGE is overexpressed in dogs with CIE and (2) gastrointestinal epithelial RAGE expression and serum sRAGE concentrations correlate with the severity of clinical, clinicopathologic, and histologic evidence of canine CIE.

3 OWN PUBLICATIONS

My contribution to this part of the dissertation consisted of staining tissue samples and evaluating the intestinal mucosal expression of RAGE (receptor for advanced glycation end-products), analyzing patient data and disease outcomes, and interpreting the results of the statistical analyses. I wrote these manuscripts.

3.1 FIRST PUBLICATION

Association between serum soluble receptor for advanced glycation end-products (RAGE) deficiency and severity of clinicopathologic evidence of canine chronic inflammatory enteropathy

Angela Isabel Cabrera-García,[1] Jan S. Suchodolski[2], Jörg M. Steiner[2], Romy M. Heilmann[1]

[1]Department for Small Animals, Veterinary Teaching Hospital, College of Veterinary Medicine, University of Leipzig, Leipzig, Saxony, Germany (Cabrera-García, Heilmann); [2]Gastrointestinal Laboratory, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX (Suchodolski, Steiner).

[1]Corresponding author: Angela Isabel Cabrera-García, Department for Small Animals, Veterinary Teaching Hospital, College of Veterinary Medicine, University of Leipzig, An den Tierkliniken 23, Leipzig, SN, DE-04103, Germany. isabel.garcia@kleintierklinik.uni-leipzig.de

Citation: Angela Isabel Cabrera-García, Jan S. Suchodolski, Jörg M. Steiner, Romy M. Heilmann. Association between serum soluble receptor for advanced glycation end-products (RAGE) deficiency and severity of clinicopathologic evidence of canine chronic inflammatory enteropathy. J Vet Diagn Invest. 2020; 32(5): 664–674.

Editor: Paula Krimer-Rollison, DVM, DVSc, DACVP (Clinical Pathology)
Received: November 18, 2019
Accepted: April 24, 2020
Published: July 27, 2020

Abstract. Innate immunity plays a central role in the pathogenesis of chronic inflammatory enteropathies (CIE) in dogs, and further evaluation of the innate immune receptor for advanced glycation end-products (RAGE) is warranted. We measured serum concentrations of decoy receptor soluble RAGE (sRAGE) in 102 dogs diagnosed with CIE, and evaluated relationships with clinical disease severity, histologic lesion severity, concentrations of serum C-reactive protein (CRP), and serum and fecal calprotectin, S100A12, and alpha$_1$-proteinase inhibitor (α_1PI). Serum sRAGE levels were not associated with clinical disease activity, serum CRP, serum and fecal α_1PI, calprotectin, or S100A12 concentrations. Microscopic lesions in the du-

odenum were more severe in dogs with serum sRAGE concentration ≤340ng/L (p=0.013). Serum sRAGE levels were weakly and inversely correlated with the severity of lymphoplasmacytic infiltration in the gastric antrum and duodenum, and with crypt dilation and the neutrophilic infiltrate in the duodenum, in univariate analysis (all p<0.05), but none of the correlations remained statistically significant after correction for multiple comparisons. Our study confirms that CIE in dogs is associated with decreased serum sRAGE concentrations, suggesting a dysregulated sRAGE/RAGE axis.

Key words: canine; ELISA; inflammatory bowel disease; innate immunity; pattern recognition receptor; RAGE; receptor for advanced glycation end-products.

Introduction

Chronic inflammatory enteropathies (CIE) are important diseases in dogs, the diagnosis of which requires the presence of chronic gastrointestinal signs (≥ 2 wk), histopathologic evidence of intestinal mucosal inflammation, and the exclusion of other underlying causes.[2,8,34] Canine CIE are classified based on response to dietary changes (food-responsive enteropathy, FRE) and antibiotic trials (antibiotic-responsive enteropathy), or the response to anti-inflammatory or immunosuppressive treatment (immunosuppressant-responsive or -refractory enteropathy, IRE).[2,8,10] Several biomarkers of inflammation have been evaluated in dogs with CIE,[21] of which C-reactive protein (CRP), calprotectin (S100A8/A9 protein complex), S100A12 (calgranulin C) protein, and alpha$_1$-proteinase inhibitor (α_1PI) appear to have clinical utility.[14,17,20,23] Similar to inflammatory bowel disease (IBD) in humans, medical treatment of dogs with IRE usually involves corticosteroids and/or other immunosuppressive drugs;[2,8,10] more targeted treatment strategies would be desirable but are currently lacking.

Dysregulations of the innate immune response, in particular the signaling cascades of pattern recognition receptors (PRRs), have been linked to the development of chronic autoimmune diseases.[3,32] The innate immune system plays a key role in the complex pathogenesis of CIE,[16,19,27,34] and there is also evidence of PRR dysregulation in dogs with IRE.[19] Further research in this area is needed to better characterize the pathogenesis of CIE and to aid in the development of more targeted treatment strategies.[19]

The receptor of advanced glycation end-products (RAGE) is a PRR that recognizes pathogen-associated molecular patterns and endogenous molecular structures released at sites of inflammation or tissue damage.[3,19,32] Full-length RAGE is a type I transmembrane glycoprotein that is constitutively expressed on the surface of certain cells (e.g., alveolar cells); its expression in other cells (e.g., macrophages) is induced by the accumulation of RAGE ligands (i.e., existence of a proinflammatory microenvironment) or the activation of certain transcription factors (e.g., nuclear factor–κB).[3,19,25] Sustained RAGE-ligand interaction was suggested to be associated with the perpetuation and amplification of the proinflammatory immune response in autoimmune diseases, such as human IBD.[3,26] Shortened isoforms of RAGE, such as the decoy receptor soluble RAGE (sRAGE), which results from alternative splicing or proteolytic cleavage,[19,25]

can abrogate the RAGE-ligand interaction and thus modulate downstream signaling.[3,26] A 2019 experimental study also indicated that the RAGE signaling pathway plays a key role in intestinal inflammation as it promotes oxidative stress and endothelial activation, and that interference with the RAGE pathway presents a promising therapeutic target in patients with IBD.[4]

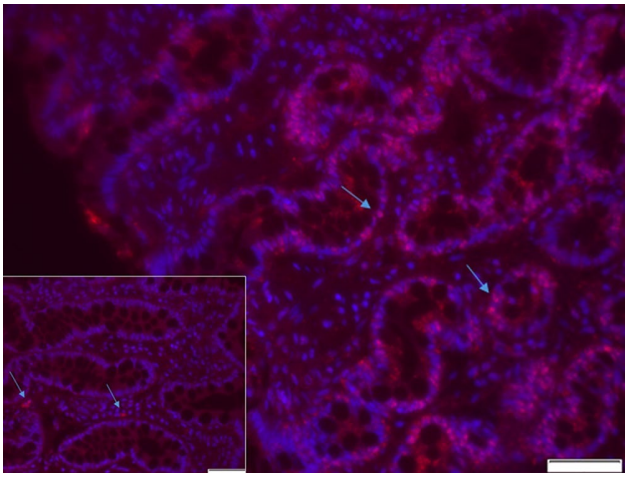

Figure 1. Intestinal mucosal expression of the receptor for advanced glycation end-products (RAGE). Fluorescence microscopy image of a colonic mucosal biopsy obtained from a dog diagnosed with moderate diffuse lymphoplasmacytic and eosinophilic chronic colitis. The image shows multifocal positive immunostaining (pink fluorescence; arrows) for RAGE that is located predominantly in the colonic epithelium. Bar = 50µm. Inset: representative negative control (primary antibody omitted) confirming absence of nonspecific staining. Arrows indicate artificial autofluorescence caused by erythrocytes. Bar = 50 µm.

The canine *RAGE* gene and its gene products have been characterized,[28] and the expression of RAGE in canine lymphoma and histiocytic sarcoma has been evaluated.[29,30] RAGE is also expressed in the intestinal mucosa in dogs (Fig. 1). In a pilot study, serum sRAGE concentrations were decreased in a small group of dogs with CIE compared to healthy controls and normalized only in those dogs that experienced full clinical remission during the induction phase of therapy.[15] However, the association between serum sRAGE, disease severity, and other inflammatory biomarkers has not been studied extensively in canine CIE. Thus, further evaluation of the sRAGE/RAGE axis in canine CIE is warranted. We hypothesized that serum sRAGE concentrations in dogs with CIE correlate with 1) the severity of clinical signs, 2) the severity of microscopic lesions, and 3) with the concentrations of serum and fecal biomarkers of inflammation or protein loss. To test these hypotheses, we evaluated serum sRAGE concentrations in a large group of dogs with CIE and correlated serum sRAGE concentration with 1) a clinical disease activity score, 2) a histologic disease score, and 3) the concentrations of serum CRP and serum and fecal calprotectin, S100A12, and α_1PI.

Materials and methods

Ethics approval

Our study was approved by the Clinical Research Review Committee (CRRC approval TAMU 2009-06, approved 01-15-2009) and the Institutional Animal Care and Use Committee at Texas A&M University (IACUC approval TAMU 2012-083, approved 05-22-2012). A letter of informed consent was signed by the owner to enroll dogs in the study.

Study population

We included 102 dogs diagnosed with CIE in our study (Fig. 2). These dogs were enrolled over a 61-mo period (Sept 2009– Oct 2014) and were recruited at the Veterinary Teaching Hospital at Texas A&M University (College Station, TX; $n = 6$) or at one of several small animal referral hospitals across the United States ($n = 96$). Inclusion criteria were the presence of gastrointestinal clinical signs for at least 2–3 wk, exclusion of other identifiable causes (i.e., atypical hypoadrenocorticism, exocrine pancreatic insufficiency), and intestinal mucosal infiltration with inflammatory cells confirmed by histopathology.[34] There were no restrictions on canine breed or age. The exclusion criteria were the diagnosis of other causes of the gastrointestinal clinical signs (e.g., alimentary lymphoma), the lack of gastrointestinal tissue biopsies, or insufficient sample material for serum sRAGE analysis. Some of the data from these dogs have been published previously.[20]

Routine diagnostic investigation of dogs included a physical examination; hematology (performed at the institution recruiting the case or at the Texas Veterinary Medical Diagnostic Laboratory), serum biochemistry profile (LiquiColor, Sirrus clinical chemistry analyzer; Stanbio Laboratory) with a bile acid stimulation test or an ACTH stimulation test if indicated, and urinalysis (with a urine culture and/or a urine protein-to- creatinine ratio if indicated); fecal endoparasite screen; abdominal diagnostic imaging (ultrasonography and/or radiographs); a gastrointestinal profile including serum canine trypsin-like immunoreactivity (cTLI), specific canine pancreatic lipase (Spec cPL), cobalamin ($n = 101$), folate ($n = 101$), and gastrin concentration. Hypoalbuminemia was defined as mild (serum albumin concentration 15–20 g/L), moderate (12–14.9 g/L), or severe (< 12 g/L), and dogs with a serum cobalamin concentration < 300 ng/L were classified as hypocobalaminemic.

At the time of evaluation, each patient (except for one dog) was assigned a canine chronic enteropathy clinical activity index (CCECAI) score,[1] which considers the general attitude and activity of the dog, appetite, frequency of vomiting, stool consistency and frequency of defecation, weight loss, serum albumin concentration, peripheral edema or ascites, and pruritus. Individual CCECAI criteria can range from 0–3 (0 = normal, 1 = slightly abnormal, 2 = moderately abnormal, 3 = severely abnormal), and the cumulative CCE- CAI score is interpreted as clinically insignificant disease (score 0–3), mild disease (score of 4–5), moderate disease (score of 6–8), severe disease (score of 9–11), or very severe disease (score of ≥ 12).[1]

Endoscopy of the gastrointestinal tract ($n = 94$) or laparotomy ($n = 8$) with collection of tissues biopsies was performed on each dog. Several biopsies were taken from different locations in

the gastrointestinal tract (stomach— range: 1–24, median: 12; duodenum—range: 1–29, median: 13; ileum—range: 1–22, median: 4; colon—range: 1–44, median: 14). The tissue samples were subjected to routine histologic evaluation,[9,34] through the Texas A&M University. Gastrointestinal Laboratory Histopathology service and were evaluated by 1 of 9 board-certified anatomic pathologists with special expertise in small animal gastrointestinal pathology. Tissue biopsies were histologically evaluated using the structural and inflammatory criteria of the World Small Animal Veterinary Association Gastrointestinal Standardization grading system.[9,34] A score of 0 was assigned to normal tissues, 1 for mild histologic lesions, 2 for moderate histologic lesions, and 3 for severe histologic changes. Individual and cumulative lesion scores (calculated as the sum of individual lesion scores of the stomach, duodenum, ileum, and colon) were considered for data and statistical analyses.

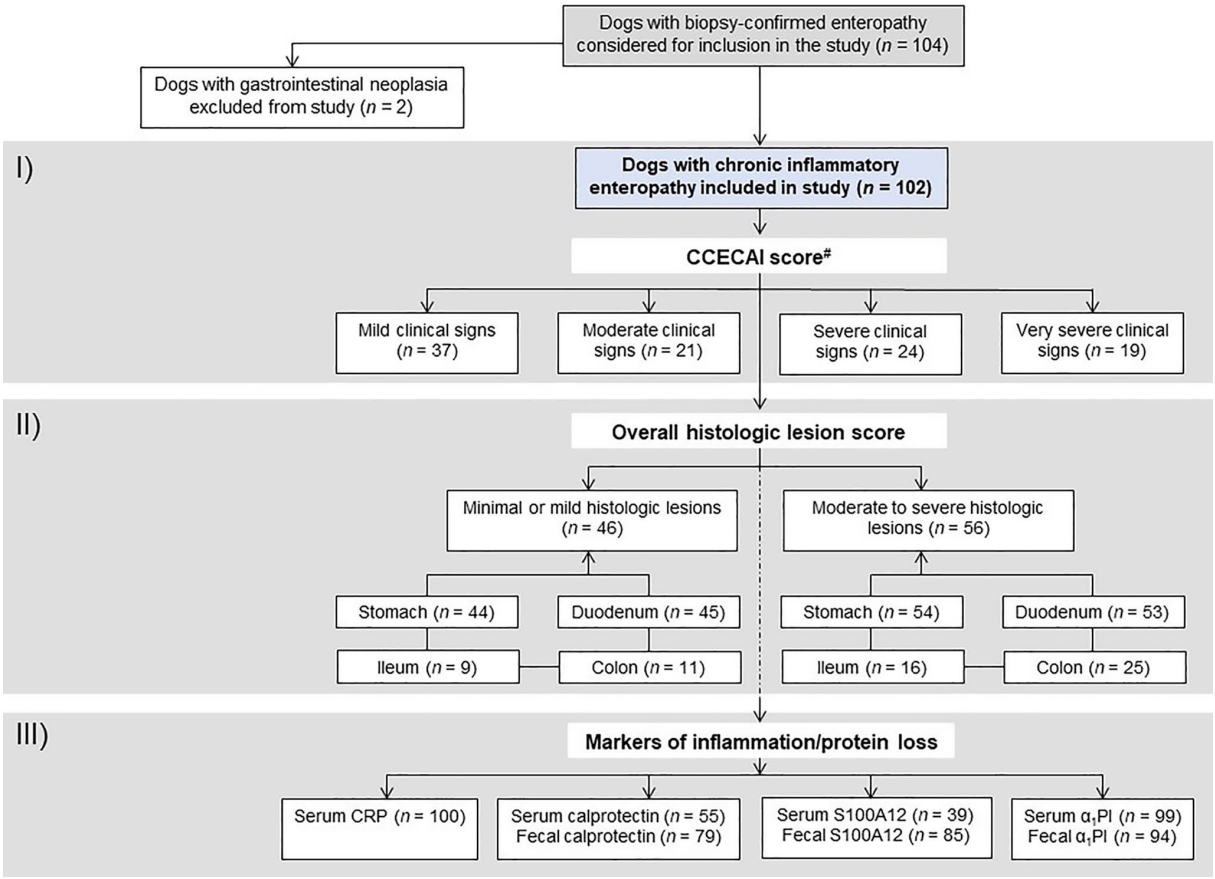

Figure 2. Study flowchart. Of the 104 dogs considered for inclusion in the study, 102 dogs were included. Data from all 102 dogs were included in the first part of the study (I: correlation with clinical disease severity) and in the second part of the study (II: correlation with the severity of histologic lesions); data from a subset of these dogs were included in the third part of the study (III: correlation with biomarkers of gastrointestinal inflammation or protein loss). Available from 101 dogs.

Follow-up samples and/or information were available for 20 dogs and for a period of 1–10 mo. Patients were diagnosed with FRE based on the clinical response to an elimination diet with a protein hydrolysate or a novel protein and carbohydrate source (2 of these 5 dogs had received antibiotic treatment without a clinical response), whereas dogs that required anti-inflammatory

or immunosuppressive treatment were classified as IRE (8 of these 15 dogs also received metronidazole or tylosin as part of their treatment plan).

Sample collection and analysis

Serum samples from dogs included in our study were also used for measurement of CRP (n = 100), calprotectin (n = 55), S100A12 (n = 39), and α PI (n = 99). In addition, calprotectin 1 (n = 79), S100A12 (n = 39), and α_1PI concentrations (n = 94) were measured in fecal samples from 3 consecutive days. Not all markers could be analyzed in samples from all dogs given the amount of sampling material available.

Serum sRAGE concentrations were measured in archived samples (stored at −80°C for 1–72 mo) by ELISA as described previously.[15] Briefly, 96-well plates were coated with 150 ng of polyclonal anti-canine RAGE antibody (sheep anti- recombinant canine RAGE; R&D Systems). Following a wash step, nonspecific binding sites were blocked with 30% (v/v) newborn calf serum and 1% (v/v) Triton X-100 (Thermo Fisher) in phosphate-buffered saline (PBS; assay buffer). Plates were then incubated with test samples (diluted 1 in 2 [50% each] in assay buffer, each sample tested in duplicate), calibrator solutions with different canine RAGE concentrations (5,000, 2,000, 1,000, 500, 200, 100, and 20 ng/L in assay buffer), and assay blanks (assay buffer). After another wash step, plates were incubated with a biotinylated poly- clonal anti-canine RAGE antibody (60 ng/well; R&D Systems) and were washed again. NeutrAvidin–horseradish peroxidase (NA-HRP; Thermo Fisher Scientific) in PBS with 1% (w/v) bovine serum albumin (20 ng/well) was added to each plate, and after a wash step each well received a stabilized 3,3',5,5'-tetramethylbenzidine substrate. After 10min, the reaction was stopped by adding 4M acetic acid, and the absorbance in each well was measured at 450 nm by use of an automated plate reader. A 5-parameter logistic curve fit was used to determine canine serum sRAGE concentrations in test samples. The limit of detection (LOD) of the assay has previously been reported at 52 ng/L.[15]

Serum CRP concentrations were measured using a commercial ELISA (Phase CRP; Tri-Delta Diagnostic). Calprotectin and S100A12 concentrations in serum and fecal samples were tested using previously established and analytically validated species-specific sandwich ELISAs;[11,18] serum and fecal α_1PI were measured using an in-house radioimmunoassay.[12,13]

Statistical analysis

Normality of the data and equality of variances were tested by using a Shapiro–Wilk test and a Brown–Forsythe test, respectively. Summary statistics are presented as median and interquartile ranges (IQR) or ranges. Serum sRAGE concentrations were compared among the different groups of dogs using nonparametric group comparisons (Wilcoxon rank-sum test or Kruskal–Wallis test with Dunn post-hoc comparisons). A potential relationship of serum sRAGE levels with clinical disease activity (CCECAI scoring system), histologic lesion severity (4-point semi-quantitative grading system), and serum and fecal inflammatory marker concentrations was tested using a likelihood ratio test for association or calculation of a Spearman rank-sum correlation coefficient ρ. Sensitivity and specificity were calculated as the true-positive and true-negative rate, respectively (dichotomous data), or by construction of a receiver operating

characteristic curve with a Youden index for determination of the optimum cutoff value (continuous data). Statistical significance was set at $p \leq 0.05$, and a Holm sequential Bonferroni correction was applied for multiple comparisons.[22] A commercial statistical software package (JMP v.13; SAS) was used for all statistical analyses.

Results

Study population

A total of 102 dogs met the inclusion criteria (Fig. 2, Table 1). Dogs were 4–9y old, and there was an approximately even sex distribution. Most dogs were purebred (82%). Clinical disease activity (CCECAI) scores and histologic lesions scores varied from mild to severe or very severe. Approximately one third of the dogs were hypoalbuminemic (29%), and 33% of the dogs were hypocobalaminemic.

Serum sRAGE concentrations

Serum sRAGE concentrations were 52–3,260 ng/L (median: 287ng/L) in all CIE dogs, with 65 dogs (64%) having a serum sRAGE concentration ≤340ng/L (cutoff value that has previously been shown to provide the best separation of CIE dogs from healthy control dogs;[15] Table 1). Twelve dogs (12%) had a serum sRAGE concentration below the LOD of 52 ng/L. Prior corticosteroid treatment (p=0.150, Wilcoxon rank-sum test) or disease duration (p = 0.387, Spearman correlation analysis) did not affect serum sRAGE concentrations. Age was also not correlated with serum sRAGE concentrations (ρ=0.16, p=0.108), and serum sRAGE concentrations did not differ between purebred (median: 287ng/L, IQR: 153–432ng/L) and mixed-breed (median: 275 ng/L, IQR: 178–637 ng/L; p = 0.772) dogs.

Table 1. Characteristics of the 102 dogs included in our study of biomarkers of inflammation in cases of canine chronic inflammatory enteropathy.

Group characteristic	Value
Total number	102
Age in years, median (IQR)	6.8 (4–9)
Sex, male (neutered)/female (spayed)	50 (44)/52 (50)
Body weight in kg, median (IQR)	14.6 (6.5–24.8)
Dog Breed, n	
Purebreed	84 (82%)
German Shepherd	14 (14%)
Yorkshire Terrier	7 (7%)
Mixed breed	18 (18%)
Disease duration in months, median (IQR)	3 (1-8)
Prior glucocorticosteroid treatment	11 (11%)
Biopsy type, n	

Endoscopic	94 (92%)
Surgical	8 (8%)
No. of biopsies per site, median (IQR)	
Stomach (n = 89)	12 (10-16)
Endoscopic	13 (10-17)
Surgical	2 (1-4)
Duodenum (n = 90)	13 (8- 17)
Endoscopic	13 (11-17)
Surgical	1 (1)
Ileum (n = 22)	4 (1–11)
Endoscopic	8 (4-14)
Surgical	1 (1-2)
Colon (n = 28)	14 (10-17)
Endoscopic	15 (10-17)
Surgical	2 (1-2)
Histologic lesion score, median (IQR)	
Stomach	1 (0–2)
Duodenum	1 (1–2)
Ileum	2 (0–2)
Colon	1 (1–2)
Overall lesion score	2 (1–2)

CCECAI score, median (IQR)*	7 (5–11)
Clinical disease severity, n (%)	
Mild†	37 (37%)
Moderate‡	21 (21%)
Severe §	24 (24%)
Very severe‖	19 (19%)

Serum sRAGE concentration in ng/L, median (IQR)	287 (154–472)
Serum sRAGE concentration >340 ng/L, n (%)	37 (36%)
Serum sRAGE concentration ≤340 ng/L, n (%)	65 (64%)
Serum sRAGE concentration 52–340 ng/L, n (%)	53 (52%)
Serum sRAGE concentration ≤52 ng/L, n (%)	12 (12%)
Serum albumin concentration in g/L, median (IQR)	26 (19–30)
Hypoalbuminemia, n	29 (29%)
Mild#	14 (14%)
Moderate	9 (9%)
Severe**	6 (6%)
Serum cobalamin concentration in ng/L, median (IQR)*	319 (212–678)
Hypocobalaminemia††, n	33 (33%)
Serum folate concentration in µg/L, median (IQR)*	12.4 (9.4–16.0)

Hypofolatemia, n	16 (16%)
Hyperfolatemia, n	5 (5%)
Serum CRP concentration in mg/L, median (IQR) (n=100)	8.6 (1.2–26.6)
Fecal calprotectin concentration in µg/g, median (IQR) (n=79)	1.5 (0.5–17.0)
Serum calprotectin concentration in mg/L, median (IQR) (n=55)	6.3 (4.1–8.9)
Fecal S100A12 concentration in ng/g, median (IQR) (n=85)	131 (21.3–864)
Serum S100A12 concentration in µg/L, median (IQR) (n=39)	211 (148–339)
Fecal α_1PI concentration in µg/g, median (IQR) (n=94)	7.4 (3.8–23.8)
Serum α_1PI concentration in mg/L, median (IQR) (n=99)	1290 (1005–1493)

CCECAI = canine chronic enteropathy clinical disease activity index; CRP = C-reactive protein; IQR = interquartile range. [*]available from 101 dogs; [†]CCECAI score of 4–5, [‡]CCECAI score of 6–8, [§]CCECAI score of 9–11, [|]CCECAI score of ≥12; [#]defined as a serum albumin concentration 15–20 g/L; [¶]defined as a serum albumin concentration 12–14.9 g/L; [**]defined as a serum albumin concentration <12 g/L; [††]defined as a serum cobalamin concentration <300 ng/L

Correlation of serum sRAGE concentrations with clinical and histologic disease severity

Serum sRAGE concentrations were not associated with the severity of clinical signs (CCECAI scores; $\rho = -0.07$, $p = 0.460$). A higher maximum overall histologic lesion score was associated with a serum sRAGE concentration ≤ 340 ng/L (p=0.028; sensitivity: 73%, specificity: 48%), and microscopic lesions in the duodenum were more severe in dogs with serum sRAGE levels ≤340ng/L (p=0.013; Fig. 3). A cumulative microscopic lesion score in the duodenum of ≥ 4 was detected by a serum sRAGE concentration of ≤ 340 ng/L with a sensitivity of 57% and a specificity of 68%. Serum sRAGE was significantly and inversely correlated with the severity of lymphoplasmacytic infiltration in the gastric antrum ($\rho = -0.22$, $p = 0.038$) and duodenum ($\rho = -0.25$, $p = 0.017$), and with crypt dilation ($\rho = -0.23$, $p = 0.027$) and neutrophilic infiltrate in the duodenum ($\rho = -0.21$, $p = 0.044$), in univariate analysis, but none of the results remained statistically significant after correction for multiple comparisons (Table 2). No other correlations of histologic criteria with serum sRAGE concentrations were identified.

Correlation of serum sRAGE concentrations with other inflammatory biomarkers

Serum sRAGE concentrations were not associated with serum CRP concentrations ($n = 100$; $\rho = 0.04$, $p = 0.708$), nor with the concentration of serum ($n = 55$; $\rho = -0.08$, $p = 0.551$), or fecal calprotectin ($n = 79$; $\rho = 0.12$, $p = 0.277$), or fecal S100A12 ($n = 85$; $\rho = 0.17$, $p = 0.132$). Serum sRAGE and S100A12 concentrations were also not significantly correlated ($n = 39$; $\rho = -0.17$, $p = 0.095$; Fig. 4).

Correlation of serum sRAGE concentrations with other prognostic markers

Dogs with more moderate or severe hypoalbuminemia (i.e., a serum albumin concentration < 15 g/L) had numerically lower serum sRAGE concentrations (median: 242 ng/L, IQR: 76–340 ng/L) compared to dogs with normoalbuminemia or mild hypoalbuminemia (i.e., a serum albumin con- centration ≥ 15 g/L; median: 297 ng/L, IQR: 161–519 ng/L; Fig. 5), but this difference was not statistically significant ($p = 0.095$). There was also no significant difference in serum

sRAGE concentrations between dogs with hypocobalaminemia (i.e., a serum cobalamin concentration < 300 ng/L; median: 266 ng/L, IQR: 201–422 ng/L) and dogs with normocobalaminemia (i.e., serum cobalamin concentrations ≥ 300 ng/L; median: 296 ng/L, IQR: 140– 499 ng/L; $p = 0.891$). Also, there was no correlation between serum sRAGE and serum ($n = 99$; $\rho = -0.05$, $p = 0.640$) or fecal α_1PI concentrations ($n = 94$; $\rho = -0.15$, $p = 0.154$).

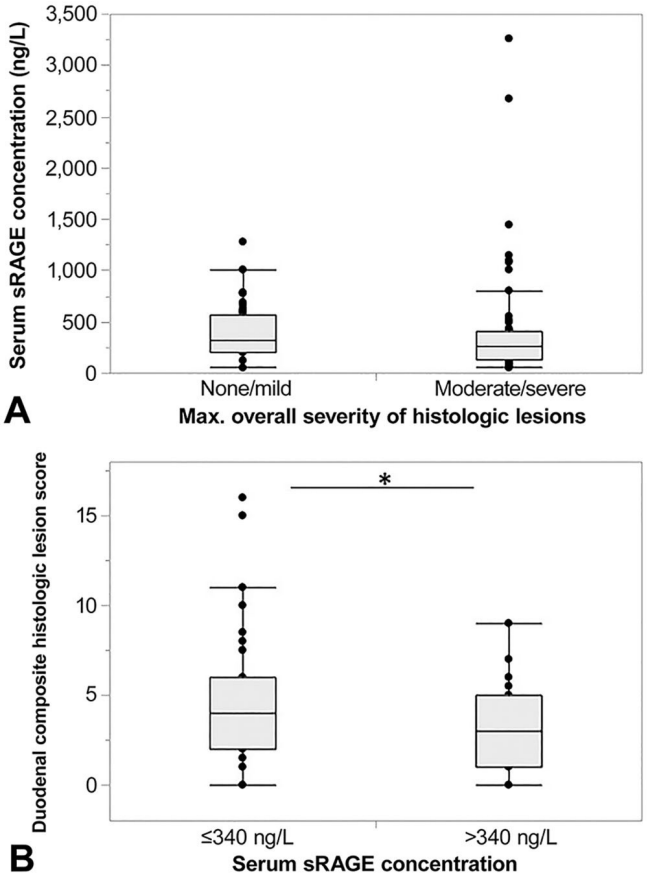

Figure 3. Association of gastrointestinal histologic lesions with serum soluble RAGE (sRAGE) concentrations in dogs with chronic inflammatory enteropathy (CIE; $n = 102$). **A.** Patients with moderate-to-severe histologic lesions (score 2–3) had numerically lower serum sRAGE concentrations (median: 257 ng/L, interquartile range [IQR]: 126–413ng/L; n=56) compared to dogs with no more than mild histologic lesions (score: 0–1; median: 326 ng/L, IQR: 207–573 ng/L; n=46), but no significant difference in serum sRAGE concentration was found between the groups of dogs (p=0.157). **B.** Cumulative histologic lesion scores in the duodenum in dogs with CIE and a serum sRAGE concentration ≤ 340 ng/L were significantly higher (median: 4, IQR: 2–6; $n = 61$) compared to dogs with a serum sRAGE concentration >340ng/L (median: 3, IQR: 1–5; n=34; p=0.013). Asterisk (*) indicates significant difference at p<0.05.

Table 2. Correlation between serum soluble RAGE (receptor for advanced glycation end-products) and histologic lesions in dogs with chronic inflammatory enteropathy ($n = 102$) included in the study.

Parameter correlated with serum sRAGE concentrations	**Spearman ρ**	***P*-value**	**P_{corr}-value**
Histologic lesions (composite score)	-0.15	0.136	0.136
Morphologic criteria (composite score)	-0.13	0.198	0.396
Inflammatory criteria (composite score)	-0.09	0.357	0.714
Stomach			
Morphologic criteria	0.01	0.973	ns
Fundus			
▪ Surface epithelial injury	0.04	0.721	ns

▪ Gastric pit epithelial injury	-0.13	0.249	0.747
▪ Mucosal fibrosis	-0.04	0.722	ns
Antrum			
▪ Surface epithelial injury	-0.03	0.770	ns
▪ Gastric pit epithelial injury	0.15	0.162	0.486
▪ Mucosal fibrosis	-0.01	0.903	ns
Inflammatory criteria	-0.12	0.269	0.269
Fundus			
▪ Intraepithelial lymphocytes	-0.07	0.509	ns
▪ Lamina propria LPC	-0.13	0.228	ns
▪ Lamina propria eosinophils	0.14	0.204	ns
▪ Lamina propria neutrophils	N/A	N/A	N/A
▪ Lamina propria MΦ	0.07	0.486	ns
▪ Lymphoid follicular hyperplasia	-0.01	0.978	ns
Antrum			
▪ Intraepithelial lymphocytes	-0.10	0.359	ns
▪ **Lamina propria LPC**	**-0.22**	**0.038**	0.228
▪ Lamina propria eosinophils	-0.03	0.789	ns
▪ Lamina propria neutrophils	0.14	0.209	ns
▪ Lamina propria MΦ	-0.03	0.812	ns
▪ Lymphoid follicular hyperplasia	-0.10	0.369	ns
Duodenum (composite score)	-0.19	0.070	0.210
Morphologic criteria (sum)	-0.14	0.189	0.378
▪ Villus stunting	-0.10	0.354	ns
▪ Epithelial injury	0.03	0.759	ns
▪ **Crypt distension**	**-0.23**	**0.027**	0.135
▪ Lacteal dilatation	-0.11	0.310	ns
▪ Mucosal fibrosis	0.07	0.512	ns
Inflammatory criteria (sum)	-0.14	0.189	0.378
▪ Intraepithelial lymphocytes	-0.05	0.602	ns
▪ **Lamina propria LPC**	**-0.25**	**0.017**	0.085
▪ Lamina propria eosinophils	0.01	0.902	ns
▪ **Lamina propria neutrophils**	**-0.21**	**0.044**	0.176
▪ Lamina propria MΦ	0.12	0.256	0.768
Ileum (composite score)	0.11	0.629	ns
Morphologic criteria (sum)	0.09	0.670	ns
▪ Villus stunting	0.13	0.545	ns
▪ Epithelial injury	0.20	0.346	ns
▪ Crypt distension	0.24	0.254	ns
▪ Lacteal dilatation	-0.25	0.238	ns
▪ Mucosal fibrosis	N/A	N/A	N/A
Inflammatory criteria (sum)	0.10	0.645	ns
▪ Intraepithelial lymphocytes	0.04	0.843	ns
▪ Lamina propria LPC	0.17	0.417	ns
▪ Lamina propria eosinophils	0.06	0.781	ns
▪ Lamina propria neutrophils	-0.06	0.786	ns

▪ Lamina propria MΦ	0.20	0.341	ns
Colon (composite score)	-0.07	0.692	ns
Morphologic criteria (sum)	-0.20	0.262	0.524
▪ Epithelial injury	-0.11	0.550	ns
▪ Crypt distension	-0.23	0.177	0.708
▪ Change in goblet cells	0.04	0.833	ns
▪ Mucosal fibrosis	-0.11	0.542	ns
Inflammatory criteria (sum)	0.08	0.649	ns
▪ Intraepithelial lymphocytes	-0.14	0.418	ns
▪ Lamina propria LPC	0.08	0.668	ns
▪ Lamina propria eosinophils	0.15	0.389	ns
▪ Lamina propria neutrophils	-0.06	0.731	ns
▪ Lamina propria MΦ	0.02	0.910	ns

LPC = lymphocytes/plasma cells; MΦ = macrophages; N/A = not applicable; ns = not significant; P_{corr} = Holm-Bonferroni corrected *P*-value (n = 2, 3, 4, 5, or 6). Values in bold font indicate significance at $P < 0.05$.

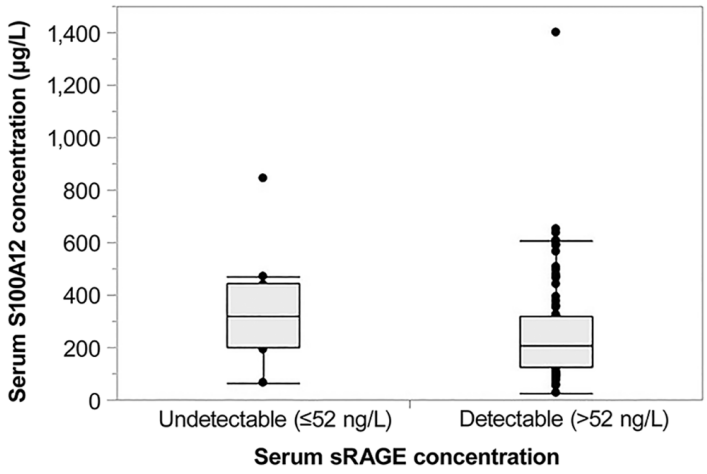

Figure 4. Association of serum sRAGE and S100A12 concentrations in dogs with chronic inflammatory enteropathy (CIE; *n*=93). Dogs with an undetectable serum sRAGE concentration (≤ 52 ng/L; *n* = 10) had numerically higher serum S100A12 concentrations (median: 316 µg/L, interquartile range [IQR]: 197–447µg/L) compared to dogs with detectable serum sRAGE concentrations (median: 207µg/L, IQR: 127–320µg/L; *n*=83) but there was no significant difference between the groups ($p = 0.081$).

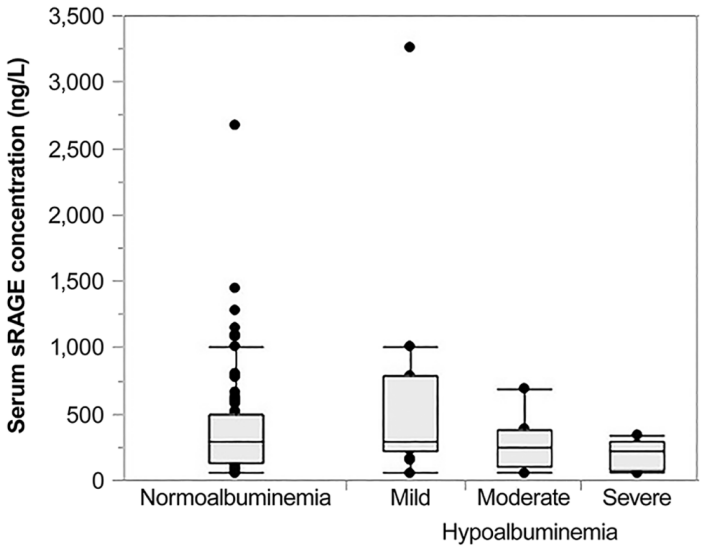

Figure 5. Association of hypoalbuminemia and serum sRAGE concentrations in dogs with chronic inflammatory enteropathy (CIE). Dogs with more severe hypoalbuminemia (*n*=15) had numerically lower serum sRAGE concentrations (moderate hypoalbuminemia—median: 252ng/L, interquartile range [IQR]: 103–381 ng/L; severe hypoalbuminemia—median: 215 ng/L, IQR: 70–286ng/L) than dogs with normoalbuminemia (median: 297ng/L, IQR: 137–499ng/L) or mild hypoalbuminemia (median: 285 ng/L, IQR: 212–782 ng/L; *n* = 86), but there were no significant differences among those groups of dogs ($p = 0.303$)

Association of serum sRAGE concentrations with response to treatment

Serum sRAGE concentrations were lower in dogs diagnosed with IRE (n = 15; median: 248 ng/L, IQR: 52–554 ng/L) com- pared to dogs with FRE (n = 5; median: 415 ng/L, IQR: 217–565 ng/L), but the difference was not statistically significant (p = 0.189; Table 3).

Table 3. Characteristics of dogs with food-responsive enteropathy (FRE) or immunosuppressant-responsive/-refractory enteropathy (IRE) with available case outcomes (n = 20).

Group characteristic	FRE	IRE
Total number	5	15
Age (y), median (IQR)	8 (1–10)	8 (7–10)
Sex, male/female	3/2	8/7
Body weight (kg), median (IQR)	19.4 (9.1–37.6)	12.4 (4.4–25.0)
Follow-up time (mo), median (IQR)	15 (6-18)	9 (4-12)
CCECAI score, median (IQR)	6 (3–8)	9 (5–10)
Clinical disease severity, n (%)		
Mild*	2 (40%)	4 (27%)
Moderate†	2 (40%)	2 (13%)
Severe‡	1 (20%)	7 (47%)
Very severe§	0	2 (13%)
Serum sRAGE (ng/L), median (IQR)	415 (217–565)	248 (52–554)
>340 ng/L, n (%)	3 (60%)	4 (27%)
≤340 ng/L, n (%)	2 (40%)	11 (73%)
52–340 ng/L, n (%)	2 (40%)	7 (47%)
≤52 ng/L, n (%)	0 (0%)	4 (27%)
Serum albumin (g/L), median (IQR)	31 (30–35)	22 (17–29)
Hypoalbuminemia, n (%)	0	6 (40%)
Mild‖	–	4 (27%)
Moderate#	–	0
Severe¶	–	2 (13%)
Serum cobalamin (ng/L), median (IQR)	367 (293–872)	226 (178–331)
Hypocobalaminemia, n (%)**	0	8 (53%)
Serum folate (µg/L), median (IQR)	15.1 (12.7–21.2)	15.8 (9.4–19.7)
Hypofolatemia, n (%)	0	3 (20%)
Hyperfolatemia, n (%)	0	1 (7%)
Serum CRP (mg/L), median (IQR)	1.4 (0.6–2.8)	14.3 (8.5–37.8)††
Fecal calprotectin (µg/g), median (IQR)	0.9 (0.5–1.6)	1.6 (0.8–17.7)
Serum calprotectin (mg/L), median (IQR)	6.1 (2.6–9.6)‡‡	9.3 (4.8–11.1)§§
Fecal S100A12 (ng/g), median (IQR)	72 (35–193)	205 (59–1087)
Serum S100A12 (µg/L), median (IQR)	93 (40–233)	232 (152–393)‖‖
Fecal α_1PI (µg/g), median (IQR)	8.6 (4.7–20.9)	11.0 (4.0–23.5)

Serum α_1PI (mg/L), median (IQR)	1478 (1178–1781)	1173 (855–1571)[##]

CCECAI = canine chronic enteropathy clinical disease activity index; CRP = C-reactive protein; FRE = food-responsive enteropathy; IQR = interquartile range; IRE = immunosuppressant-responsive/-refractory enteropathy. [*]CCECAI score of 4–5, [†]CCECAI score of 6–8, [‡]CCECAI score of 9–11, [§]CCECAI score of ≥12; [‖]defined as a serum albumin concentration 15–20 g/L; [#]defined as a serum albumin concentration 12–14.9 g/L; [¶]defined as a serum albumin concentration <12 g/L; [**]defined as a serum cobalamin concentration <300 ng/L; [††]available from 14 dogs diagnosed with IRE; [‡‡]available from 2 dogs with FRE; [§§]available from 5 dogs with IRE; [‖‖]available from 11 dogs with IRE; [##]available from 12 dogs with IRE.

Discussion

The weak correlation between the severity of histologic lesions in the proximal gastrointestinal tract (i.e., duodenal lymphoplasmacytic and neutrophilic infiltrate, duodenal crypt dilation, and lymphoplasmacytic infiltration in the gastric antrum)—which did not remain significant after correction for multiple comparisons—together with the decrease in serum sRAGE concentrations might suggest a dysregulation of the sRAGE/RAGE axis in canine CIE, suggesting that sRAGE and also intestinal RAGE expression is of interest to further study the pathology of CIE. The serum sRAGE decoy receptor deficiency might signal a perturbation in membrane-bound RAGE signaling (i.e., an increased activation of the receptor causing proinflammatory intracellular signaling), either as a cause or consequence of the disease. This finding is also interesting, considering the potential worse prognosis of CIE dogs with more severe mucosal lesions in the duodenum[1] and our previous finding that serum sRAGE concentrations subsequently increased only in those dogs with a complete response to treatment.[15] These findings differ slightly from the results of our previous pilot study in which no correlation was seen between histologic lesions and serum sRAGE concentrations,[15] but a limitation of that study was the small sample size. Also, endoscopic lesion scores were not obtained in our current study and could not be evaluated for a possible association with serum sRAGE concentrations. Our results agree with the inverse relationship between serum sRAGE concentrations and the severity of histologic lesions in patients with Crohn disease,[7] although the primary disease location in human Crohn disease differs from the distribution of lesions in dogs with CIE.[5] Intestinal expression of RAGE at the protein level was also shown to correlate with inflammatory lesions in human patients with IBD.[31] Although histologic lesion severity was not evaluated, another study found that plasma sRAGE concentrations correlated with the severity of endoscopic lesions in patients with IBD.[25] Whether genetic defects in the *RAGE* gene also contribute to a dysregulation in the sRAGE/RAGE axis[33] in canine CIE is unknown and requires further investigation.

In contrast to the severity of histologic lesions, the severity of clinical signs was not correlated with serum sRAGE concentrations in our study. This finding is consistent with the lack of correlation between RAGE positivity and clinical disease activity index in human IBD patients, but serum sRAGE concentrations were not evaluated in that study.[6] However, our findings are in contrast with another study that showed an inverse correlation between clinical disease ac-

tivity and serum sRAGE concentrations in Crohn disease and ulcerative colitis patients.[7] Similar to our results, a study[6] also found no correlation with prior treatment or disease duration in people.

Serum sRAGE concentrations were not correlated with any of the serum or fecal biomarkers of inflammation or protein loss that we evaluated, and only a trend of an association was seen for serum S100A12 concentrations. This could be explained by spatial and/or temporal differences in the expression of these molecules and also by the recognition of various 3-dimensional molecular structures by RAGE, which includes exogenous ligands and also endogenous molecules such as the S100/calgranulin proteins.[3] Consistent with the results of our study, a correlation of serum sRAGE with fecal calprotectin or serum S100A12 concentrations was also not found in patients with Crohn disease, whereas an inverse correlation existed between serum sRAGE and fecal calprotectin concentration in patients with ulcerative colitis.[7] Lack of a relationship between serum sRAGE and CRP concentrations in canine CIE also agrees with the results in patients with ulcerative colitis[7] but differs from findings in patients with Crohn disease.[7,25] Thus, further evaluation of the ligand-RAGE pathways and the sRAGE/RAGE axis is warranted.

We acknowledge that our study had some limitations. First, the possibility of a concurrent disease process (e.g., chronic or subclinical pancreatitis) being present and affecting serum sRAGE concentrations in some dogs can- not be excluded entirely. Also, fecal viral screening tests are not included in the standard diagnostic work-up of patients with a suspicion of CIE.[2,8,10,24] Thus, the possibility of a (concurrent) infectious (e.g., chronic viral or occult parasitic) gastrointestinal disease cannot be entirely excluded in the dogs in our study. Although we included many dogs with CIE, the smaller size of the subgroups of dogs with FRE and dogs with SRE or IRE presents another limitation. Thus, long-term outcome and prognosis could not be evaluated for most dogs in our study. Third, a limited number of dogs were included in the analysis of some markers in serum and fecal samples. Thus, the possibility of a type II error for finding no significant differences or associations cannot be excluded. Further, histopathology of gastrointestinal tissue biopsies was evaluated by 9 different board-certified pathologists (albeit with special expertise in canine gastrointestinal pathology), which might be associated with a high interobserver variation despite the use of standardized criteria.[24] Last, sRAGE concentrations in serum were analyzed after sample storage for up to 6 y. The stability of sRAGE in canine serum is unknown and could also affect the results of our study.

Our study confirms our previous finding that serum sRAGE concentrations are significantly decreased in dogs with CIE. Our current study further suggests that serum sRAGE might be associated with the severity of histologic lesions. It remains to be determined whether the decrease detected in the systemic concentrations of this decoy receptor is a cause (decreased systemic production contributing to the pathogenesis of canine CIE) or consequence (consumption in the context of inflammation) of CIE in these patients. With the hypothesis that the expression of full-length transmembrane RAGE in the intestinal epithelium correlates with the serum sRAGE level, further research is now needed to investigate the expression of the RAGE receptor along the gastrointestinal tract in dogs with CIE and in healthy controls.

Acknowledgments

We thank Drs. Martina Protschka and Gottfried Alber for their assistance with acquiring the fluorescence image for RAGE immunostaining. Part of the data was presented as an abstract at the 2018 Annual Congress of the European College of Veterinary Internal Medicine, Sept 2018, Rotterdam, The Netherlands.

Declaration of conflicting interests

Drs. Suchodolski and Steiner are affiliated with the Gastrointestinal Laboratory at the Texas A&M University College of Veterinary Medicine and Biomedical Sciences, where serum CRP and fecal α_1PI testing are offered on a fee-for-service basis. The authors declared no other potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support from a formal grant for the research, authorship, and/or publication of this article. Materials and supplies for the study were generously provided by the Gastrointestinal Laboratory at Texas A&M University; publication costs were paid by the Leipzig University Department for Small Animals.

References

1. Allenspach K, et al. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med 2007;21:700–708.

2. Allenspach K, et al. Long-term outcome in dogs with chronic enteropathies: 203 cases. Vet Rec 2016;178:178–368.

3. Bierhaus A, et al. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med 2005;83: 876–886.

4. Body-Malapel M, et al. The RAGE signaling pathway is involved in intestinal inflammation and represents a promising therapeutic target for inflammatory bowel disease. Mucosal Immunol 2019;12:468–478.

5. Cerquetella M, et al. Inflammatory bowel disease in the dog: differences and similarities with humans. World J Gastroenterol 2010;16:1050–1056.

6. Ciccocioppo R, et al. Role of the advanced glycation end products receptor in Crohn's disease inflammation. World J Gastroenterol 2013;19:8269–8281.

7. Ciccocioppo R, et al. The circulating level of soluble receptor for advanced glycation end products displays different patterns in ulcerative colitis and Crohn's disease: a cross-sectional study. Dig Dis Sci 2015;60:2327–2337.

8. Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? J Small Anim Pract 2016;57:589–599.

9. Day MJ, et al. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. J Comp Pathol 2008;138:S1–S43.

10. Erdmann C, Heilmann RM. Diagnostic and therapeutic approach to chronic inflammatory enteropathies in dogs. Tierarztl Prax Ausg Kleintiere Heimtiere 2017;45:317–327.

11. Heilmann RM, et al. Development and analytical validation of an enzyme-linked immunosorbent assay for the quantification of canine calprotectin in serum and feces from dogs [abstract]. J Vet Intern Med 2011;25:693.

12. Heilmann RM, et al. Development and analytical validation of a radioimmunoassay for the measurement of alpha$_1$-proteinase inhibitor concentrations in feces from healthy puppies and adult dogs. J Vet Diagn Invest 2011;23:476–485.

13. Heilmann RM, et al. Serum alpha$_1$-proteinase inhibitor concentrations in healthy dogs—method validation and determination of reference interval and intra-individual variation. Vet Clin Pathol 2013;42:190–195.

14. Heilmann RM, et al. Association between fecal S100A12 concentration and histologic, endoscopic, and clinical disease severity in dogs with idiopathic inflammatory bowel disease. Vet Immunol Immunopathol 2014;158:156–166.

15. Heilmann RM, et al. Systemic levels of the anti-inflammatory decoy receptor soluble RAGE (receptor for advanced glycation end products) are decreased in dogs with inflammatory bowel disease. Vet Immunol Immunopathol 2014;161:184–192.

16. Heilmann RM, Suchodolski JS. Is inflammatory bowel disease in dogs and cats associated with a Th1 or Th2 polarization? Vet Immunol Immunopathol 2015;168:131–134.

17. Heilmann RM, et al. Fecal S100A12 concentration predicts a lack of response to treatment in dogs affected with chronic enteropathy. Vet J 2016;215:96–100.

18. Heilmann RM, et al. Validation of an enzyme-linked immunosorbent assay (ELISA) for the measurement of canine S100A12. Vet Clin Pathol 2016;45:135–147.

19. Heilmann RM, Allenspach K. Pattern-recognition receptors: signaling pathways and dysregulation in canine chronic enteropathies—brief review. J Vet Diagn Invest 2017;29:781–787.

20. Heilmann RM, et al. Association of fecal calprotectin concentrations with disease severity, response to treatment, and other biomarkers in dogs with chronic inflammatory enteropathies. J Vet Intern Med 2018;32:679–692.

21. Heilmann RM, Steiner JM. Clinical utility of current biomarkers in canine chronic inflammatory enteropathies. J Vet Intern Med 2018;32:1495–1508.

22. Holm S. A simple sequentially rejective multiple test procedure. Scand J Stat 1979;6:64–70.

23. Jergens AE, et al. Comparison of oral prednisone and prednisone combined with metronidazole for induction therapy of canine inflammatory bowel disease: a randomized-controlled trial. J Vet Intern Med 2010;24:269–277.

24. Jergens AE, et al. Design of a simplified histopathologic model for gastrointestinal inflammation in dogs. Vet Pathol 2014;51:946–950.

25. Meijer B, et al. Total soluble and endogenous secretory receptor for advanced glycation end products (RAGE) in IBD. J Crohns Colitis 2014;8:513–520.

26. Schmidt AM, et al. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest 2001;108:949–955.

27. Schmitz S, et al. Effects of ex-vivo and in-vivo treatment with probiotics on the inflammasome in dogs with chronic enteropathy. PLoS One 2015;10:e0120779.

28. Sterenczak KA, et al. Cloning, characterisation, and comparative quantitative expression analyses of receptor for advanced glycation end products (RAGE) transcript forms. Gene 2009;434:35–42.

29. Sterenczak KA, et al. High-mobility group B1 (HMGB1) and receptor for advanced glycation end-products (RAGE) expression in canine lymphoma. Anticancer Res 2010;30: 5043–5048.

30. Sterenczak KA, et al. Quantitative PCR and immunohistochemical analyses of HMGB1 and RAGE expression in canine disseminated histiocytic sarcoma (malignant histiocytosis). Anticancer Res 2011;31:1541–1548.

31. Stintzing S, et al. Role of cannabinoid receptors and RAGE in inflammatory bowel disease. Histol Histopathol 2001;26:735–745.

32. Walsh D, et al. Pattern recognition receptors—molecular orchestrators of inflammation in inflammatory bowel disease. Cytokine Growth Factor Rev 2013;24:91–104.

33. Wang ZT, et al. RAGE gene three polymorphisms with Crohn's disease susceptibility in Chinese Han population. World J Gastroenterol 2014;20:2397–2402.

34. Washabau RJ, et al. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med 2010;24: 10–26.

3.2 SECOND PUBLICATION

Dysregulation of gastrointestinal RAGE (receptor for advanced glycation end products) expression in dogs with chronic inflammatory enteropathy

Cabrera-García AI[1], Protschka M[2], Alber G[2], Kather S[1], Dengler F[3], Müller U[2], Steiner JM[4], Heilmann RM[1,*]

[1.] Department for Small Animals, Veterinary Teaching Hospital, College of Veterinary Medicine, University of Leipzig, Leipzig, SN, Germany. [2.] Institute of Immunology, College of Veterinary Medicine, Biotechnological-Biomedical Center, University of Leipzig, Leipzig, SN, Germany. [3.] Institute of Veterinary Physiology, University of Leipzig, Leipzig, SN, Germany. [4.] Gastrointestinal Laboratory, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA.

[*]Corresponding author: Prof. Dr. med. vet. Romy M. Heilmann, DACVIM (SAIM), DECVIM-CA, MANZCVS, PhD; Department for Small Animals, Veterinary Teaching Hospital, College of Veterinary Medicine, University of Leipzig, An den Tierkliniken 23, DE-04103 Leipzig, SN, Germany. romy.heilmann@kleintierklinik.uni-leipzig.de

Citation: Angela Isabel Cabrera-García, Martina Protschka, Gottfried Alber, Stefanie Kather, Franziska Dengler, Uwe Müller, Jörg M. Steiner, Romy M. Heilmann. Dysregulation of gastrointestinal RAGE (receptor for advanced glycation end products) expression in dogs with chronic inflammatory enteropathy. Vet Immunol Immunopathol. 2021; 234:110216 (online ahead of print).

Editor: Dr. Elisabeth J. Glass
Received: October 19, 2020
Accepted: January 11, 2021
Published: February 13, 2021

Abstract: The pathogenesis of chronic inflammatory enteropathies (CIE) in dogs involves dysregulated innate immune responses. The receptor for advanced glycation end products (RAGE), a pattern recognition receptor, plays a role in chronic inflammation. Abrogation of proinflammatory RAGE signaling by ligand binding (e.g., S100/calgranulins) to soluble RAGE (sRAGE) might also be a novel therapeutic avenue. Serum sRAGE levels are decreased in canine CIE, but gastrointestinal tissue RAGE expression has not been investigated in dogs. Thus, the study aimed to evaluate the gastrointestinal mucosal RAGE expression in dogs with CIE.

Further, the potential binding of RAGE to canine S100/calgranulin ligands was investigated. Epithelial RAGE expression was quantified in gastrointestinal (gastric, duodenal, ileal, and colonic) biopsies from 12 dogs with CIE and 9 healthy control dogs using confocal laser scanning microscopy. RAGE expression was compared between both groups of dogs and was tested for an association with patient characteristics, clinical variables, histologic lesion severity, and biomarkers of extra-gastrointestinal disease, systemic or gastrointestinal inflammation, function, or protein loss. Statistical significance was set at $p < 0.05$.
RAGE:S100/calgranulin binding was assessed by immunoassay and electrophoretic techniques. RAGE expression was detected in all 59 biopsies from diseased and healthy control dogs evaluated. Epithelial RAGE expression in the duodenum and colon was significantly higher in dogs with CIE than in healthy controls ($p < 0.04$). Compared to healthy controls, RAGE expression in dogs with CIE also tended to be higher in the ileum but lower in the stomach. A slight (statistically not significant) shift towards more basal intestinal epithelial RAGE expression was detected in CIE dogs. Serum sRAGE was proportional to epithelial RAGE expression in the duodenum ($p < 0.04$), and RAGE expression in the colon inversely correlated with biomarkers of protein loss in serum (both $p < 0.04$). Several histologic morphologic and inflammatory lesion criteria and markers of inflammation (serum C-reactive protein and fecal calprotectin concentration) were related to epithelial RAGE expression in the duodenum, ileum, and/or colon. In vitro canine RAGE:S100A12 binding appeared more pronounced than RAGE:S100A8/A9 binding. This study showed a dysregulation of epithelial RAGE expression along the gastrointestinal tract in dogs with CIE. Compensatory regulations in the sRAGE/RAGE axis are an alternative explanation for these findings. The results suggest that RAGE signaling plays a role in dogs with CIE, but higher anti-inflammatory decoy receptor sRAGE levels paralleled RAGE overexpression. Canine S100/calgranulins were demonstrated to be ligands for RAGE.

Keywords: Canine; immunofluorescence; inflammatory bowel disease; innate immunity; pattern recognition receptor; receptor for advanced glycation end products.

1. Introduction

Chronic inflammatory enteropathies (CIE) are a group of diseases in dogs that are further classified based on the response to treatment as either food-responsive enteropathy (FRE; if patients show significant improvement or resolution of their clinical signs following a dietary trial with an elimination diet) or steroid- and/or immunosuppressant-responsive enteropathy (SRE/IRE; if dogs need to be treated with corticosteroids such as prednisolone or budesonide, and/or other immunosuppressive medication such as cyclosporine, azathioprine, or chlorambucil) (Allenspach, 2016; Dandrieux, 2016). Finally, dogs with a CIE not responding to steroid- or immunosuppressant treatment are classified as having a steroid- and/or immunosuppressant-refractory enteropathy (NRE) (Allenspach, 2016; Dandrieux, 2016). Histologically, there appears to be no clear distinction between these three groups of dogs with CIE (Day et al., 2008; Washabau et al., 2010), and all CIE subclasses require the exclusion of other underlying causes for the presence of chronic gastrointestinal signs (Dandrieux, 2016; Erdmann & Heilmann, 2017).

Thus, the clinical work-up for dogs suspected to have CIE – particularly those dogs with SRE/IRE – aims for a diagnosis of exclusion, and arriving at this diagnosis can be challenging. The existence of a previously proposed fourth subgroup of CIE, antibiotic-responsive enteropathy (ARE; in which affected dogs show a marked and lasting improvement in their clinical signs following antibiotic administration), is currently being questioned (Erdmann and Heilmann, 2017; Cerquetella et al., 2020). The etiology of canine CIE appears to be complex (Dandrieux, 2016; Erdmann and Heilmann, 2017). Recent studies suggest that dysregulated signaling mechanisms within the innate immune system and its cross-talk with other acquired immune responses play a relevant role in the pathogenesis of canine CIE (Jergens & Simpson, 2012; Heilmann & Allenspach, 2017). Pattern recognition receptors (PRR) play an important part in the signaling mechanisms of the innate immune response. These PRRs recognize exogenous pathogen-associated molecular pattern (PAMP) molecules as well as endogenous damage-associated molecular pattern (DAMP) molecules and regulate innate immune responses (Heilmann and Allenspach, 2017). The hypothesis that defects and dysregulations in innate immune PRRs contribute to the complex pathogenesis of CIEs in dogs has become an important focus of research in recent years (Heilmann and Allenspach, 2017).

The receptor for advanced glycation end products (RAGE) is a PRR of the innate immune system (Hofmann et al., 1999) and plays a central role in the pathogenesis of chronic inflammatory diseases, including gastrointestinal inflammation, in humans (Hofmann et al., 1999; Bierhaus et al., 2005; Ciccocioppo et al., 2013; Body-Malapel et al., 2019; Bramhall et al., 2020). Following the detection and binding of DAMP molecules (such as the S100/calgranulin proteins), transmembrane RAGE can induce and even maintain an inflammatory cascade (Hofmann et al., 1999; Heilmann and Allenspach, 2017). The full-length (transmembrane) form of RAGE consists of 5 domains (Heilmann and Allenspach, 2017). In addition to the complete transmembrane receptor, there are several isoforms of this molecule, including the soluble forms of RAGE (sRAGE) from which the membrane and cytoplasmic part is missing or has been cleaved (Heilmann et al., 2014). sRAGE acts as a decoy receptor because it can bind to its ligands and abrogate the transmembrane RAGE-ligand interaction, thus contributing to a downregulation of the inflammatory cascade (Taguchi et al., 2000; Bierhaus et al., 2005; Maillard-Lefebvre et al., 2009; Bramhall et al., 2020).

Previous analysis showed the highest level of RAGE expression in human tissues to be present in the lung (Schmidt et al., 2000). A recent study demonstrated that full-length RAGE is expressed in the small intestine as well as the colon in people and mice and that intestinal RAGE expression is upregulated in patients with chronic inflammatory conditions (Body- Malapel et al., 2018). Furthermore, this study suggests that interference with the sRAGE/RAGE axis is a potential avenue for developing of novel therapeutic targets (Body-Malapel et al., 2018). Two different functional allelic and genotypic polymorphisms of RAGE were associated with a diagnosis of inflammatory bowel disease (Crohn's disease or ulcerative colitis) in humans (Ciccocioppo et al., 2019). These findings further suggest a role for RAGE in the regulation of chronic intestinal inflammation.

There are currently 24 known variants of RAGE in dogs (Sterenczak et al., 2009; Heilmann & Allenspach, 2017). Expression of RAGE has been investigated in dogs with malignant lymphoma (Sterenczak et al., 2010) and dogs with histiocytic sarcoma showing that RAGE dysregulation is associated with the progression of histiocytic disorders (Sterenczak et al., 2011). A previous study in dogs showed that serum sRAGE concentrations are significantly lower in dogs with CIE than in healthy dogs, and sRAGE concentrations increased only in those dogs with CIE that showed complete clinical remission following the initiation of treatment (Heilmann et al., 2014). Serum sRAGE concentrations were associated with microscopic lesions in the duodenum in dogs with CIE, further suggesting that the sRAGE/RAGE axis is dysregulated in dogs with CIE (Cabrera-García et al., 2020). However, the gastrointestinal mucosal expression of RAGE in dogs with CIE and its association with serum sRAGE concentrations, as well as other disease markers, has not yet been investigated.

We hypothesized that the RAGE receptor is overexpressed in the gastrointestinal mucosa of dogs diagnosed with CIE and that the increased cellular RAGE expression correlates with decreased serum sRAGE concentrations and the severity of the disease. We further hypothesized that canine S100/calgranulin proteins bind to sRAGE in vitro. Thus, the aims of this study were (i) to immunohistologically investigate and quantify mucosal RAGE expression along the different segments of the gastrointestinal tract (i.e., stomach, duodenum, ileum, and colon) and (ii) to evaluate the possibility of an association of gastrointestinal RAGE expression with serum sRAGE concentration, clinical and histological disease severity, and the concentrations of other biomarkers of gastrointestinal inflammation or protein loss. The secondary aim of this study was to assess the in vitro binding of canine sRAGE to the S100/calgranulin proteins.

2. Materials and methods

2.1. Ethics approval

The enrollment of dogs with CIE into the study of several markers was approved by the Clinical Research Review Committee (CRRC approval #TAMU 2009-06, approved 01-15- 2009) and the Institutional Animal Care and Use Committee at Texas A&M University (IACUC approval #TAMU 2012-083, approved 05-22-2012). Owners were informed, and all samples were collected following their written consent.

2.2. Sampling population

2.2.1. Dogs with CIE

Tissue biopsies of the gastrointestinal tract (i.e., stomach, duodenum, ileum, and colon), whole blood, serum, urine, and fecal samples (from 3 consecutive days) were collected from 12 dogs recruited between May 2011 and October 2014 at the Gastrointestinal Laboratory and the Veterinary Teaching Hospital at Texas A&M University (Table 1). Information about some of the dogs has previously been reported (Cabrera-García et al., 2020). Patients were included in the study if they showed clinical signs of chronic enteropathy (i.e., vomiting, diarrhea, and/or weight loss for at least 3 weeks), if other possible causes of chronic gastrointestinal signs (e.g.,

atypical hypoadrenocorticism, exocrine pancreatic insufficiency, or alimentary lymphoma) had been excluded, and no anti-inflammatory and/or immunosuppressive medication had been administered within at least 2 weeks prior to enrollment into the study. There were no restrictions on breed, age, diet, or other medications (e.g., proton pump inhibitors) or supplements (e.g., cyanocobalamin, probiotics) for inclusion in this study.

Table 1. Clinical characteristics of the dogs with chronic inflammatory enteropathy (CIE; *n* = 12) and healthy control dogs (*n* = 9) included in the study (*n* = 21).

Group characteristic	**CIE dogs**	**Healthy dogs**
Total number	12	9
Age in years, median (IQR)	5.3 (1.6–8.1)	8.4 (1.5–8.9)
Sex, male/female	9/3	2/7
Body weight in kg, median (IQR)	16.9 (10.3–26.7)	25.8 (20.1–26.4)
Breed, n (%)		
Pure-bred dogs	11 (92%)	9 (100%)
Mixed breed dogs	1 (8%)	0
Disease duration in months, median (IQR)	4 (2–12)	–
Follow-up time in months, median (IQR)	13 (4–18)	–
CIE classification based on follow-up, n (%)[†]		
FRE	2 (67%)	–
SRE/IRE	1 (33%)	–
CCECAI score, median (IQR)[*]	6 (4–9)	–
Clinical disease severity, n (%)[*]		
Mild clinical disease (CCECAI score: 4–5)	4 (36%)	–
Moderate clinical disease (CCECAI score: 6–8)	4 (36%)	–
Severe clinical disease (CCECAI score: 9–11)	2 (19%)	–
Very severe clinical disease (CCECAI score: ≥12)	1 (9%)	–

CCECAI = canine chronic enteropathy clinical disease activity index; CIE = chronic inflammatory enteropathy; FRE = food-responsive enteropathy; IQR = interquartile range; IRE = immunosuppressant-responsive/-refractory enteropathy. [*]available from 11 dogs diagnosed with CIE; [†]available from 3 dogs diagnosed with CIE.

The severity of clinical disease displayed by these dogs was assessed using an established clinical scoring system (i.e., canine chronic enteropathy clinical activity index, CCECAI [Allenspach et al., 2007]). The individual clinical parameters included in the CCECAI (i.e., appetite, frequency of vomiting, stool consistency and frequency of defecation, weight loss, serum albumin concentration, peripheral edema or ascites, and pruritus) are graded from 0–3 (0: normal, 1: slightly abnormal, 2: moderately abnormal, and 3: severely abnormal). The sum of these individual scores is interpreted as the cumulative CCECAI score, where a score of 0– 3 reflects clinically insignificant disease, a score of 4–5 mild clinical disease, a score of 6–8 moderate

clinical disease, a score of 9–11 severe clinical disease, and a score of ≥12 very severe clinical disease (Allenspach et al., 2007).

In addition to a complete blood cell count and a serum biochemistry profile, serum concentrations of specific canine pancreatic lipase (Spec cPL, a marker used for the diagnosis of pancreatitis), cobalamin and folate (markers of gastrointestinal function), and C-reactive protein (CRP), calprotectin, and S100A12 as markers of systemic inflammation were measured. Markers of gastrointestinal inflammation (i.e., fecal calprotectin and S100A12 concentrations) or protein loss (i.e., fecal and serum alpha1-proteinase inhibitor [α_1PI] concentrations) were also determined. Serum sRAGE concentrations were measured by ELISA as previously described (Heilmann et al., 2014; Cabrera-García et al., 2020).

Gastrointestinal tissue biopsies (endoscopic biopsies from 11 dogs, surgical biopsies from 1 dog) were histopathologically evaluated by one of 6 board-certified pathologists using the criteria of the World Small Animal Veterinary Association Gastrointestinal Standardization grading system (Day et al., 2008; Washabau et al., 2010). Individual structural and inflammatory lesions were graded as absent (score = 0), mild (score =1), moderate (score = 2), or severe (score = 3), and cumulative lesion scores were calculated as the sum of individual lesion scores of each segment of the gastrointestinal tract.

2.2.2. Healthy controls

Full-thickness gastrointestinal tissue biopsies (i.e., stomach, duodenum, ileum, and colon) were also obtained from 9 purpose-bred healthy Hound dogs (Table 1) that were euthanized for an unrelated project at the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University (AUP approval #TAMU 2009-0123). To be included as a healthy control dog in this study, the dog could not have any clinical signs of gastrointestinal disease, receive any medication known to affect the gastrointestinal tract, and needed to be regularly vaccinated and dewormed.

2.3. Quantitative evaluation of RAGE in gastrointestinal tissue biopsies

2.3.1. Immunofluorescence staining protocol

Tissue biopsy samples from canine stomach and intestines (i.e., duodenum, ileum, colon) as well as canine lung (a tissue with very high expression levels of RAGE in the naïve state [Schmidt et al., 2000]) as positive control tissue (all surplus material from unrelated necropsies) were fixed in formalin, paraffin-embedded, and cut at 5 µm-thickness, and served to establish and optimize the RAGE immunofluorescence staining protocol (Fig. 1).

After deparaffination in xylol and a decreasing ethanol series, the tissues were permeabilized using 0.1% (v/v) Triton X-100[1] in PBS. Antigen demasking was achieved by hot citric acid with 0.025% (v/v) Tween-20 (PBST) (approximately 95°C; 10 mM, pH 6.0). Endogenous peroxidase was blocked with 3% (v/v) H_2O_2 for 1 h. Blocking of non-specific binding sites was achieved with 2.5% (v/v) bovine serum albumin in PBST for 1 h at room temperature (approximately 23°C). Two polyclonal anti-recombinant canine RAGE (Asp25–Val339) antibodies[2,3]

were tested for their suitability as a primary antibody for tissue RAGE staining, and antibody specificity was confirmed by Western blot (data not shown). Tissue sections 1:20 anti-recombinant canine RAGE), using a non-specific goat (α-armenian hamster) antibody (diluted at 1:1500), served as a negative control. For all tissues, rabbit anti-goat IgG_4 was used as secondary antibody at a 1:500 dilution and were then incubated overnight at 4°C with the primary antibody selected based on the anti-recombinant canine RAGE[3] diluted at signal in Western blot analysis (polyclonal incubation period of 1 h at room temperature. A polyclonal horseradish peroxidase-labeled goat anti-rabbit IgG[5] was used as tertiary detection antibody. Following the removal of excess antibody, the sections were incubated with Alexa Fluor™488 nm tyramide reagent[6] for 5 min. Nuclei were counterstained with Hoechst 33342[7]. The presence of tissue RAGE expression was analyzed in gastrointestinal and pulmonary tissue samples by screening the slides using a fluorescence microscope[8] and the cellSens Dimension software[9].

2.3.2. Confocal laser scanning microscopic (CLSM) data acquisition and analysis

For gastrointestinal tissues (stomach, duodenum, ileum, and colon), RAGE expression was quantitatively determined by measuring the fluorescence intensity applying photon counting (in voxels) on a confocal laser scanning microscope (CLSM).[10] Alexa Fluor™488 was excited at 514 nm, and photon counting was performed using a hybrid detector (HyD)[11] at an emission range of 550–603 nm and a 63×/1.30 Glyc objective[12]. CLSM was conducted following a two-step protocol. First, an overview (tile scan at 600 Hz bidirectional, pinhole: 2 airy units [AU]) of the entire tissue section was created with a scan resolution of 256×256 pixels. The overview scan, allowing equal pre-illumination of the fluorescent tissue prior to photon counting, provided a virtual slide of the section. This scan served as the basis for selecting 4 regions (scan positions) in each gastrointestinal tissue (including up to 4 different biopsies for endoscopically collected tissues) that met the structural requirements (i.e., intact epithelial surface, proper orientation of the tissue, distinction between villus and crypt area in the duodenum and ileum, and distinction between apical and basal portion of the epithelium in the duodenum, ileum, and colon) for further evaluation of tissue RAGE immunoreactivity (Fig. 2).

2.3.2. Confocal laser scanning microscopic (CLSM) data acquisition and analysis

For gastrointestinal tissues (stomach, duodenum, ileum, and colon), RAGE expression was quantitatively determined by measuring the fluorescence intensity applying photon counting (in voxels) on a confocal laser scanning microscope (CLSM).[10] Alexa Fluor™ 488 was excited at 514 nm, and photon counting was performed using a hybrid detector (HyD)[11] at an emission range of 550–603 nm and a 63×/1.30 Glyc objective[12]. CLSM was conducted following a two-step protocol. First, an overview (tile scan at 600 Hz bidirectional, pinhole: 2 airy units [AU]) of the entire tissue section was created with a scan resolution of 256×256 pixels. The overview scan, allowing equal pre-illumination of the fluorescent tissue prior to photon counting, provided a virtual slide of the section. This scan served as the basis for selecting 4 regions (scan positions) in each gastrointestinal tissue (including up to 4 different biopsies for endoscopically collected tissues) that met the structural requirements (i.e., intact epithelial surface, proper orientation of the tissue, distinction between villus and crypt area in the duodenum and ileum, and

distinction between apical and basal portion of the epithelium in the duodenum, ileum, and colon) for further evaluation of tissue RAGE immunoreactivity (Fig. 2).

Second, photon counting was applied at the previously selected scan positions using a resolution of 512×512 pixels at 200 Hz unidirectional (pinhole: 1 AU). The transmission value of the 514 nm-laser line, once appropriately set using the saturation control within the counting mode of the HyD, was kept unchanged for all photon counting records of the study. To assure that intensity measurements were carried out at corresponding focus planes all over the tissue section, a focus map (maximum intensity mode) of the tissue section was created prior to photon counting. Thus, at each scan position data recording was conducted in the previously detected focus plane.

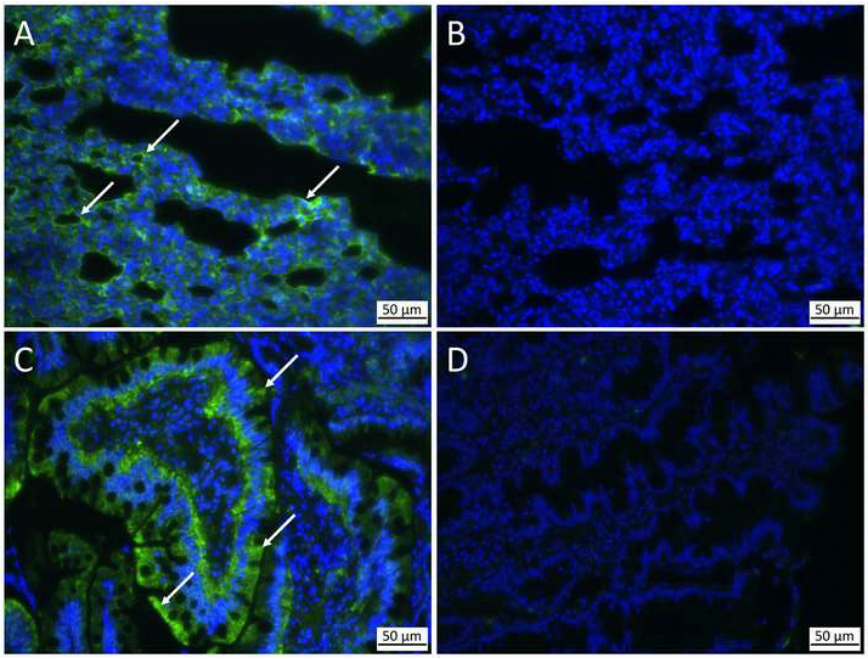

Fig. 1. Representative fluorescence images show the localization of RAGE expression in canine pulmonary (panels A-B) and duodenal (panels C-D) tissue biopsy sections. Canine RAGE is displayed in green (Alexa Fluor™ 488), and nuclei are stained blue (Hoechst 33342). RAGE is expressed in several sections of the lung of a healthy dog (panel A; indicated by white arrows) and sections of the duodenum of a dog with chronic intestinal inflammation (CIE, panel C; indicated by white arrows). Negative controls for non-specific reactions are also shown (Alexa Fluor™ 488; panels B and D), with the nuclei stained blue (Hoechst 33342). Staining for RAGE in gastrointestinal tissues was performed with an enhancement kit due to the weak expression of RAGE in these tissues in the naïve state. In contrast, RAGE expression in the lung (used as positive control tissue to demonstrate adequacy of the staining protocol) is very strong in the naïve state, yielding a strong signal for RAGE over the entire tissue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Photon counting images were analyzed using the Leica software LAS-X 3.4.2[13] A total number of 59 sections, each at four positions, were analyzed: 12 obtained from gastric, 14 from duodenal, 16 from ileal, and 17 from colonic tissues obtained from 12 dogs with CIE and from 9 healthy dogs. In duodenal, ileal, and colonic tissue, photon counting related to epithelial Alexa Fluor™ 488 labeling was quantified at the apical and basal part of the epithelial cells, each at

10 regions of interest (ROI; each with an area of 3.14 µm^2 or a 2-µm diameter). In addition, section-specific control measurements were obtained by positioning 10 ROI in the center of goblet cell secretion, a portion of the epithelium supposed to show no RAGE expression, and was used for normalization against this section-specific background staining. In gastric biopsies, photon counting was determined with 10 ROI each in the cellular (extranuclear) part of epithelial cells (any portion because apical and basal regions could not be reliably distinguished) and, as control, in adjacent subepithelial soft tissue (lamina propria). To ensure the standardized positioning of the ROI at the brightest spots within the epithelium and the darkest spots within the lamina propria, images were displayed as greyscale pictures. Then, the upper and lower histogram thresholds were modified for the highest photon counts (brightest pixel) to be displayed as red pixels and the lowest photon counts (darkest pixel) as blue pixels (Fig. 3 and 4).

The average photon counts obtained from the 10 apical epithelial ROI, the 10 basal epithelial ROI, and the 10 cellular ROI were each normalized against the average photon count within the control region by calculation of their ratios (i.e., normalized apical epithelial photon count = [average photon count of the 10 apical epithelial ROI/ average photon count of the 10 control ROI] and normalized basal epithelial photon count = [average photon count of the 10 basal epithelial ROI/ average photon count of the 10 control ROI]). The average of all normalized apical (AEC_n), basal (BEC_n), and, for the gastric localizations, cellular (CEC_n) epithelial photon counts and the ratio between AECn and BECn (AEC_n-to-BEC_n ratio, ABR) were then calculated for each segment of the gastrointestinal tract of an individual patient and were used for statistical analyses.

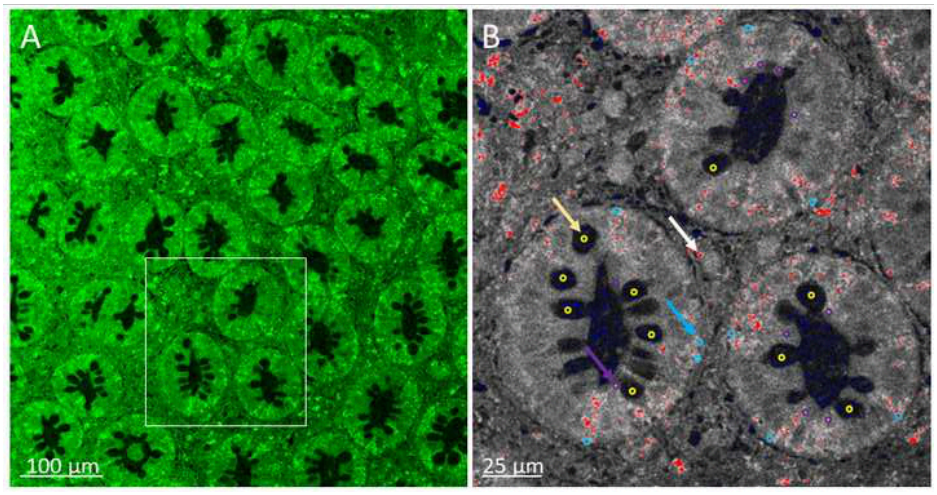

Fig. 2. Quantitative evaluation of RAGE expression in gastrointestinal tissue biopsies. (A) Overview scan of a colonic tissue biopsy by confocal laser scanning microscopy (CLSM) where green staining areas reflect RAGE expression. (B) Example of a defined area (position, white square in panel A) for evaluating tissue RAGE expression in an endoscopic colonic biopsy from a dog. The colored circles are the regions of interest (ROI) with the high-est (purple and blue circles) or lowest (yellow circles) signal intensity and in which the expression of RAGE was quantified (also indicated by colored arrows): the apical area of the epithelium (purple circles, one example indicated by the purple arrow), basal area of the epithelium (blue circles, one example indicated by the blue arrow), and the control region (yellow circles, one example indicated by the yellow arrow). The white arrow indicates

small areas of artificial staining of macrophages and erythrocytes (CLSM, original magnification 63×/1.3 Glyc objective). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

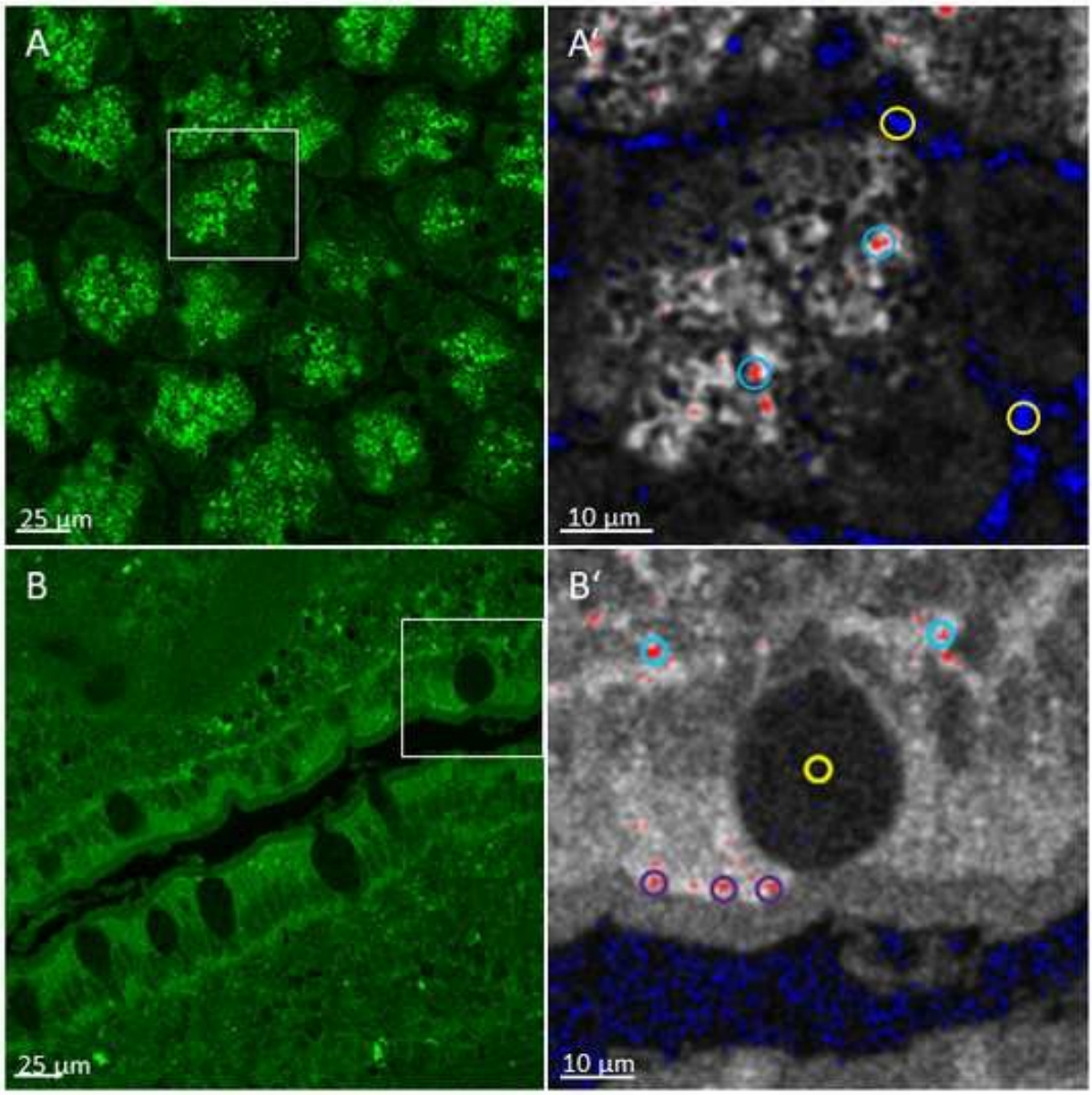

Fig. 3. Quantitative evaluation of RAGE in proximal gastrointestinal tissue biopsies. Left panel: examples of defined areas (positions) for evaluating gastric (panel A) and duodenal (panel B) tissue RAGE expression in endoscopic tissue biopsies from a dog. Right panel: magnified view of an area within that position (white squares in panels A and B). The colored circles reflect the regions of interest (ROI) in which the expression of RAGE was quantified: cellular (extranuclear, blue circles) and control region (lamina propria, yellow circles) in the stomach (panel A'); and the apical area of the epithelium (purple circles), basal area of the epithelium (blue circles), and the control region (yellow circles) in the duodenum (panel B'). Original images of the positions converted to grayscale; red pixels indicate the highest photon counts (brightest pixel) and blue pixels the lowest photon counts (darkest pixel) (CLSM, 63×/1.3 Glyc objective). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

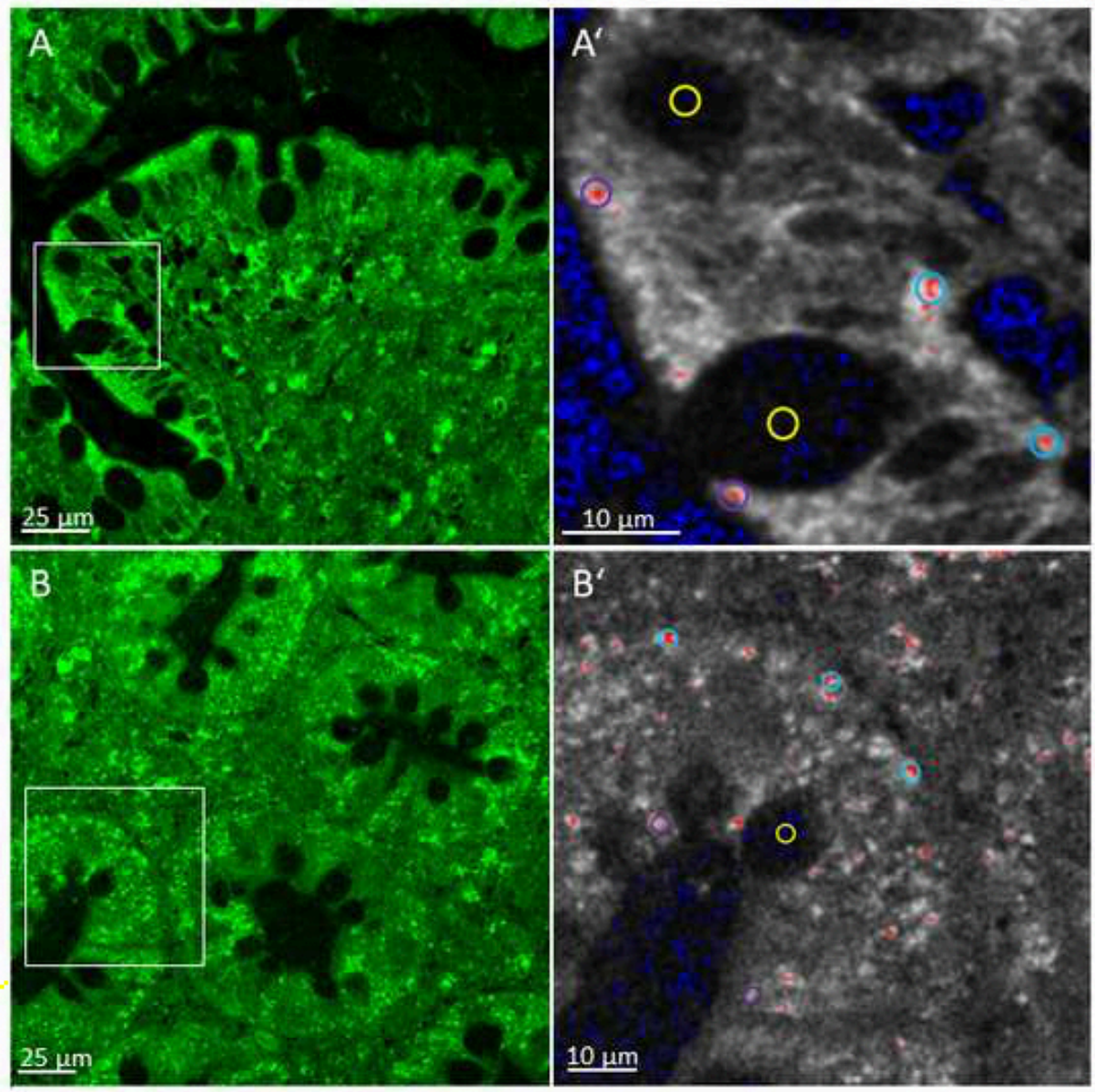

Fig. 4. Quantitative evaluation of RAGE in distal gastrointestinal tissue biopsies. Left panel: examples of defined areas (positions) for evaluating ileal (panel A) and colonic (panel B) tissue RAGE expression in endoscopic tissue biopsies from a dog diagnosed with CIE. Right panel: magnified view of an area within that position (white squares in panels A and B). The colored circles reflect the regions of interest (ROI) in which the expression of RAGE was quantified: the apical area of the epithelium (purple circles), basal area of the epithelium (blue circles), and the control region (yellow circles) (panels A' and B'). Original images of the positions converted to grayscale; red pixels indicate the highest photon counts (brightest pixel) and blue pixels the lowest photon counts (darkest pixel) (CLSM, 63×/1.3 Glyc objective). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.4. Assessment of in vitro binding of RAGE to S100/calgranulin proteins

2.4.1. Radiolabeling of purified canine S100/calgranulin proteins

Labeling of canine S100A12 and calprotectin (S100A8/A9) with radioactive iodine (^{125}I) was performed by the chloramine T method as previously described (Heilmann et al., 2008; Heilmann et al., 2011) with the modification of adding 1.0 µL Na^{125}I (0.1 mCi at the time of production) to 10 µg of purified protein followed by an incubation for 60 s. ^{125}I-tagged protein was then separated from free ^{125}I by size exclusion, and the ^{125}I-tagged protein fractions were supplemented with $CaCl_2$ (2.4 mM in the final solution).

2.4.2. Canine RAGE:S100/calgranulin protein binding ELISA

In 3 independent experiments, wells of a flat-bottom enhanced binding ELISA plate[14] were coated with recombinant canine RAGE[15] (2.5 µg/well), affinity-purified polyclonal anti-canine S100A12 (1.5 µg/well) or anti-canine S100A8/A9 antibody (1.5 µg/well; positive controls), or bovine serum albumin (BSA; negative controls) dissolved in 200 mM carbonate-bicarbonate (pH 9.4), and were incubated for 20 h at 4°C. Wells were then washed three times with 25 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) polyoxyethylene-20 sorbitan monolaurate, pH 8.0. Non-specific binding sites were blocked with 25 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) polyoxyethylene-20 sorbitan monolaurate, 10% (w/v) BSA, pH 8.0 for 1 h at 37°C. Wells were hen incubated with 1.1 µg of ^{125}I-tagged canine S100A12 (5.6 µCi/well) or ^{125}I-tagged canine S100A8/A9 (3.2 µCi/well) in 25 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) polyoxyethylene-20 sorbitan monolaurate, 0.5% (w/v) BSA, pH 8.0 supplemented with 2.4 mM $CaCl_2$ and 15 (or 150) µM $ZnSO_4$ (assay buffer) for 2 h at 37°C, and were then washed as described (with the supernatant being separately collected). Bound or captured ^{125}I-S100A12 or ^{125}I-S100A8/A9 was detected by an automated -counter[16].

2.4.3. Canine RAGE:S100/calgranulin protein native polyacrylamide gel electrophoresis (PAGE)

Canine RAGE[15] (2.1 µM; molecular mass: 42–50 kDa) and increasing molarities of purified canine S100A12 and canine S100A8/A9 protein (0, 4.2, 10.5, 21.0, 31.5, and 42.0 µM) in 2.4 mM $CaCl_2$ were incubated for 4 h at room temperature (approximately 23°C) followed by native PAGE in a discontinuous buffer system and a separating gel with a 7% acrylamide concentration. Electrophoresis (buffers supplemented with 2.4 mM $CaCl_2$) was performed at a constant voltage of 125 V for 120 min at approximately 23°C, followed by Coomassie brilliant blue staining[17]. Scanned images were analyzed using the Quantity One® software[18].

2.4.4. Canine RAGE:S100A12 size exclusion chromatography (SEC)

Canine RAGE[15] (15 µg) and purified canine S100A12 protein (100 µg) in 20 mM HEPES-NaOH (pH 8.0), 100 mM NaCl, 2.4 mM $CaCl_2$, 15 µM $ZnSO_4$ (running buffer) were incubated overnight at 4°C and were then applied to a calibrated sephacryl SEC column[19] (marker proteins: ribonuclease A, chymotrypsinogen A, ovalbumin, BSA, and blue dextran 2000) and equilibrated with 5 column volumes (V_C) of running buffer at a flow rate of 0.5 ml/min at room temperature on a fast protein liquid chromatography (FPLC) system[20]. Protein fractions eluted

from the column were analyzed by reducing sodium dodecyl sulfate (SDS)-PAGE in a vertical mini-gel format (10% Bis/Tris), where protein bands were visualized by staining with Coomassie brilliant blue. Scanned images were analyzed using the Quantity One® software[18].

2.5. Statistical analyses

A statistical software package was used for all statistical analyses[21]. Continuous variables were first assessed for normality of their distribution and equality of the variances using a Shapiro-Wilk *W* test and a Brown-Forsythe test, respectively. Summary statistics for continuous variables are reported as medians and ranges. Categorical data are presented as counts (*n*) and percentages.

A non-parametric Wilcoxon rank-sum test was used to compare the degree of gastric, duodenal, ileal, and colonic RAGE expression (AEC_n, BEC_n, CEC_n, and ABR) between dogs with CIE and healthy control dogs. A non-parametric Spearman rank correlation coefficient rho (ρ) was calculated to test the possibility of a correlation between tissue RAGE expression (AEC_n, BEC_n, CEC_n, and ABR) and patient characteristics (age, body weight), clinical variables (CCECAI score, disease location), biomarkers of concurrent disease (Spec cPL), systemic inflammation (CRP, serum calprotectin, and S100A12), and gastrointestinal function (serum cobalamin and folate), inflammation (fecal calprotectin and S100A12), or protein loss (fecal and serum $_1$PI), and the severity of histological lesions in the stomach, duodenum, ileum, and colon. The Spearman was interpreted as indicating a very strong (0.8–1.0), strong (0.6–0.8), moderate (0.4–0.6), weak (0.2–0.4), or very weak (0–0.2) correlation. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Study population

A total of 21 dogs were included in the study: 12 dogs diagnosed with CIE and 9 healthy control dogs (Table 1). There were no differences in age (p = 0.5937) or body weight (p = 0.1627) between the two groups of dogs, but significantly more male dogs were present in the group of dogs with CIE (75%) compared to the healthy control group (22%, p = 0.0140) (Table 1). The CCECAI scores in dogs with CIE ranged from indicating mild to very severe clinical disease, with a median CCECAI score of 6 (moderate clinical disease activity) (Table 1). In the group of dogs with CIE, 3 dogs (25%) received antisecretory treatment (antihistamine and/or proton pump inhibitor) at the time of study enrollment and tissue sample collection; 3 dogs (25%) were given a probiotic and 3 dogs (25%) antimicrobials (metronidazole and/or tylosin).

3.2. Gastrointestinal tissue RAGE expression

3.2.1. RAGE expression along the gastrointestinal tract in healthy dogs and dogs with CIE

Immunofluorescence staining of gastrointestinal tissue biopsies indicated that RAGE is expressed in the epithelium of the stomach, duodenum, ileum, and colon of all healthy dogs and dogs with CIE (Fig. 3 and 4, Table 2). Immunofluorescence analysis by CLSM showed a strong correlation between apical and basal epithelial RAGE expression in the ileum (ρ = 0.83, p <

0.001), duodenum (ρ = 0.75, p = 0.0022), and colon (ρ = 0.67, p = 0.0030). These correlations remained for the ileum (ρ = 0.86, p = 0.0065) and colon (ρ = 0.73, p = 0.0158) in the group of dogs with CIE. In contrast, in healthy dogs, apical and basal epithelial RAGE expression were only correlated in the duodenum (ρ = 0.89, p = 0.0068). Among the different segments, there was a strong correlation between the basal compartments of the duodenum and colon (ρ = 0.83, p = 0.0017).

Comparative immunofluorescence analysis showed a significantly higher epithelial RAGE expression in the duodenum and colon in dogs with CIE than in healthy dogs (Table 2). Compared to healthy controls, epithelial RAGE expression in dogs with CIE was numerically higher in the ileum but lower in the stomach. However, neither of these differences reached statistical significance (Table 2). A minimal shift towards more basal than apical epithelial RAGE expression (i.e., an increased ABR) was detected in the duodenum, ileum, and colon of dogs with CIE; but this was not statistically significant in any of these gastrointestinal segments.

Table 2. Comparison of gastrointestinal tissue RAGE expression in dogs with CIE (*n* = 12) and healthy control dogs (*n* = 9) included in the study.

Parameter	CIE	Healthy	*p*-value
Stomach			
CEC_n, median (IQR)	5.36 (3.06–6.83)	7.33 (5.19–8.81)	0.1439
Number of biopsies	5	7	–
Duodenum			
AEC_n, median (IQR)	10.36 (8.14–15.20)	6.75 (4.70–8.67)	**0.0152**
BEC_n, median (IQR)	12.72 (10.93–14.32)	6.09 (4.51–7.73)	**0.0033**
ABR, median (IQR)	1.02 (0.62–1.11)	1.15 (1.02–1.17)	0.2502
Number of biopsies	7	7	–
Ileum			
AEC_n, median (IQR)	13.00 (8.93–16.95)	11.66 (6.71–20.98)	0.8748
BEC_n, median (IQR)	12.30 (8.02–19.34)	12.21 (7.08–17.58)	0.7929
ABR, median (IQR)	1.04 (0.82–1.13)	1.08 (0.91–1.17)	0.7929
Number of biopsies	8	8	–
Colon			
AEC_n, median (IQR)	10.24 (8.18–16.42)	6.32 (5.90–7.17)	**0.0029**
BEC_n, median (IQR)	11.64 (7.86–27.82)	7.65 (5.63–9.17)	**0.0359**
ABR, median (IQR)	0.94 (0.62–1.16)	0.98 (0.69–1.12)	0.9611
Number of biopsies	10	7	–

CEC_n = normalized cellular epithelial photon counts; AEC_n = normalized apical epithelial photon counts; BEC_n = normalized basal epithelial photon counts; ABR = ratio between AEC_n and BEC_n. *P*-values in bold font indicate significance at $p < 0.05$.

3.2.2. Correlation of gastrointestinal tissue RAGE expression with serum sRAGE levels

Serum sRAGE concentrations ranged from 52–804 ng/L (median: 311 ng/L) in the group of dogs with CIE (Table 3). About half of the dogs (58%) had a serum sRAGE concentration ≤340

ng/L, the previously determined cut-off concentration that best separated dogs with CIE from healthy controls (median: 448 ng/L, interquartile range: 326–536 ng/L [Heilmann et al., 2014]). Serum sRAGE concentrations were strongly correlated with the degree of apical epithelial RAGE expression in the duodenum ($\rho = 0.79$, $p = 0.0362$) (Table 4) but not with the expression of tissue RAGE or compartmental shift (ABR) in any of the other gastrointestinal segments evaluated in this study.

Table 3. Clinicopathological and histological data for the dogs with CIE ($n = 12$) included in the study.

Parameter	**Value**
Serum sRAGE concentration in ng/L, median (IQR)	311 (159–490)
sRAGE concentration >340 ng/L, *n* (%)	5 (42%)
sRAGE concentration ≤340 ng/L, *n* (%)	7 (58%)
sRAGE concentration 52–340 ng/L, *n* (%)	6 (50%)
sRAGE concentration ≤52 ng/L, *n* (%)	1 (8%)
Serum CRP concentration in mg/L, median (IQR)	5.9 (0.9–15.4)
Fecal calprotectin concentration in µg/g, median (IQR)*	1.5 (0.6–23.4)
Serum calprotectin concentration in mg/L, median (IQR)†	8.2 (6.1–10.9)
Fecal S100A12 concentration in ng/g, median (IQR)*	94 (33–2601)
Serum S100A12 concentration in µg/L, median (IQR)	261 (196–440)
Fecal α_1PI concentration in µg/g, median (IQR)‡	10.5 (5.1–36.3)
Serum α_1PI concentration in mg/L, median (IQR)	1485 (1303–1609)
Serum albumin concentration in g/L, median (IQR)	28 (24–31)
Hypoalbuminemia, *n* (%)	1 (8%)
Mild hypoalbuminemia§	1 (8%)
Moderate hypoalbuminemia|	0 (0%)
Severe hypoalbuminemia#	0 (0%)
Serum total calcium concentration in md/dL, median (IQR)	9.6 (9.1–10.7)
Serum cobalamin concentration in ng/L, median (IQR)	423 (299–782)
Hypocobalaminemia¶, *n* (%)	2 (17%)
Serum folate concentration in µg/L, median (IQR)	10.7 (8.9–20.1)
Hypofolatemia**, *n* (%)	2 (17%)
Hyperfolatemia††, *n* (%)	1 (8%)
Serum Spec cPL concentration in µg/L, median (IQR)	43 (30–79)
Serum gastrin concentration in µg/L, median (IQR)	13.0 (10.0–37.3)
Histologic lesion score, median (IQR)	
Stomach	1 (1–2)
Duodenum	1.5 (1–2)
Ileum‡	2 (1–2)
Colon	1 (1–2)
Overall lesion score	2 (1–2)

CRP = C-reactive protein; IQR = interquartile range; Spec cPL = specific canine pancreatic lipase. *available from 8 dogs; †available from 6 dogs; ‡available from 11 dogs; §defined as a serum albumin concentration 15–20 g/L; |defined as a serum albumin concentration 12–14.9 g/L; #defined as a serum albumin concentration <12 g/L; ¶defined as a serum cobalamin concentration <300 ng/L; **defined as a serum folate concentration <7.7 µg/L; ††defined as a serum folate concentration >24.4 µg/L.

3.2.3. Correlation of gastrointestinal tissue RAGE expression with patient characteristics, clinical variables, and histopathologic lesions

Gastrointestinal RAGE expression was not associated with the dogs' age, body weight, disease duration, or CCEAI scores (Table 4). Histologic lesion scores ranged from 0 (no abnormalities) to 3 (severe lesions), with a median overall histologic lesion score indicating moderate histologic changes (Table 3). Segmental tissue RAGE expression was strongly correlated with the degree of villus stunting ($\rho = 0.76$, $p = 0.0487$) and crypt dilation/distortion ($\rho = 0.78$, $p = 0.0399$) in the basal compartment of the duodenum. The degree of eosinophilic infiltration ($\rho = -0.80$, $p = 0.0319$) and the overall ($\rho = -0.94$, $p = 0.0048$) and segmental histologic score ($\rho = -0.78$, $p = 0.0399$) were also strongly but inversely correlated with the epithelial RAGE expression in the apical compartment of the duodenum (Table 4). The compartmental shift (ABR) of epithelial RAGE expression in the ileum was strongly associated with the degree of neutrophilic infiltration ($\rho = 0.76$, $p = 0.0300$), in the duodenum was inversely correlated with the severity of histologic lesions in that segment ($\rho = -0.84$, $p = 0.0189$), and in the colon it was inversely correlated with the degree of colonic intraepithelial lymphocyte infiltration ($\rho = -0.70$, $p = 0.0240$) and the overall inflammatory criteria ($\rho = -0.73$, $p = 0.0166$). Basal epithelial RAGE expression in the colon was related to the overall inflammatory score ($\rho = 0.71$, $p = 0.0227$).

Table 4. Correlation (Spearman ρ) between gastrointestinal tissue RAGE expression and histological, clinical, and clinicopathological variables in dogs with CIE ($n = 12$).

Parameter correlated	**Stomach**	**Duodenum**			**Ileum**			**Colon**		
with	CEC_n	AEC_n	BEC_n	ABR	AEC_n	BEC_n	ABR	AEC_n	BEC_n	ABR
Histologic lesions (composite score)	0.30	**-0.94**	-0.09	-0.77	0.57	0.29	0.41	0.41	0.58	-0.47
Morphologic criteria (composite score)	0.40	-0.71	0.26	-0.66	0.60	0.44	0.12	0.38	0.28	-0.17
Inflammatory criteria (composite score)	-0.20	-0.77	-0.37	-0.43	0.14	-0.05	0.45	0.34	**0.71**	**-0.73**
	Stomach	Duodenum			Ileum			Colon		
Segment composite score	0.11	**-0.78**	0.30	**-0.84**	0.61	0.62	-0.17	0.12	0.39	-0.43
Morphologic criteria	-0.35	-0.36	0.62	-0.49	0.10	-0.08	0.45	0.01	0.00	0.01
▪ Surface epithelial injury	n/a	-0.63	-0.32	-0.16	-0.08	-0.25	0.41	0.00	-0.17	0.35
▪ Gastric pit epithelial injury	n/a									
▪ Villus stunting		-0.09	**0.76**	-0.54	-0.08	-0.25	0.41			
▪ Crypt dilation/distortion		-0.54	**0.78**	-0.72	0.17	0.03	0.35	-0.28	-0.22	0.10
▪ Lacteal dilatation		0.23	0.14	0.13	n/a	n/a	n/a			
▪ Change in goblet cells								0.12	0.16	-0.16
▪ Mucosal fibrosis	-0.35	n/a	n/a	n/a	n/a	n/a	n/a	0.53	0.42	-0.23
Inflammatory criteria	0.63	-0.73	0.09	-0.46	0.01	-0.10	0.32	0.18	0.52	-0.54
▪ Intraepithelial lymphocytes	0.00	-0.66	0.22	-0.44	0.06	0.17	-0.17	0.24	0.51	**-0.70**
▪ Lamina propria LPC	-0.31	-0.47	0.00	-0.19	-0.50	-0.32	0.06	0.45	0.43	-0.20
▪ Lamina propria eosinophils	0.21	**-0.80**	0.06	-0.60	0.65	0.34	0.18	-0.27	0.27	-0.51
▪ Lamina propria neutrophils	n/a	-0.61	0.20	-0.41	-0.25	-0.50	**0.76**	n/a	n/a	n/a
▪ Lamina propria MΦ	n/a	n/a	n/a	n/a	-0.08	-0.25	0.41	0.41	0.17	0.06
	0.26									

▪ Lymphoid follicular hyper-plasia										
Age	0.10	-0.07	-0.15	-0.15	0.12	-0.01	0.07	0.19	-0.10	0.14
Body weight	-0.04	-0.32	-0.38	0.18	-0.37	-0.32	0.10	-0.07	-0.07	-0.06
Disease duration	-0.40	0.00	-0.02	0.25	-0.41	-0.33	0.25	0.20	-0.19	0.07
CCECAI score	0.32	-0.44	-0.38	-0.44	0.36	0.18	0.31	-0.01	-0.14	0.22
Serum sRAGE concentration	0.00	**0.79**	0.21	0.39	-0.21	-0.19	-0.12	-0.10	0.30	-0.44
Serum CRP concentration	0.10	-0.29	0.29	-0.29	0.05	-0.26	**0.71**	0.52	0.10	0.07
Fecal calprotectin concentration	-0.20	0.09	0.14	0.26	-0.55	-0.43	0.14	0.43	**0.89**	**-1.00**
Serum calprotectin concentration	0.50	-0.40	-0.40	0.20	-0.26	0.09	-0.14	-0.30	-0.30	-0.30
Fecal S100A12 concentration	-0.10	0.32	0.11	0.43	-0.60	-0.31	-0.31	0.49	0.71	-0.66
Serum S100A12 concentration	0.00	0.32	0.46	0.21	-0.41	-0.02	-0.33	-0.10	-0.35	0.21
Fecal α_1PI concentration	-0.10	0.32	0.36	0.21	-0.48	-0.10	-0.29	0.43	-0.12	0.25
Serum α_1PI concentration	-0.10	0.46	-0.36	0.50	-0.31	-0.07	-0.48	**-0.72**	-0.53	0.06
Serum albumin concentration	-0.30	0.00	-0.49	0.25	-0.08	0.08	-0.43	**-0.64**	-0.21	-0.15
Serum total calcium concentration	-0.67	0.31	-0.58	0.67	-0.32	-0.20	-0.32	-0.32	0.31	-0.58
Serum cobalamin concentration	0.00	-0.68	0.14	-0.68	0.45	0.19	0.24	-0.06	0.48	-0.62
Serum folate concentration	-0.30	0.25	-0.50	0.50	-0.50	-0.36	-0.12	-0.33	-0.26	0.22
Serum Spec cPL concentration	**0.90**	-0.18	0.20	-0.54	0.50	0.47	-0.10	0.10	-0.13	0.34
Serum gastrin concentration	-0.67	0.19	-0.07	0.33	-0.58	-0.44	-0.07	-0.12	-0.17	0.01

α_1PI = alpha$_1$-proteinase inhibitor; ABR = ratio between AEC_n and BEC_n; AEC_n = normalized apical epithelial photon counts; BEC_n = normalized basal epithelial photon counts; CCECAI = canine chronic enteropathy clinical disease activity index; CEC_n = normalized extranuclear (cytosolic) epithelial photon counts; LPC = lymphocytes/plasma cells; MΦ = macrophages; n/a = not applicable; Spec cPL = specific canine pancreatic lipase. Spearman ρ in bold font with green background indicate significance at $p < 0.05$.

3.2.4. Correlation between gastrointestinal tissue RAGE expression and other biomarkers

RAGE expression in the basal segment of the colon was very strongly associated with fecal calprotectin concentrations ($\rho = 0.89$, $p = 0.0188$), and in the apical segment of the colon was inversely correlated with serum α_1PI ($\rho = -0.72$, $p = 0.0186$) and albumin concentrations ($\rho = -0.64$, $p = 0.0470$). The compartmental shift (ABR) of epithelial RAGE expression in the ileum was strongly associated with the systemic inflammatory biomarker CRP ($\rho = 0.71$, $p = 0.0465$), and in the colon was very strongly and inversely correlated with fecal calprotectin concentrations ($\rho = -1.00$, $p < 0.0001$). A direct relationship was observed between epithelial RAGE expression in the stomach and serum Spec cPL concentrations ($\rho = 0.90$, $p = 0.0374$). Tissue RAGE expression in the gastrointestinal segments evaluated was not correlated with any other biomarkers (Table 4).

3.3. Assessment of in vitro binding of RAGE to S100/calgranulin proteins

Normalized against the non-specific adherence to BSA (negative control, set at 100%), binding of 125I-S100A12 protein to recombinant canine RAGE was higher (149%) than that of the 125I-S100A8/A9 complex (117%) as measured by ELISA (Fig. 5); statistical comparison could not be performed due to the experiment size ($n = 3$). Native PAGE to evaluate canine RAGE:S100/calgranulin protein binding revealed multiple large protein bands between approximately 50–150 kDa (not shown), whereby the protein bands appeared more distinct for the canine RAGE:S100A8/A9 solutions, suggesting more or stronger canine RAGE:S100A12 than canine RAGE:S100A8/A9 binding.

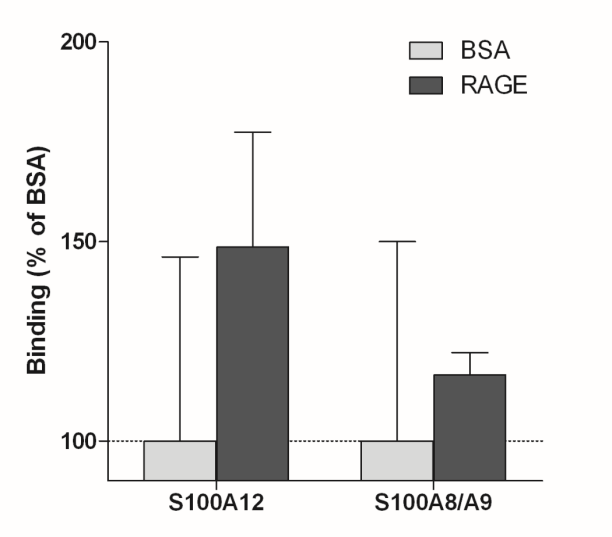

Fig. 5 – *In vitro* binding of the canine S100/calgranulins to canine RAGE assessed by ELISA. Compared to BSA as a reference (arbitrarily set to 100%; grey bars), more S100A12 protein was bound to recombinant canine RAGE (149%, left black bar) compared to the S100A8/A9 complex (117%, right black bar). Bars: means and standard deviations (3 different experiments; statistical comparison could not be performed).

4. Discussion

This study aimed at evaluating and quantifying the gastrointestinal mucosal expression of RAGE in dogs with CIE. This was accomplished by immunofluorescence detection and CLSM-guided quantification of RAGE in the apical and basal regions of the epithelial layer in different segments of the gastrointestinal tract (i.e., stomach, duodenum, ileum, and colon) in dogs with CIE in comparison to healthy control dogs. In contrast to conventional light microscopy, a fraction of tissue specimens is illuminated during CLSM. The standardized procedure of photon counting using CLSM and the help of ROI is currently considered as the gold standard method.

Similar to the results in human studies (Ciccocioppo et al., 2013; Body-Malapel et al., 2019), tissue RAGE was expressed along the gastrointestinal tract in both groups of dogs, those affected with CIE and healthy control dogs. This finding suggests gastrointestinal expression of RAGE to play a regulatory role in canine health and disease, and the expression levels might depend on the presence and spectrum of its ligands. RAGE is constitutively expressed in all tissues during embryonic development (Schmidt et al., 2000; Bierhaus et al., 2005), whereas postnatal RAGE expression decreases or ceases in most tissues except for a strong expression of the receptor in the skin, lungs, and certain cells of the immune system (Oldfield et al., 2001; Bierhaus, 2005). RAGE expression increases at sites of inflammation where the receptor is primarily localized to inflammatory and epithelial cells (Sparvero et al., 2009). This concept warrants consideration for interpreting RAGE expression in the gastrointestinal tract because gastrointestinal mucosal immunity is a delicate balance between immune responses and immunotolerance. Resulting from RAGE interaction with its ligands, proinflammatory responses are produced that also promote oxidative stress (Teissier et al., 2019), and RAGE blockade can protect against such conditions, including inflammaging (Teissier et al., 2019).

Gastric expression of RAGE was generally higher in healthy dogs than in dogs with CIE, whereas an opposite trend was seen in the ileum. Significantly higher tissue RAGE expression in affected dogs was seen in the duodenum and colon. This suggests a role of RAGE signaling

pathways in canine CIE, but the biological relevance of these results warrants further findings are mostly consistent with studies in humans with IBD, where tissue RAGE was consistently upregulated (Cioccociopo et al. 2013, Body-Malapel 2019). However, in one study, the majority of RAGE positivity was localized to the peri-ulcerative areas of the epithelium (Ciccocioppo et al., 2013), which is not a characteristic feature in canine CIE (Washabau et al., 2010, Slovak et al., 2015). Crohn's disease (CD) and ulcerative colitis (UC), the most common forms of IBD in humans, are characterized by predominantly granulomatous inflammation (Jumani et al., 2020), which might explain species differences in RAGE expression because the inflammatory infiltrate in dogs is predominantly lymphoplasmacytic (Day et al., 2008). Differences in location and mucosal penetration of the disease (Ortigosa, 2005; Body-Malapel et al., 2019; Jumani et al., 2020) might also contribute to the differences seen. In contrast to our results, RAGE expression is generally poor in the small intestine and colon in healthy humans and mice (Body-Malapel et al. 2019). This could be a species-specific difference, but the use of different techniques might also explain the discrepancy. As opposed to traditional semi-quantitative immunofluorescence analyses in the previous study (Body-Malapel et al. 2019), quantitative CLSM was used in our study and is a more sensitive technique. Quantification of mRNA expression could help to verify these findings.

RAGE expression in the apical and basal regions of the gastrointestinal epithelium was highly correlated along the gastrointestinal tract in dogs with CIE, but not in the control group of healthy dogs. This result is consistent with those in human IBD patients, pointing to a discontinuous expression of RAGE with low concentrations in non-inflamed gastrointestinal portions to reaching maximum levels in inflamed segments where RAGE ligands accumulate Ciccocioppo et al., 2013). A trend for an apical-to-basal shift in the expression of epithelial RAGE (expressed as ABR) was seen in the duodenum, ileum, and colon of dogs with CIE compared to healthy dogs. This finding might point to an attempt to decrease the activation of gastrointestinal epithelial transmembrane RAGE associated with the presence or accumulation of luminal stimuli (e.g., PAMPs) or even protect the apical portion of the epithelium from such antigens in the face of chronic inflammation. Whether the differences observed between health and disease in dogs are linked to the accumulation of RAGE ligands and the impact on functionality (e.g., the possibility of perpetuated proinflammatory signaling induced by RAGE ligands) warrant further study. Differences in tissue RAGE expression between the stomach and intestines could reflect differences between gastric and intestinal mucosa in dogs with CIE (Allenspach et al., 2018). However, the possibility of an effect of antisecretory treatment (e.g., proton pump inhibitors) or supplements (e.g., probiotics) on RAGE expression cannot be definitively excluded in dogs with CIE.

The positive relationship of the (primarily apical epithelial) duodenal RAGE expression and serum sRAGE concentrations (reflecting a splice-variant or de-anchoring of transmembrane [full-length] RAGE) in dogs with CIE further supports a dysregulation or compensatory regulation in the sRAGE/RAGE axis in canine CIE. Provision of increased anti-inflammatory ligand-capturing capacity to abrogate proinflammatory transmembrane RAGE signaling (Taguchi et al., 2000; Maillard-Lefebvre et al., 2009) might explain the (unexpected) parallel trends in circulating decoy receptor (serum sRAGE) levels and small intestinal tissue RAGE expression. Alternatively, serum sRAGE levels could merely reflect the degree of intestinal tissue RAGE

expression. The sRAGE/RAGE axis derangements in canine CIE appear only partially to depend on histologic lesions because primarily duodenal (but not ileal and to some extent colonic) epithelial RAGE expression was linked to the severity of histologic changes. Opposite trends in the correlation of tissue RAGE expression and histologic architectural duodenal lesions, compared to duodenal inflammatory lesions, are an interesting finding. This is in line with our previous observation of serum sRAGE concentrations to inversely correlate with the severity of proximal intestinal microscopic lesions (Cabrera-García et al., 2020) and further suggests that perturbations in the RAGE axis depend on the type, severity, and/or chronicity of histologic lesions. However, careful interpretation of these correlations is needed as cause-and-effect relationships cannot be definitively determined through the results of this study. Also, tissue RAGE expression was evaluated only in the epithelial layer and in some segments of the gastrointestinal tract in the dogs included in this study. Thus, a contribution of gastrointestinal segments not evaluated due to being outside reach of the endoscope, or extra-intestinal organs (e.g., exocrine pancreas with concurrent pancreatitis), or even a combination of these to the serum sRAGE concentrations cannot be excluded or evaluated. Further, lamina propria infiltrating or resident cells (e.g., macrophages) could also contribute to gastrointestinal tissue expression of RAGE (Ciccocioppo et al., 2013), but this warrants further study.

Gastrointestinal epithelial RAGE expression was not linked to the clinical disease severity (as determined by CCECAI score) in dogs with CIE in this study, although very high CCECAI scores (Allenspach et al., 2007) and also persistent sRAGE deficiency (Heilmann et al., 2014) were associated with poor outcomes. This agrees with the results in a human study on IBD, where no correlation was seen between RAGE expression and the severity of clinical signs (Ciccocioppo et al., 2013) but contrasts with pulmonary disease studies in humans showing the downregulation of RAGE to be an indicator of poor prognosis (Bierhaus et al., 2005). Large intestinal mucosal expression of RAGE was negatively associated with albumin and α_1PI (but not total calcium) concentrations in serum and suggests upregulated epithelial RAGE expression with hypoalbuminemia (e.g., due to protein-losing enteropathy), which is a negative prognostic factor in canine CIE (Allenspach et al., 2007). This result is consistent with the lack of a link between tissue RAGE expression and CCECAI scores because hypoalbuminemia is one of the nine criteria included in the CCECAI scoring system. Hypocalcemia is also a negative prognostic factor in canine CIE (Craven et al., 2004; Titmarsh et al., 2015). Associated with hypovitaminosis D, hypocalcemia can contribute to intestinal inflammation (Titmarsh et al., 2015b; Allenspach, 2017). Hypovitaminosis D and calcitriol treatment also, directly and indirectly, affect the sRAGE/RAGE axis in people (Sung et al., 2013; Lee et al., 2014; Torino et al., 2017), but this relationship requires further study in dogs with CIE.

Except for the apical-to-basal shift in the ileum, gastric or intestinal mucosal expression of RAGE was not associated with serum CRP concentrations in dogs with CIE. It was also not correlated with any other serum or fecal biomarkers of gastrointestinal inflammation, except for fecal calprotectin concentrations. Once again, this finding would indicate a role of tissue RAGE (i.e., RAGE upregulation) and the sRAGE/RAGE axis in the inflammatory process in canine CIE. Except for these few correlations between tissue RAGE expression and serum or fecal biomarker concentrations, the present findings agree with our recent results, showing that

serum sRAGE concentrations are also not associated with serum cobalamin or folate concentrations, nor with serum calprotectin, S100A12, fecal 1PI, or S100A12 concentrations (Cabrera-García et al., 2020). The lack of such associations between biomarker levels and the expression of RAGE might point to spatial and/or temporal differences in their expression and is also consistent with RAGE (transmembrane and decoy receptor) presenting a non-specific multi-ligand receptor as has been demonstrated in human studies (Schmidt et al., 2001; Bierhaus et al., 2005; Heilmann and Allenspach, 2017; Teissier et al., 2019).

We attempted to compare tissue RAGE expression between dogs with FRE and SRE/IRE dogs, which generally have higher CCECAI scores (Allenspach et al., 2007; Volkmann et al., 2017). However, complete follow-up information was only available for 3 dogs (2 dogs diagnosed with FRE and 1 dog with SRE/IRE). In the dog with SRE/IRE, tissue RAGE expression compared to the FRE dogs was higher in the apical compartment (SRE/IRE: AEC_n= 8.68; FRE: AEC_n = 6.72) but lower in the basal compartment of the colon (SRE/IRE: BEC_n = 6.49; FRE: BEC_n = 10.13). A basal-to-apical shift in colonic RAGE expression was seen in the dog with SRE/IRE (ARB = 1.34) but an apical-to-basal shift with FRE (ARB = 0.66). Statistical comparisons were not possible given the small number of dogs in the two subgroups.

The results of this study provide a basis for further elucidating the immune mechanisms and the role of PRRs in the pathogenesis of canine CIE. The standard treatment for human IBD and also canine SRE/IRE is based on corticosteroids and/or other immunosuppressive drugs (e.g., azathioprine, cyclosporine) with the risk of severe adverse effects, especially with prolonged administration (Sulz et al., 2020; Dandrieux, 2016; Erdmann and Heilmann, 2017). Further study of the pathways involved in chronic intestinal inflammation (canine CIE and human IBD) might lead to novel treatment strategies. Such a possible role of RAGE has been evaluated in a rodent model of IBD by placebo-controlled administration of the RAGE-specific inhibitor N-Benzyl-N-cyclohexyl-4-chlorobenzamide (FPS-ZM1), which dampened intestinal inflammation (Body-Malapel et al., 2019). Further studies into such pathway-based treatment strategies could benefit from utilizing canine CIE as a spontaneous model for human IBD.

The in vitro binding assays in this study suggest binding of the canine S100/calgranulins S100A12 protein and S100A8/A9 protein complex) to canine RAGE, indicating that both S100A12 and calprotectin (S100A8/A9) are ligands for RAGE.

We acknowledge that this study has some limitations. First, the time between the preparation of the samples (i.e., deparaffinization and immunofluorescence staining) and their evaluation by CLSM was between 6–11 months. This delay was inevitable given the time for evaluation of tissue specimens by this modality. Another limitation is that, because endoscopic tissue biopsies were used, each tissue sample was of slightly different size, orientation, and overall quality (Willard et al., 2008). Particularly endoscopic tissue biopsies from the ileum can be of lower quality because endoscopic intubation of the ileum from the colon is difficult in some dogs, requiring a smaller number of blind biopsies collected in some cases. Finally, pre-illumination of the slides due to screening immediately after immunofluorescence staining to evaluate the quality of the stained slides prior to CLSM is a limitation of the study. This procedure can weaken the signal intensity obtained during the CLSM analysis.

5. Conclusions

The results of this study suggest a role of RAGE pathways and the sRAGE/RAGE axis in the inflammatory process in dogs with CIE. Our findings largely agree with the results of studies in humans and experimental models of IBD (Ciccocioppo et al., 2013; Body-Malapel et al., 2019), further supporting the hypothesis that transmembrane RAGE expression and intracellular RAGE signaling are involved in the chronic inflammatory response. RAGE antagonization (e.g., abrogation of transmembrane RAGE activation and intracellular signaling by synthetic anti-inflammatory receptor decoys) appears to be a reasonable novel therapeutic strategy in chronic gastrointestinal inflammation. Further research into such targeted (pathway-specific) treatment options in dogs with canine CIE may serve as a good model for human IBD.

Footnotes

[1]Triton X-100, Roth, Karlsruhe, Germany
[2]Polyclonal sheep anti-canine RAGE antibody, R&D Systems, Minneapolis, MN, USA
[3]Polyclonal sheep anti-recombinant canine RAGE, Thermo Fisher Scientific, Dreieich, Germany
[4]Rabbit anti-goat IgG, Dianova, Hamburg, Germany
[5]Tyramide SuperBoostTM Kit, Thermo Fisher Scientific, Dreieich, Germany
[6]Alexa Fluor™ 488 nm tyramide reagent, Thermo Fisher Scientific, Dreieich, Germany
[7]Hoechst 33342 (Molecular probes, Eugene, OR, USA)
[8]Olympus IX71 fluorescence microscope, Olympus, Hamburg, Germany
[9]CellSens Dimension software, Olympus, Hamburg, Germany
[10]Leica TCS SP8 and LAS-X 3.5.5, Leica Microsystems, Mannheim, Germany
[11]HyD, Leica Microsystems, Mannheim, Germany
[12]HC PL APO CS2 63×/1.30 Glyc, Leica Microsystems, Mannheim, Germany
[13]LAS-X 3.4.2 software, Leica Microsystems, Mannheim, Germany
[14]MaxiSorpTM Nunc-ImmunoTM Plates, Thermo Scientific, Rockford, IL, USA
[15]Recombinant canine RAGE, R&D Systems, Minneapolis, MN, USA
[16]Wallac 1470 WIZARD, Perkin Elmer Life and Analytical Sciences, Wellesley, USA
[17]ImperialTM Protein Stain, BioTek®, Thermo Scientific, Rockford, IL, USA
[18]Quantity One® software (v4.6.5), Bio-Rad Laboratories, Hercules, CA, USA
[19]HiPrepTM Sephacryl® S-100 HR, GE Healthcare, Piscataway, NJ, USA
[20]ÄKTA basic, GE Healthcare, Piscataway, NJ, USA
[21]JMP® v13.1.0, SAS Institute, Cary, NC, USA

Acknowledgments

Part of the data has been presented as a research abstract (poster) at the 30th Annual Congress of the European College of Veterinary Internal Medicine (ECVIM), Barcelona, Spain (3–5 September, 2020). The authors greatly appreciate Prof. Andreas Holzenburg and Dr. Stanislav Vitha for their assistance and advice regarding CLSM data interpretation. We also thank the BioImaging Core Facility of Leipzig University for support on confocal laser scanning microscopy.

Declaration of conflicting interests

The authors declare that they have no competing interests.

Funding

The authors received no financial support from a formal grant for the research, authorship, and/or publication of this article. Materials and supplies for the study were generously provided by the Gastrointestinal Laboratory at Texas A&M University, the Institute of Immunology, and the Department for Small Animals at Leipzig University, Germany.

References

Allenspach, K., Wieland, B., Gröne, A., Gaschen, F., 2007. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J. Vet. Intern. Med. 21, 700–708. Allenspach, K., Culverwell, C., Chan, D., 2016. Long-term outcome in dogs with chronic enteropathies: 203 cases. Vet. Rec. 178, 368.

Allenspach, K. Rizzo, J., Jergens, A.E., Chang, Y.M., 2018. Hypovitaminosis D is associated with negative outcome in dogs with protein losing enteropathy: a retrospective study of 43 cases. BMC Vet. Res. 13, 96.

Allenspach, K.A., Mochel, J.P., Du, Y., Priestnall, S.L., Moore, F., Slayter, M, Rodrigues, A., Ackermann, M., Krockenberger, M., Mansell, J.; WSAVA GI Standardization Working Group, Luckschander, N., Wang, C., Suchodolski, J.S., Berghoff, N., Jergens, A.E., 2019. Correlating gastrointestinal histopathologic changes to clinical disease activity in dogs with idiopathic inflammatory bowel disease. Vet. Pathol. 56, 435–443.

Bierhaus, A., Humpert, P.M., Morcos, M., Wendt, T., Chavakis, T., Arnold, B., Stern, DM., Nawroth, P.P., 2005. Understanding RAGE, the receptor for advanced glycation end products. J. Mol. Med. 83, 876–886.

Body-Malapel, M., Djouina., M., Waxin, C., Langlois, A., Gower- Rousseau, C., Zerbib, P., Schmidt, A.M., Desreumaux, P., Boulanger, E., Vignal, C., 2019. The RAGE signaling pathway is involved in intestinal inflammation and represents a promising therapeutic target for inflammatory bowel diseases. Mucosal Immunol. 12, 468–478.

Bramhall, M., Rich, K., Chakraborty, A., Logunova, L., Han, N., Wilson, J., McLaughlin, J., Brass, A., Cruickshank, S.M., 2020. Differential expression of soluble receptor for advanced glycation end products in mice susceptible or resistant to chronic colitis. Inflamm. Bowel Dis. 26, 360–368.

Cabrera-García, I.A., Suchodolski, J.S., Steiner, J.M., Heilmann, R.M., 2020. Association between serum soluble receptor for advanced glycation end products (RAGE) deficiency

and severity of clinicopathologic evidence of canine chronic inflammatory enteropathy. J. Vet. Diagn. Invest. 32, 664–674.

Cerquetella, M., Rossi, G., Suchodolski, J.S., Schmitz, S.S., Allenspach, K., Rodríguez-Franco, F., Furlanello, T., Gavazza, A., Marchegiani, A., Unterer, S., Burgener, I.A., Pengo, G., Jergens, A.E., 2020. Proposal for rational antibacterial use in the diagnosis and treatment of dogs with chronic diarrhoea. J. Small Anim. Pract. 62, 211–215.

Ciccocioppo, R., Vanoli, A., Klersy, C., Imbesi, V., Boccaccio, V., Manca, R., Betti, E., Cangemi, G.C., Strada, E., Besio, R., Rossi, A., Falcone, C., Ardizzone, S., Fociani, P., Danelli, P., Corazza, G.R., 2013. Role of the advanced glycation end products receptor in Crohn's disease inflammation. World J. Gastroenterol. 19, 8269–8281.

Ciccocioppo, R., Bozzini, S, Betti, E., Imbesi, V., Klersy, C., Lakyova, L.S., Sukovsky, L., Benacka, J., Kruzliak, P., Corazza, G.R., Di Sabatino, A., Falcone, C., 2019. Functional polymorphisms of the receptor for the advanced glycation end product promotor gene in inflammatory bowel disease: a case-control study. Clin. Exp. Med. 19, 367–375.

Craven, M., Simpson, J.W., Ridyard, A.E., Chandler, M.L., 2004. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995–2002). J. Small Anim. Pract. 45, 336–342.

Dandrieux, J.R., 2016. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? J. Small Anim. Pract. 57, 589–599.

Day, M.J., Bilzer, T., Mansell, J., Wilcock, B., Hall, E.J., Jergens, A., Minami, T., Willard, M., Washabau, R., 2008. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. J. Comp. Pathol. 138, S1–S43.

Erdmann, C., Heilmann, R.M., 2017. Diagnostic and therapeutic approach to chronic inflammatory enteropathies of dogs. Tierarztl. Prax. Ausg. K. Kleintiere Heimtiere 45, 317–327.

Heilmann, R.M., Suchodolski, J.S., Steiner, J.M., 2008. Development and analytic validation of a radioimmunoassay for the quantification of canine calprotectin in serum and feces from dogs. Am. J. Vet. Res. 69, 845–853.

Heilmann, R.M., Lanerie, D.J., Ruaux, C.G., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2011. Development and analytic validation of an immunoassay for the quantification of canine S100A12 in serum and fecal samples and its biological variability in serum from healthy dogs. Vet. Immunol. Immunopathol. 144, 200–209.

Heilmann, R.M., Otoni, C.C., Jergens, A.E., Grützner, N., Suchodolski, J.S., Steiner, J.M., 2014. Systemic levels of the anti-inflammatory decoy receptor soluble RAGE (receptor for advanced glycation end products) are decreased in dogs with inflammatory bowel disease. Vet. Immunol. Immunopathol. 161, 184–192.

Heilmann, R.M., Allenspach, K., 2017. Pattern-recognition receptors: signaling pathways and dysregulation in canine chronic enteropathies – brief review. J. Vet. Diagn. Invest. 29, 781–787.

Hofmann, M., Drury, S., Fu, C., Qu, W., Taguchi, A., Lu, Y., Avila, C., Kambham, N., Bierhaus, A., Nawroth, P., Neurath, M.F., Slattery, T., Beach, D., McClary, J., Nagashima, M., Morser, J., Stern, D., Schmidt, A.M., 1999. RAGE mediates a novel proinflammatory axis: A central cell surface receptor for S100/calgranulin polypeptides. Cell. 97, 889–901. Jergens, A.E., Simpson, K.W., 2012. Inflammatory bowel disease in veterinary medicine. Front. Biosci. (Elite Ed). 4, 1404–1419.

Jumani, L., Kataria, D., Ahmed, M.U., Shah, M.A.A., Raja, K., Shaukat, F., 2020. The spectrum of extra-intestinal manifestation of Crohn's disease. Cureus. 12, e6928.

Lee, T.W., Kao, Y.H., Lee, T.I., Chang, C.J., Lien, G.S., Chen, Y.J., 2014. Calcitriol modulates receptor for advanced glycation end products (RAGE) in diabetic hearts. Int. J. Cardiol. 173, 236–241.

Maillard-Lefebvre, H., Boulanger, E., Daroux, M., Gaxatte, C., Hudson, B.I., Lambert, M., 2009. Soluble receptor for advanced glycation end products: a new biomarker in diagnosis and prognosis of chronic inflammatory diseases. Rheumatology. 48, 1190–1196.

Oldfield, M.D., Bach, L.A., Forbes, J.M., Nikolic-Paterson, D., McRobert, A., Thallas, V., Atkins, R.C., Osicka, T., Jerums, G., Cooper, M.E., 2001. Advanced glycation end products cause epithelial-myofibroblast transdifferentiation via the receptor for advanced glycation end products (RAGE). J. Clin. Invest. 108, 1853–1863.

Ortigosa, M.D., 2005. Concepto actual y aspectos cl nicos de la enfermedad de Crohn y la colitis ulcerosa. Colomb. Med. 36, S16–24.

Schmidt, A.M., Yan, S.D., Yan S.F., Stern, D.M., 2000. The biology of receptor for advanced glycation end products and its ligands. Biochim. Biophys. Acta. 1498, 99–111. Slovak, J.E., Wang, C., Sun, Y., Otoni, C., Morrison, J., Deitz, K., LeVine, D., Jergens, A.E., 2015. Development and validation of an endoscopic activity score for canine inflammatory bowel disease. Vet. J. 203, 290–295.

Sparvero, L.J., Asafu-Adjei, D., Kang, R., Tang, D., Amin, N., Im, J., Rutledge, R., Lin, B., Amoscato, A.A., Zeh, H.J., Lotze, M.T, 2009. RAGE (receptor for advanced glycation endproducts), RAGE ligands, and their role in cancer and inflammation. J. Transl. Med. 7, 17.

Sterenczak, K.A., Willenbrock, S., Barann, M., Klemke, M., Soller, J.T., Eberle, N., Nolte, I., Bullerdiek, J., Murua Escobar, H., 2009. Cloning, characterization, and comparative quantitative expression analyses of receptor for advanced glycation end products (RAGE) transcript forms. Gene. 434, 35–42.

Sterenczak, K.A., Joetzke, A.E., Willenbrock, S., Eberle, N., Lange, S., Junghanss, C., Nolte, I., Bullerdiek, J., Simon, D., Murua Escobar, H., 2010. High-mobility group B1 (HMGB1) and receptor for advanced glycation end-products (RAGE) expression in canine lymphoma. Anticancer Res. 30, 5043–5048.

Sterenczak, K.A., Kleinschmidt, S., Wefstaedt, P., Eberle, N., Hewicker-Trautwein, M., Bullerdiek, J., Nolte, I., Murua Escobar, H., 2011. Quantitative PCR and immunohistochemical analyses of HMGB1 and RAGE expression in canine disseminated histiocytic sarcoma (malignant histiocytosis). Anticancer Res. 31, 1541–1548.

Sulz MC., Burri, E., Michetti, P., Rogler, G., Peyrin-Biroulet. L., Seibald, F., 2020. Treatment algorithms for Crohn's disease. Digestion. 101 Suppl. 1, 43–57.

Sung, J.Y., Chung, W., Kim, A.J., Kim, H.S., Ro, H., Chang, J.H., Lee, H.H., Jung, J.Y., 2013. Calcitriol treatment increases serum levels of the soluble receptor of advanced glycation end products in hemodialysis patients with secondary hyperparathyroidism. Tohoku J. Exp. Med. 230, 59–66.

Taguchi, A., Blood, D.C., del Toro, G., Canet, A., Lee, D.C., Qu, W., Tanji, N., Lu, Y., Lalla, E., Fu, C., Hofmann, M.A., Kislinger, T., Ingram, M., Lu, A., Tanaka, H., Hori, O., Ogawa, S., Stern, D.M., Schmidt, A.M., 2000. Blockade of RAGE-amphoterin signaling suppresses tumour growth and metastases. Nature. 405, 354–360.

Teissier, T., Boulanger, E., 2019. The Receptor for advanced glycation end-products (RAGE) is an important pattern recognition receptor (PRR) for inflammaging. Biogerontology. 20, 279–301.

Titmarsh, H.F., Gow, A.G., Kilpatrick, S., Cartwright, J.A., Milne, E.M., Philbey, A.A., Berry, J., Handel, J., Mellanby, R.J., 2015. Low vitamin D status is associated with systemic and gastrointestinal inflammation in dogs with a chronic enteropathy. PLoS One 10, e0137377.

Titmarsh, H.F., Gow, A.G., Kilpatrick, S., Sinclair, J., Milne, E., Philbey, A.A., Berry, J., Handel, J., Mellanby, R.J., 2015b. Association of vitamin D status and clinical outcome in dogs with a chronic enteropathy. J. Vet. Intern. Med. 29, 1473–1478.

Torino, C., Pizzini, P., Cutrupi, S., Tripepi, R., Vilasi, A., Tripepi, G., Mallamaci, F., Zoccali, C., 2017. Effect of vitamin D receptor activation on the AGE/RAGE system and myeloperoxidase in chronic kidney disease patients. Oxid. Med. Cell Longev. 2017, 2801324.

Volkmann, M., Steiner, J.M., Fosgate, G.T., Zentek, J., Hartmann, S., Kohn, B., 2017. Chronic diarrhea in dogs – retrospective study in 136 cases. J. Vet. Intern. Med. 31, 1043–1055.

Washabau, R.J., Day, M.J., Willard, M.D., Hall, E.J., Jergens, A.E., Mansell, J., Minami, T., Bilzer, T., 2010. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J. Vet. Intern. Med. 24, 10–26.

Willard, M.D., Mansell, J., Fosgate, G.T., Gualtieri, M., Olivero, D., Lecoindre, P., Twedt, D.C., Collett, M.G., Day, M.J., Hall, E.J., Jergens, A.E., Simpson, J.W., Else, R.W., Washabau, R.J., 2008. Effect of sample quality on the sensitivity of endoscopic biopsy for detecting gastric and duodenal lesions in dogs and cats. J. Vet. Intern. Med. 22, 1084–1089.

4 DISCUSSION

4.1 Objective of the study

The goal of the first part of this study was to evaluate serum sRAGE concentrations in a large group of dogs with CIE and to assess the possibility of a relationship of serum sRAGE concentrations with the clinical disease activity score (CCECAI), histologic lesion severity, serum C-reactive protein (CRP) concentrations, and serum and fecal calprotectin, S100A12, and α_1PI concentrations. We hypothesized that (i) serum sRAGE concentrations are severely decreased in dogs with CIE, and (ii) serum sRAGE concentrations correlate with the severity of clinical disease, the severity of microscopic lesions, and the concentrations of biomarkers reflecting gastrointestinal function, inflammation, or protein loss.

The second part of this study aimed at immunohistologically detecting and quantifying mucosal RAGE expression along the different segments of the gastrointestinal tract (i.e., stomach, duodenum, ileum, and colon) and assessing the possibility of an association of gastrointestinal RAGE expression with the serum sRAGE concentration, clinical disease and histologic lesion severity, and biomarkers of gastrointestinal function, inflammation, or protein loss. A secondary objective of this second part of the study was to determine the *in vitro* binding of sRAGE to the S100/calgranulin proteins in dogs. We hypothesized that (i) RAGE is overexpressed in the gastrointestinal mucosal epithelium in dogs with CIE and (ii) increased gastrointestinal RAGE expression correlates with the serum sRAGE concentration and indicators of disease severity. We also hypothesized that, *in vitro,* the S100/calgranulin proteins bind to sRAGE.

The materials & methods and the results of these studies, have been discussed and are published in two independent publications. The following discussion summarizes the key points, results, and conclusions of both studies.

4.2 Discussion of the results

This study revealed an overall serum sRAGE deficiency and dysregulations in the gastrointestinal mucosal expression of RAGE in dogs with CIE. In addition, sRAGE:S100/calgranulin protein binding was confirmed in dogs.

Tissue RAGE was expressed along the gastrointestinal tract in dogs with CIE and healthy control dogs, similar to human and experimental animal studies (CICCOCIOPPO et al. 2013, BODY-MALAPEL et al. 2019) and suggesting that postnatal gastrointestinal expression of

RAGE plays a regulatory role in canine health and disease (SCHMIDT et al. 2000, OLDFIELD et al. 2001, BIERHAUS et al. 2005). The expression levels of RAGE in dogs likely depend on the presence and spectrum of its ligands because a trend for an apical-to-basal shift in the expression of epithelial RAGE was seen in the duodenum, ileum, and colon of dogs with CIE, and RAGE expression was shown to increase at sites of inflammation in human patients (SPARVERO et al. 2009). Protection against inflammatory responses, oxidative stress, and inflammaging by RAGE blockade by administering a RAGE-specific inhibitor or $RAGE^{-/-}$ (BODY-MALAPEL et al. 2019, TEISSIER et al. 2019) adds to the concept of gastrointestinal mucosal immunity presenting a delicate balance between immune responses and immunotolerance.

Significantly higher tissue RAGE expression was seen in the duodenum and colon of dogs with CIE – with the same trend in the ileum but opposite trends in the stomach – and a strong correlation of RAGE expression between the apical and basal regions of the gastrointestinal epithelium. This further suggests a role of RAGE signaling pathways in canine CIE. In contrast to human IBD, where RAGE is primarily localized to the peri-ulcerative areas of the epithelium (CICCOCIOPPO et al. 2013, BODY-MALAPEL 2019), RAGE positivity was consistently upregulated in dogs with CIE. Crohn's disease (CD) and ulcerative colitis (UC), the most common forms of IBD in humans, are characterized by predominantly granulomatous inflammation (JUMANI et al. 2020), which might explain the species differences in RAGE expression because the inflammatory infiltrate in dogs is predominantly lymphoplasmacytic (DAY et al. 2008). Differences in disease location and mucosal penetration (ORTIGOSA 2005, BODY-MALAPEL et al. 2019, JUMANI et al. 2020) might also contribute to the differences seen.

In contrast to this study, RAGE expression is generally very low in the small intestine and colon in healthy humans and experimental animals (BODY-MALAPEL et al. 2019), reflecting either a species-specific difference or might be explained by the use of different techniques. As opposed to traditional semi-quantitative immunofluorescence analyses in the study by BODY-MALAPEL et al. (2019), quantitative confocal laser scanning microscopy (CLSM) was used in this study and is a more sensitive technique.

Together with the positive relationship between duodenal RAGE expression and serum sRAGE concentrations, the decrease in serum sRAGE decoy receptor levels and its correlation with the severity of proximal gastrointestinal histologic lesions in dogs with CIE supports a dysregulation in the sRAGE/RAGE axis and transmembrane RAGE signaling (i.e., increased activity of the cellular receptor leading to pro-inflammatory intracellular signaling) in canine CIE, either presenting a cause or consequence of this condition. Provision of increased anti-inflammatory

ligand-capturing capacity to abrogate proinflammatory transmembrane RAGE signaling (TAGUCHI et al. 2000, MAILLARD-LEFEBVRE et al. 2009) is a possible explanation for the parallel trends in circulating decoy receptor (serum sRAGE) levels and small intestinal tissue RAGE expression. Alternatively, serum sRAGE levels could merely reflect the degree of intestinal tissue RAGE expression. Opposite trends in the correlation of tissue RAGE expression and histologic architectural duodenal lesions, compared to duodenal inflammatory lesions, are in line with the observation of serum sRAGE concentrations to inversely correlate with the severity of proximal intestinal microscopic lesions and suggests that perturbations in the RAGE axis depend on the type, severity, and/or chronicity of histologic lesions. Endoscopic lesion scores were not obtained in this study and could not be evaluated for a possible association with serum sRAGE concentrations or gastrointestinal epithelial RAGE expression.

The severity of clinical signs in dogs with CIE (as determined by CCECAI score) was not correlated with serum sRAGE concentrations or the gastrointestinal epithelial expression of RAGE, although very high CCECAI scores (ALLENSPACH et al. 2007) and also persistent sRAGE deficiency (HEILMANN et al. 2014) were associated with poor outcomes. This finding is compatible with a lack of correlation between RAGE positivity and the clinical activity index in human patients with IBD (CICCOCIOPPO et al. 2013). Our findings contrast those of another study, showing an inverse association between clinical disease activity and serum sRAGE concentrations in Crohn‘s disease and ulcerative colitis patients (CICCOCIOPPO et al. 2015). Like this study, CICCOCIOPPO et al. (2013) found no correlation with previous treatment or disease length in human patients. Comparison of tissue RAGE expression between dogs with FRE and SRE/IRE dogs was attempted. However, complete follow-up information was only available for a small number of dogs limiting statistical comparisons between the two subgroups.

The negative association of large intestinal mucosal RAGE expression with serum albumin and α_1PI (markers of gastrointestinal protein loss) concentrations suggests an upregulated epithelial RAGE expression with hypoalbuminemia (e.g., due to protein-losing enteropathy [PLE]), which is a negative prognostic factor in canine CIE (ALLENSPACH et al. 2007). This result agrees with the lack of a link between tissue RAGE expression and CCECAI scores because hypoalbuminemia is one of the nine criteria included in the CCECAI scoring system. Lack of an association between serum sRAGE concentrations and any biomarkers of gastrointestinal function or inflammation in serum or fecal specimens – despite a trend of an association seen for serum S100A12 concentrations – might be explained by a spatial and/or temporal variation

in the expression of these molecules and by identifying several different three-dimensional molecular structures by RAGE. The latter involves exogenous ligands and endogenous molecules like the S100/calgranulin proteins (BIERHAUS et al. 2005). Consistent with this study‘s results, an association of serum sRAGE with concentrations of fecal calprotectin or serum S100A12 was also not observed in patients with Crohn‘s disease (CICCOCIOPPO et al. 2015). In contrast, there was an inverse relationship between serum sRAGE and fecal calprotectin in patients with ulcerative colitis (CICCOCIOPPO et al. 2015). Lack of a correlation between serum concentrations of sRAGE and CRP in canine CIE also agrees with the findings in ulcerative colitis patients but differs from findings in patients with Crohn‘s disease (MEIJER et al. 2014, CICCOCIOPPO et al. 2015). Similarly, gastric or intestinal mucosal expression of RAGE was also not associated with serum CRP concentrations or any other serum or fecal biomarkers of gastrointestinal inflammation in dogs with CIE, indicating a role of tissue RAGE (i.e., RAGE upregulation) and the sRAGE/RAGE axis in the inflammatory process in canine CIE. The lack of such associations between biomarker levels and RAGE expression also points to spatial and/or temporal differences in their expression. It is also consistent with RAGE (transmembrane and decoy receptor) presenting a non-specific multi-ligand receptor (SCHMIDT et al. 2001, BIERHAUS et al. 2005, HEILMANN and ALLENSPACH 2017, Teissier et al. 2019). Therefore, further examination of the ligand-RAGE pathways and the sRAGE/ RAGE axis is warranted. Whether genetic defects in the *RAGE* gene contribute to a disruption in sRAGE/RAGE signaling (WANG et al. 2014) in canine CIE is unknown and requires further investigation.

The *in vitro* binding assays in this study suggest binding of the canine S100/calgranulins (S100A12 protein and S100A8/A9 protein complex) to canine RAGE, indicating that both S100A12 and calprotectin (S100A8/A9) are ligands for RAGE.

4.3 Limitations of the study

Some limitations of the study warrant to be acknowledged. The risk of a concurrent disease process (e.g., chronic or subclinical pancreatitis) affecting concentrations of serum sRAGE in some dogs cannot be entirely excluded.

Tissue RAGE expression in dogs with CIE was evaluated based on endoscopic tissue biopsies, where each tissue sample is of slightly different size, orientation, and overall quality (WILLARD et al. 2008). Unlike conventional light microscopy, where whole tissue specimens are evaluated, only fractions of such specimens are illuminated during confocal laser scanning microscopy (CLSM). However, the standardized procedure of photon counting using CLSM

and the help of ROI is currently considered as the gold standard method for quantitative tissue expression analyses. The delay between preparation of the tissue samples (i.e., deparaffinization and immunofluorescence staining) and their evaluation by CLSM was between 6–11 months, which was inevitable given the time for evaluation of tissue specimens by CLSM. Preillumination of the slides due to screening immediately after immunofluorescence staining to evaluate the quality of the stained slides prior to CLSM can weaken the signal intensity obtained during the CLSM analysis. Infiltrating or resident cells of the lamina propria may contribute to gastrointestinal tissue RAGE expression (CICCOCIOPPO et al. 2013), but this warrants further study.

Histopathologic examination of gastrointestinal tissue biopsies was done by 9 separate board-certified pathologists, with the risk of significant interobserver variability despite using standardized criteria (JERGENS et al. 2014). Also, some gastrointestinal segments were not assessed due to being outside the reach of the endoscope.

The study of certain protein biomarkers in serum and fecal samples involved a small number of dogs. Hence, the possibility of a type II error to find no major variations or correlations remains a potential risk.

4.4 Conclusions

This study confirms that serum sRAGE concentrations are significantly decreased in dogs with CIE and correlate with the severity of histologic lesions. The results also suggest a role of RAGE pathways and the sRAGE/RAGE axis in the chronic inflammatory responses in dogs with CIE. Whether the observed deficiency in systemic concentrations of this decoy receptor is a cause (decreased systemic development leading to the pathogenesis of canine CIE) or consequence of CIE (inflammatory consumption) warrants further investigation. This study also shows that S100A12 and calprotectin (S100A8/A9 complex) are RAGE ligands. These results provide a basis for further elucidating the immune mechanisms and the role of pattern-recognition receptors (PRRs) in the pathogenesis of canine CIE. Further study of these pathways in canine CIE (and human IBD) is expected to bring about novel treatment strategies with potentially less severe adverse effects than traditional immunosuppressive drugs (e.g., corticosteroids) (DANDRIEUX 2016, ERDMANN and HEILMANN 2017). Further studies into such pathway-based treatment strategies, particularly RAGE antagonization (e.g., abrogation of transmembrane RAGE activation and intracellular signaling by synthetic anti-inflammatory receptor decoys), are expected to benefit from utilizing canine CIE as a spontaneous model for human IBD.

5 Summary

Angela Isabel Cabrera García

Evaluation of RAGE (receptor for advanced glycation end products) in dogs with chronic enteropathy

Department for Small Animals, Faculty of Veterinary Medicine, Leipzig University

Submitted in March 2021

(83 pages, 22 figures, 8 tables, 83 references)

Keywords: chronic inflammatory enteropathy, damage-associated molecular pattern, dog, inflammatory bowel disease, pattern recognition receptor, receptor for advanced glycation end products.

Background: Chronic inflammatory enteropathies (CIE) are an important group of gastrointestinal diseases in dogs, and dogs with CIE are subclassified as having either food-responsive (FRE) or immunosuppressive-responsive/-refractory enteropathy (IRE). The innate immune system has been shown to play a central role in the pathogenesis of CIE in dogs. Innate immune dysregulations in canine CIE involve the signaling of innate immune receptors, such as the pattern-recognition receptors (PRRs). The receptor for advanced glycation end products (RAGE) is a PRR that has not been investigated extensively in canine CIE. The anti-inflammatory decoy receptor soluble RAGE (sRAGE) can compete for ligand-binding to transmembrane (full-length) RAGE and thus abrogate pro-inflammatory cellular post-receptor responses. A previous study in dogs showed that serum soluble RAGE (sRAGE) concentrations are decreased in dogs with CIE, leading to the hypothesis that the RAGE/sRAGE axis is dysregulated in canine CIE. This hypothesis was further investigated in this study.

Objectives: This study aimed to determine serum sRAGE concentrations in canine CIE and their association with histological and clinicopathological evidence of CIE, including serum and fecal biomarkers of gastrointestinal inflammation or protein loss. Also, the expression of full-length RAGE along the gastrointestinal tract (stomach, duodenum, ileum, and colon) was evaluated in canine CIE using immunofluorescence analysis. Quantitative gastrointestinal epithelial expression of RAGE in dogs with CIE was compared to that in healthy control dogs and was evaluated for an association with the serum sRAGE concentration and with clinical, clinicopathological, and histological markers of gastrointestinal disease.

Material and Methods: Serum and patient data from a total of 102 dogs diagnosed with CIE were used in the first part of this study. All dogs underwent a standard diagnostic work-up, which included a minimum database (hematology, serum biochemistry, urinalysis, fecal examination, gastrointestinal profile, and biomarkers of gastrointestinal inflammation or protein loss), further diagnostics to exclude other gastrointestinal and extra-gastrointestinal disorders that can mimic CIE (e.g., serum baseline cortisol), abdominal diagnostic imaging, histopathological evaluation to document inflammation, and sequential treatment trials. Dogs that showed a complete clinical response to an elimination diet (novel or hydrolyzed protein diet) were classified as FRE; those that required anti-inflammatory/immunosuppressive treatment (prednisolone, cyclosporine) were diagnosed as IRE. Gastrointestinal tissue biopsies from 15 dogs with CIE and 9 healthy dogs were subjected to RAGE immunofluorescence detection and quantification by laser-scanning microscopy. Statistical analyses included non-parametric two- or multiple group comparison tests and correlation coefficients (Spearman ρ) with multiple-comparison corrections if needed. Statistical significance was set at $P < 0.05$.

Results: Serum sRAGE concentrations were not correlated with biomarkers of gastrointestinal function (cobalamin, folate), inflammation (C-reactive protein, calprotectin, S100A12), or protein loss (α_1-proteinase inhibitor, albumin). However, serum soluble RAGE decreases were linked to more severe microscopic lesions in the proximal small intestine.
RAGE positivity was detected in all biopsies from dogs with CIE and healthy controls. Duodenal and colonic epithelial RAGE expression was significantly higher in dogs with CIE than in healthy dogs. RAGE expression in dogs with CIE was also higher in the ileum but lower in the stomach, though both differences did not reach statistical significance. Gastrointestinal epithelial RAGE expression was not correlated with the age or body weight of the dogs, the duration of gastrointestinal clinical signs, or the clinical disease activity (CCECAI) score. Histologic gastrointestinal lesions and biomarkers of gastrointestinal inflammation were related to segmental RAGE expression. In dogs with CIE, soluble RAGE receptor concentrations in serum were correlated with the apical epithelial expression of RAGE in the duodenum.

Conclusions: This study showed a dysregulation of epithelial RAGE expression along the gastrointestinal tract and the RAGE/sRAGE axis in canine CIE. These findings suggest that RAGE signaling plays a role in the pathogenesis of canine CIE. However, gastrointestinal overexpression of RAGE was seen with less severe disease and was also paralleled by higher anti-inflammatory decoy receptor sRAGE levels.

6 Zusammenfassung

Angela Isabel Cabrera García

Evaluation des RAGE-Rezeptors (receptor for advanced glycation end products) bei Hunden mit chronischer Enteropathie.

Klinik für Kleintiermedizin, Veterinärmedizinische Fakultät, Universität Leipzig

Eingereicht im März 2021
(83 Seiten, 22 Abbildungen, 8 Tabellen, 83 Verweise)

Schlüsselwörter: chronisch-entzündliche Enteropathie, entzündliche Darmerkrankung, Hund, Rezeptor für fortgeschrittene Glykierungsendprodukte, molekulare Gefahrensignale, Mustererkennungsrezeptor.

Einleitung: Zu den chronisch-entzündlichen Enteropathien (*chronic inflammatory enteropathies*, CIE) beim Hund zählen vor allem zwei Erkrankungsformen, die Futtermittel-responsive Enteropathie (FRE) und die Immunsuppressiva-responsive (bzw. -refraktäre) Enteropathie (IRE). Mehrere Studien deuten darauf, dass der angeborenen Immunität eine zentrale Rolle bei der Pathogenese der CIE des Hundes zukommt. Dysregulationen im angeborenen Immunsystem bei Hunden mit CIE schließen die Signalwege bestimmter Rezeptoren ein, wie die Mustererkennungsrezeptoren (*pattern recognition receptors*, PRRs). Der Rezeptor für fortgeschrittene Glykierungsendprodukte (*receptor for advanced glycation end products*, RAGE) zählt zu den PRRs. Die lösliche, nicht membranständige RAGE-Form (*soluble RAGE*, sRAGE) ist eine antiinflammatorisch wirksame Rezeptorattrappe (*decoy receptor*), die um Ligandenbindung des Transmembranrezeptors (*full-length RAGE*) konkurriert und proinflammatorische zelluläre Postrezeptorsignale aufhebt oder abschwächt. Serum sRAGE-Konzentrationen zeigten sich bei Hunden mit CIE deutlich vermindert, was zu der Hypothese führt, dass Fehlregulationen in der RAGE/sRAGE-Achse bei Hunden mit CIE eine pathogenetische Rolle spielen.

Ziele der Untersuchungen: Ziel dieser Studie ist die Bestimmung der Serum-sRAGE-Konzentrationen bei Hunden mit CIE und deren Zusammenhang mit histologischen sowie klinisch-pathologischen Befunden. Ein weiteres Ziel der Studie ist der quantitative Vergleich der Expression von Transmembran (*full-length*)-RAGE entlang des Gastrointestinaltrakts bei Hunden mit CIE sowie die Untersuchung auf deren Zusammenhang mit Serum-sRAGE-Konzentrationen sowie klinischen, klinisch-pathologischen und histologischen Befunden.

Material und Methoden: Im ersten Teil der Studie wurden 102 mit CIE diagnostizierte Hunde eingeschlossen. Alle Hunde durchliefen eine standardisierte Diagnostik (Hämatologie, Serumchemie, Urinanalyse, Kotuntersuchung, Gastrointestinalprofil und Biomarker für gastrointestinale Entzündung bzw. Proteinverlust) sowie weitere Diagnostik zum Ausschluss anderer gastrointestinaler sowie extra-gastrointestinaler Erkrankungen. Weiter erfolgte bei einigen Hunden eine bildgebende Diagnostik des Abdomens und die pathohistologische Beurteilung von gastrointestinalen Gewebeproben. Anhand des Therapieerfolgs wurden Hunde, die nach Eliminationsdiät (neuartige Proteinquelle oder Proteinhydrolysat) eine vollständige klinische Remission erfuhren, als FRE klassifiziert. Hunde, die einer entzündungshemmenden bzw. immunsuppressiven Therapie mit Prednisolon und/oder Cyclosporin bedurften, wurden als IRE diagnostiziert. Gewebebiopsien des Gastrointestinaltrakts von 12 Hunden mit CIE und 9 gesunden Kontrollhunden wurden der Immunfluoreszenzfärbung zum Nachweis von RAGE sowie dessen Quantifizierung durch konfokale Laserscanmikroskopie unterzogen. Die statistischen Auswertungen umfassten nichtparametrische Zwei- oder Mehrfachgruppen-Vergleichstests und Korrelationskoeffizienten (Spearman ρ). Die statistische Signifikanz wurde mit $P < 0,05$ festgelegt.

Ergebnisse: Die sRAGE-Konzentrationen der 102 Hunde mit CIE korrelierten nicht mit Biomarkern für eine gestörte gastrointestinale Funktion, Entzündung oder Proteinverlust. Erniedrigte sRAGE-Konzentrationen waren mit hochgradigen histologischen Veränderungen im proximalen Dünndarm verbunden. In allen Gewebebiopsien des Gastrointestinaltraktes von Hunden mit CIE sowie gesunden Kontrolltieren war ein positiver Immunfluoreszenznachweis von RAGE festzustellen. Die Gewebe-Expression von RAGE im Duodenum und Kolon war bei CIE Hunden signifikant höher als bei gesunden Tieren. Im Ileum war RAGE bei kranken Hunden ebenfalls stärker, im Magen dagegen geringer, exprimiert als bei gesunden Hunden – jedoch erreichten diese Unterschiede keine statistische Signifikanz. Die gastrointestinale RAGE-Expression korrelierte nicht mit dem Alter oder Körpergewicht der Hunde, der Dauer gastrointestinaler Symptome oder dem klinischen CCECAI-Score. Einzelne histologische Läsionen im Magen-Darm-Trakt sowie Veränderungen in gastrointestinalen Entzündungsmarkers korrelierten mit der segmentalen RAGE-Expression. Lösliche RAGE-Rezeptorkonzentrationen im Serum korrelierten bei Hunden mit CIE mit der apikalen RAGE-Expression im Duodenum.

Schlussfolgerungen: Die Ergebnisse deuten auf eine Dysregulation der RAGE/sRAGE-Achse bei der CIE des Hundes und legen nahe, dass die RAGE-Signalwege eine Rolle bei der Pathogenese dieser Erkrankung spielen.

7 References

Akira S, Uematsu S, Takeuchi O. Pathogen Recognition and Innate Immunity. Cell. 2006;124:(4)783–801.

Allenspach K, Wieland B, Gröne A, Gaschen F. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med. 2007;21:(4)700–708.

Allenspach K, House A, Smith K, McNeill FM, Hendricks A, Elson-Riggins J, Riddle A, Steiner JM, Werling D, Garden OA, Catchpole B, Suchodolski JS. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German Shepherd Dogs with chronic enteropathies. Vet Microbiol. 2010;146:(3-4)326–335.

Allenspach K, Gaschen FP. Erkrankungen des Dünndarms. In: Steiner JM, editor. Gastroenterologie bei Hund und Katze: Klinik-Diagnostik-Therapie: Hannover, Germany: Schlütersche 2011. p.193-2017.

Allenspach K, Culverwell C, Chan D. Long-term outcome in dogs with chronic enteropathies: 203 cases. Vet Rec. 2016;178:(15)368.

Arpaia N, Campbell C, Fan X, Dikiy S, Van der Veeken J, DeRoos P, Liu H, Cross JR, Pfeffer K, Coffer PK, Rudinsky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T cell generation. Nature. 2013;504:(7480)451–455.

Bartling B, Hofmann HS, Weigle B, Silber RE, Simm A. Down-regulation of the receptor for advanced glycation end- products (RAGE) supports non-small cell lung carcinoma. Carcinogenesis. 2005;26:(2)293–301.

Batt RM, Morgan JO. Role of serum folate and vitamin B_{12} concentrations in the differentiation of small intestinal abnormalities in the dog. Res Vet Sci. 1982;32:(1)17–22.

Baum B, Meneses F, Kleinschmidt S, Nolte I, Hewicker-Trautwein M. Age-related histomorphologic changes in the canine gastrointestinal tract: A histologic and immunohistologic study. World J Gastroenterol. 2007;13:(1)152–157.

Berkow R. Manual Merck: Home edition, 2nd. Edition, 2008, Océano. p. 842.

Bierhaus A, Humpert PM, Morcos M, Wendt T, Chavakis T, Arnold B, Stern DM, Nawroth P. Understanding RAGE, the receptor for advanced glycation end products. J Mol Med. 2005;83:(11)876–886.

Body-Malapel M, Djouina M, Wxin C, Langlois A, Gower- Rousseau C, Zerbib P, Schmidt AM, Desreumaux P, Boulanger E, Vignal C. The RAGE signaling pathway is involved in intestinal inflammation and represents a promising therapeutic target for inflammatory bowel diseases. Mucosal Immunol. 2019;12(2):468–478.

Buser FC, Schweighauser A, Im Hof-Gut M, Bigler B, Marti E, Mirkovitch J, Francey T. Evaluation of C-reactive protein and its kinetics as a prognostic indicator in canine leptospirosis. J Small Anim Pract. 2019;60:(8)477–485.

Bramhall M, Rich K, Chakraborty A, Logunova L, Han N, Wilson J, McLaughlin J, Brass A, Cruickshank SM. Differential expression of soluble receptor for advanced glycation end-products in mice susceptible or resistant to chronic colitis. Inflamm Bowel Dis. 2020;26:(3)360–368.

Cabrera-García AI, Suchodolski JS, Steiner JM, Heilmann RM. Association between serum soluble receptor for advanced glycation end products (RAGE) deficiency and severity of clinicopathologic evidence of canine chronic inflammatory enteropathy. J Vet Diagn Invest. 2020;32:(5)664–674.

Campbell NA, Reece JB. Biologie. In: Ernährung bei Tieren. 6th ed. Spektrum-Verlag, Berlin, 2003. p.1020–1045.

Cassmann E, White R, Atherly T, Wang C, Sun Y, Khoda S, Mosher C, Ackermann M, Jergens A. Alterations of the ileal and colonic mucosal microbiota in canine chronic enteropathies. PLoS One. 2016;11:(2)e0147321.

Cerquetella M, Rossi G, Suchodolski JS, Salavati Schmitz S, Allenspach K, Rodríguez-Franco F, Furlanello T, Gavazza A, Marchegiani A, Unterer S, Burgener IA, Pengo G, Jergens AE. Proposal for rational antibacterial use in the diagnosis and treatment of dogs with chronic diarrhoea. J Small Anim Pract. 2020;61:(4)211–215.

Craven M, Simpson JW, Ridyard AE, Chandler ML. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). J Small Anim Pract. 2004;45:(7)336–342.

Crave M, Washabau RJ. Comparative pathophysiology and management of protein-losing enteropathy. J Vet Intern Med. 2019;33:(2)383–402.

Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? J Small Anim Pract. 2016;57:(11)589–599.

Day MJ, Bilzer T, Mansell J, Wilcock B, Hall EJ, Jergens A, Minami T, Willard M, Washabau R. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. J Comp Pathol. 2008;138 Suppl 1:S1–S43.

Dossin O, Lavoué R. Protein-Losing Enteropathies in Dogs. Vet Clin North Am (Small Anim Pract). 2011;41:(2)399–418.

Engelhardt WV, Breves G. Physiologie der Haustiere. 1st Ed. Thieme-Verlag, Stuttgart 2000.

Erdmann C, Heilmann RM. Diagnostic and therapeutic approach to chronic inflammatory enteropathies in dogs. Tierarztl Prax Ausg Kleintiere Heimtiere. 2017;45:(5)317–327.

Foell D, Wittkowski H, Ren Z, Turton J, Pang G, Daebritz J, Heidemann T, Borody J, Roth J, Clancy R. Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. J Pathol. 2008;216:(2)183–192.

Foell D, Wittkowski H, Roth J. Monitoring disease activity by stool analyses: from occult blood to molecular markers of intestinal inflammation and damage. Gut. 2009;58:(6)859–868.

Furness JB, Kunze WAA, Clerc N. Nutrient tasting and signaling mechanisms in the gut II. The intestine as a sensory organ: neural, endocrine, and immune responses. Am J Physiol. 1999;277:(5)G922–928.

Goodwin LV, Goggs R, Chan DL, Allenspach K. Hypercoagulability in dogs with protein-losing Enteropathy. J Vet Intern Med. 2011; 25:(2)273–277.

Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. FEMS Microbiol Ecol. 2011;76:(2)301–310.

Hall EJ, German AJ. Diesases of the small intestina. In: Ettinger S.J, Feldmann E.C, editors. Textbook of Veterinary Internal Medicine. 7th Edition. St. Louis, Missouri, USA: Saunders Elsevier; 2010. p. 1526–1572.

Heilmann RM, Paddock CG, Ruhnke I, Berghoff N, Suchodolski JS, Steiner JM. Development and analytical validation of a radioimmunoassay for the measurement of alpha1-proteinase inhibitor concentrations in feces from healthy puppies and adult dogs. J Vet Diagn Invest. 2011;23:(3)476–485.

Heilmann RM, Grellet A, Allenspach K, Lecoindre P, Day MJ, Priestnall SL, Toresson L, Procoli F, Grützner N, Suchodolski JS, Steiner JM. Association between fecal S100A12 concentration and histologic, endoscopic, and clinical disease severity in dogs with idiopathic inflammatory bowel disease. Vet Immunol Immunopathol. 2014;158(3-4)156–166.

Heilmann RM, Suchodolski JS. Is inflammatory bowel disease in dogs and cats associated with a Th1 or Th2 polarization? Vet Immunol Immunopathol. 2015;168:(3-4)131–134.

Heilmann RM, Allenspach K. Pattern-recognition receptors: signaling pathways and dysregulation in canine chronic enteropathies – brief review. J Vet Diagn Invest. 2017;29:(6)781–787.

Helander HF, Fändriks L. Surface area of the digestive tract. Scand J Gastroenterol. 2014;49:(6)681–689.

Honneffer JB, Minamoto Y, Suchodolski JS. Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. World J Gastroenterol. 2014;20:(44)16489–16497.

Jensen P, Bjørnvad CR. Clinical effect of probiotics in the prevention or treatment of gastrointestinal disease in dogs: A systemic review. J Vet Intern Med. 2019;33:(5)1849–1864.

Jergens AE, Schreiner CA, Frank DE, Niyo Y, Ahrens FE, Eckersall PD, Benson TJ, Evans R. A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med. 2003;17:(3)291–297.

Jergens AE, Simpson KW. Inflammatory bowel disease in veterinary medicine. Front Biosci. 2012; E4:1404–1419.

Kather S, Grützner N, Kook PH, Dengler F, Heilmann RM. Review of cobalamin status and disorders of cobalamin metabolism in dogs. J Vet Intern Med. 2020;34:(1)13–28.

Kathrani A, Holder A, Catchpole B, Alvarez L, Simpson K, Werling D, Allenspach K. TLR5 risk-associated haplotype for canine inflammatory bowel disease confers hyper-responsiveness to flagellin. PLoS One. 2012;(1) 7:E30117.

Kathrani A, Lee H, White C, Catchpole B, Murphy A, German A, Werling D, Allenspach K. Association between nucleotide oligomerization domain two (Nod2) gene polymorphism and canine inflammatory bowel disease. Vet Immunol Immunopathol. 2014;161:(1-2)32–41.

Kato A, Kathryn E, Bruce K, Tan MD, Schleimer RP. B-lymphocyte lineage cells and the respiratory system. J Allerg Clin Immunol. 2013;131:(4)933–957.

Langlois DK, Koenigshof AM, Mani R. Metronidazole treatment of acute diarrhea in dogs: A randomized double blinded placebo-controlled clinical trial. J Vet Intern Med. 2020; 34:(1)98–104.

Lathan P, Thompson AL. Management of hypoadrenocorticism (Addison's disease) in dogs. Vet Med Res Rep. 2018;9:1–10.

Lecoindre A, Lecoindre P, Cadoré JL, Chevallier M, Guerret S, Derré G, Mcdonough SP, Simpson KW. Focal intestinal lipogranulomatous lymphangitis in 10 dogs. J Small Anim Pract. 2016;57:(9)465–471.

Lozano Soto F. Introducción al sistema inmunológico, sus principales elementos y la respuesta inmunitaria. Farreras; Rozman, eds. Medicina Interna (17 edición). Barcelona, España: Elsevier España. 2012 p. 2453–2488.

Mandigers PJJ, Biourge V, Van den Ingh TSGAM, Ankringa N, German AJ. A randomized, open-label, positively-controlled field trial of a hydrolyzed protein diet in dogs with chronic small bowel enteropathy. J Vet Intern Med. 2010;24:(6)1350–1357.

Meijer B, Hoskin T, Ashcroft A, Burgess L, Keenan JI, Falvey J, Gearry RB, Day AS. Total soluble and endogenous secretory receptor for advanced glycation end products (RAGE) in IBD. J Crohns Colitis. 2014;8:(6)513–520.

Minamoto Y, Otoni CC, Steelman SM, Buyukleblebici O, Steiner JM, Jergens AE, Suchodolski JS. Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut Microbes. 2015;6:(1)33–47.

Nagata N, Ohta H, Yokoyama N, Teoh TB, Nisa K, Sasaki N, Osuga T, Morishita K, Takiguchi M. Clinical characteristics of dogs with food-responsive protein-losing enteropathy. J Vet Intern Med. 2020;34:(2)659–668.

Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology. 2009;136:(1)65–80.

Otoni CC, Heilmann RM, García-Sancho M, Sainz A, Ackermann MR, Suchodolski JS, Steiner AJ, Jergens AE. Serologic and fecal markers to predict response to induction therapy in dogs with idiopathic inflammatory bowel disease. J Vet Intern Med. 2018;32:(3)999–1008.

Pereira GQ, Gomes LA, Santos IS, Alfieri AF, Weese JS, Costa MC. Fecal microbiota transplantation in puppies with canine parvovirus infection. J Vet Intern Med. 2018; 32:(2)707–711.

Rudinsky AJ, Rowe JC, Parker VJ. Nutritional management of chronic enteropathies in dogs and cats. J Am Vet Med Assoc. 2018;253:(5)570–578.

Schmitz S, Werling D, Allenspach K. Effects of ex-vivo and in-vivo treatment with probiotics on the inflammasome in dogs with chronic enteropathy. PLoS One. 2015; 10(3):e0120779.

Schummer A. Verdauungsapparat. In: Nickel, R., Schummer, A., Seiferle, E. Lehrbuch der Anatomie der Haustiere (II), 6th Ed. Parey-Verlag, Berlin-Hamburg, 1987. p. 363–393.

Suchodolski JS, Camacho J, Steiner JM. Analysis of bacterial diversity in the canine duodenum, jejunum, ileum and colon by comparative 16S rRNA gene analysis. FEMS Microbial Ecol. 2008;66:(3)567–578.

Suchodolski JS, Dowd SE, Westermack E, Steiner JM, Wolcott RD, Spillmann T, Harmonien JA. The effect of the macrolide antibiotic tylosin on microbial diversity in the canine small intestine as demonstrated by massive parallel 16S rRNA gene sequencing. BMC Microbiol. 2009;9:(1)210.

Suchodolski JS, Dowd SE, Wilke V, Steiner JM, Jergens AE. 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS One. 2012a;7(6):e39333.

Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PLoS One. 2012b;7(12):e51907.

Suchodolski JS. Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. Vet J. 2016;215:30–37.

Spahn TW, Kucharzik T. Modulating the intestinal immune system: the role of lymphotoxin and GALT organs. Gut. 2004;53:(3)456–465.

Sterenczak KA, Willenbrock S, Barann M, Klemke M, Soller JT, Eberle N, Nolte I, Bullerdiek J, Murua Escobar H. Cloning, characterization, and comparative quantitative expression analyses of receptor for advanced glycation end products (RAGE) transcript forms.Gene. 2009;434:(1-2)35-42.

Sterenczak KA, Joetzke AE, Willenbrock S, Eberle N, Lange S, Junghanss C, Nolte I, Bullerdiek J, Simon D, Murua Escobar H. High-mobility group B1 (HMGB1) and receptor for

advanced glycation end-products (RAGE) expression in canine lymphoma. Anticancer Res. 2010;30:(12)5043–5048.

Sterenczak KA, Kleinschmidt S, Wefstaedt P, Eberle N, Hewicker-Trautwein M, Bullerdiek J, Nolte I, Murua Escobar H. Quantitative PCR and immunohistochemical analyses of HMGB1 and RAGE expression in canine disseminated histiocytic sarcoma (malignant histiocytosis). Anticancer Res. 2011;31:(5)1541–1548.

Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, Barry KA, Nelson KE, Torralba M, Henrissat B, Coutinho PM, Cann IK, White BA, Fahey GC. Phylogenetic and gene-centric metagenomics of the canine microbiome reveals similarities with human and mice. ISME J. 2011;5:(4)639–649.

Szczepanski MJ, Luczak M, Olszewska E, Molinska-Glura M, Zagor M, Krzeski A, Skarzynski H, Misiak J, Dzaman K, Bilusiak M, Kopec T, Leszczynska M, Witmanowski H, Whiteside TL. Molecular signaling of the HMGB1/RAGE axis contributes to cholesteatoma pathogenesis. J Mol Med. 2015;93:(3)305–314.

Titmarsh HF, Gow AG, Kilpatrick S, Sinclair J, Milne E, Philbey AA, Berry J, Handel J, Mellanby RJ. Association of vitamin D status and clinical outcome in dogs with a chronic enteropathy. J Vet Intern Med. 2015;29:(6)1473–1478.

Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, Fehling HJ, Hardt WD, Shakhar G, Jung S. Intestinal lamina propria dendritic cell subsets have different origins and functions. Immunity. 2009;31:(3)502–512.

Volkmann M, Steiner JM, Fosgate GT, Zentek J, Hartmann S, Kohn B. Chronic Diarrhea in Dogs - Retrospective Study in 136 Cases. J Vet Intern Med. 2017;31:(4)1043–1055.

Walsh D, McCarthy J, O'Driscoll C, Melgar S. Pattern recognition receptors – molecular orchestrators of inflammation in inflammatory bowel disease. Cytokine Growth Factor Rev. 2013;24:(2)91–104.

Washabau RJ, Day MJ, Willard MD, Hall EJ, Jergens AE, Mansell J, Minami T, Bilzer T. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med. 2010;24:(1)10–26.

Watson VE, Hobday MM, Durham AC. Focal intestinal lipogranulomatous lymphangitis in 6 dogs (2008–2011). J Vet Intern Med. 2014;28:(1)48–51.

Willard MD, Jergens AE, Duncan RB, Leib MS, McCracken MD, DeNovo RC, Helman RG, Slater MR, Harbison JL. Interobserver variation among histopathologic evaluations of intestinal tissues from dogs and cats. J Am Vet Med Assoc. 2002;220:(8)1177–1182.

Willard MD, Mansell J, Fosgate GT, Gualtieri M, Olivero D, Lecoindre P, Twedt DC, Collett MG, Day MJ, Hall EJ, Jergens AE, Simpson JW, Else RW, Washabau RJ. Effect of sample quality on the sensitivity of endoscopic biopsy for detecting gastric and duodenal lesions in dogs and cats. J Vet Intern Med. 2008;22:(5)1084–1089.

Willard MD, Moore G.E, Denton BD, Day MJ, Mansell J, Bilzer T, Wilcock B, Gualtieri M, Olivero D, Lecoindre P, Twedt DC, Collet MG, Hall EJ, Simpson JW, Else RW, Washabau RJ. Effect of tissue processing on assessment of endoscopic intestinal biopsies in dogs and cats. J Vet Intern Med. 2010;24:(1)84–89.

Wissdorf H. Beitrag zur klinischen Anatomie des Verdauungstraktes der Carnivoren. Kleintierpraxis 1976;21:260–270.

Woolhead L, Whittemore JC, Stewart SA. Multicenter retrospective evaluation of ileocecocolic perforations associated with diagnostic lower gastrointestinal endoscopy in dogs and cats. J Vet Intern Med. 2020;34:(2)1–7.

Xenoulis PG, Palculict B, Allenspach K, Steiner JM, Van House AM, Suchodolski JS. Molecular-phylogenetic characterization of microbial communities imbalances in the small intestine of dogs with inflammatory bowel disease. FEMS Microbiol Ecol. 2008;66:(3)579–589.

Zentek J. Regulation der Verdauung. In: Ernährung des Hundes. 8th Ed. Enke, Stuttgart; 2016. p.35–37.

Zhao CB, Bao JM, Lu YJ, Zhao T, Zhou HH, Zheng DJ, Zhao SC. Co-expression of RAGE and HMGB1 is associated with cancer progression and poor patient outcome of prostate cancer. Am J Cancer Res. 2014;4:(4)369-377.

8 ACKNOWLEDGMENTS

My sincere thank-you goes to my mentor Prof. Dr. Romy M. Heilmann. Romy, thank you for your supervision, motivation, time, and fast and professional collaboration and mentorship. I could not have done this without your support.

My thanks also to Prof. Dr. Gerhard Oechtering, Head of the Department for Small Animals of the University of Leipzig, for the opportunity to work on my doctorate.

This project would not have been possible without the support from all my collaborators and co-authors – thank you very much.

I would also like to thank all staff and students at the Department for Small Animals for the support, collaboration, and pleasant work atmosphere. Special thanks to Carol, Frauki, Tine, Carina, and Janna R., who have always had an ear for me and have been tremendous support towards completing this work – thanks for all the kind words and hugs.

A huge thank-you goes to my partner, Jacob, for his unconditional support, encouraging words, and endless patience.

Last but not least, thank-you to Mocca, Pepe, Egon, and Rieke for their very special support given to me, each in their very own special way.