

A METHOD FOR THE MEASUREMENT OF 3-BROMOTYROSINE  
CONCENTRATIONS IN CANINE SERUM AND ITS CLINICAL APPLICATION

A Dissertation

by

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## ABSTRACT

The eosinophil count in the peripheral blood does not accurately represent eosinophil activation in tissues, as eosinophils primarily reside in tissues and not in peripheral blood. To date, a non-invasive biomarker for eosinophil activation in dogs has not yet been described. Therefore, the aims of this study were to (1) develop and analytically validate an electron ionization gas chromatography/mass spectrometry (EI-GC/MS) method to identify and measure 3-Bromotyrosine (3-BrY) concentrations in canine serum, (2) establish a reference interval for serum 3-BrY concentrations in healthy pet dogs, (3) determine the short-term stability of canine serum 3-BrY concentrations, and (4) evaluate the clinical usefulness of measuring 3-BrY concentrations in canine serum.

Left-over serum samples from dogs were used to analytically validate the assay. Serum samples from 41 healthy dogs were used to establish a reference interval. A stable isotope of 3-BrY, D<sub>3</sub>-bromotyrosine, was produced in house and added to canine serum samples. 3-BrY in serum samples was extracted and detected by solid-phase extraction and EI-GC/MS, respectively. Limit of blank and limit of detection were 0.33 and 0.63  $\mu\text{mol/L}$ , respectively. Coefficients of variation for precision and reproducibility of the 3-BrY concentrations were <13.9% and <11.0%, respectively. Linearity and accuracy recoveries ranged from 84.0% to 134.4% and 79.1% to 126.7%, respectively. The reference interval was determined to be  $\leq 1.12 \mu\text{mol/L}$ . Serum 3-BrY concentrations were stable for  $\leq 8$ , 30, and 180 days at 4°C, -20°C, and -80°C, respectively.

To evaluate the clinical usefulness of 3-BrY concentrations in canine serum, samples were collected from healthy dogs, dogs with eosinophilia, dogs with eosinophilic gastroenteritis (EGE), dogs with lymphoplasmacytic enteritis (LPE), dogs with exocrine pancreatic insufficiency, and dogs with pancreatitis. Concentrations of 3-BrY in the serum samples were significantly higher in dogs with eosinophilia, dogs with EGE, dogs with LPE, dogs with pancreatitis compared to healthy control dogs ( $P < 0.0001$ ).

In conclusion, the newly developed method for the measurement of 3-BrY concentrations in canine serum samples was precise, reproducible, linear, accurate, and stable. Moreover, serum 3-BrY concentrations may serve as a potential diagnostic marker for dogs with chronic enteropathy after the exclusion of pancreatitis.

## DEDICATION

To Pharis T. Sattasathuchana and my family

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## NOMENCLATURE

ARD	antibiotic-responsive diarrhea
3-BrY	3-bromotyrosine
CCECAI	canine chronic enteropathy activity index
CE	chronic enteropathy
CIBDAI	canine IBD activity index
cPLI	canine pancreatic lipase immunoreactivity
cTLI	canine trypsin-like immunoreactivity
CV	coefficients of variation
D <sub>3</sub> -BrY	D <sub>3</sub> -bromotyrosine
EC-NCI GC/MS	electron capture-negative chemical ionization gas chromatography/mass spectrometry
ECP	eosinophil cationic protein
EI-GC/MS	electron ionization gas chromatography/mass spectrometry
EPI	exocrine pancreatic insufficiency
EPO	eosinophil peroxidase
EDN	eosinophil-derived neurotoxin
FRD	food-responsive diarrhea
GC/MS	gas chromatography/mass spectrometry
GI	gastrointestinal
GM-CSF	granulocyte-macrophage colony-stimulating factor

H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HOBR	hypobromous acid
HPLC	reverse-phase high performance liquid chromatography
HUC	histiocytic ulcerative colitis
HYPOCBL	hypocobalaminemia
IBD	inflammatory bowel disease
LOB	limit of blank
LOD	limit of detection
LPE	lymphoplasmacytic enteritis/ lymphocytic-plasmacytic enteritis
MtBSTFA	N-methyl-N-(t-butyltrimethylsilyl)-trifluoroacetamide
m/z	mass-to-charge ratio
·O <sub>2</sub> <sup>-</sup>	superoxide
O/E	observed-to-expected ratio
SPE	solid phase extraction
TFA	trifluoroacetic acid
WSAVA	World Small Animal Veterinary Association

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## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW\*

#### **Chronic Enteropathy (CE)**

Chronic enteropathy (CE) occurs commonly in dogs. The characterization of CE is complicated and based on the dog's response to treatment in each patient (Allenspach et al., 2007; Batt and Hall, 1989; Washabau et al., 2010). Diagnosis is achieved based on the patient's response to three treatments given in a sequential order (Simpson and Jergens, 2011). The first treatment is a dietary trial of 2–3 weeks. If the patient does not respond to dietary trial, an antibiotic trial is performed. If the patient does not respond to antibiotics, a biopsy is collected and evaluated and, if an inflammatory process is confirmed, an anti-inflammatory drug is administered. The diagnosis of CE also currently relies on ruling out other causes of chronic gastrointestinal (GI) diseases, such as intestinal parasitic infestation, intestinal neoplasia, or exocrine pancreatic disease.

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CE can be divided into three major categories: food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), and idiopathic inflammatory bowel disease (IBD). FRD is a type of adverse food reaction (AFR) to a dietary component (Gaschen and Merchant, 2011; Hall and German, 2010). The reaction is either an immune response to a dietary antigen (i.e., food allergy/hypersensitivity) or a non-immunologic reaction (i.e., food intolerance) (Hall and German, 2010). Discriminating between food allergy and food intolerance is difficult because both have the same clinical GI signs and response to dietary trials. However, in dogs with food allergies, skin lesions may develop (Hillier and Griffin, 2001). There is no age or sex predilection for dogs with FRD, but it tends to occur in relatively young dogs. In dogs with FRD, signs are seen more frequently in the large intestine than in the small intestine (Allenspach et al., 2007). The diagnosis and treatment require a dietary elimination trial with novel protein or hydrolyzed protein diets. The improvement of clinical signs may be observed two weeks after starting the dietary trial. Moreover, decreased eosinophilic infiltration in the GI tract has been observed after a dietary trial in dogs with FRD (Walker et al., 2013).

ARD is an antibiotic-responsive condition with an unknown underlying cause. It commonly occurs in large breed dogs, especially German shepherds. The clinical signs of ARD resolve with antibiotic treatment (German, 2013; Hall, 2011). Tylosin and metronidazole are the most commonly used antibiotics for treating ARD (Kilpinen et al., 2011; Westermarck et al., 2005). These antibiotics have immunomodulatory and anti-inflammatory effects in addition to their antibiotic effects in the GI tract (Westermarck et al., 2005). Defects in the gut mucosal barrier, aberrant mucosal immune responses, and

qualitative changes in the enteric microbiota (intestinal dysbiosis) lead to ARD (Hall, 2011). Definitive diagnosis of ARD requires a positive response to antibiotic treatment, a relapse of clinical signs after antibiotic withdrawal, remission of clinical signs after antibiotic reintroduction, and elimination of other etiologic causes (Hall, 2011). However, this process is rather academic, as most owners would not allow discontinuation of antibiotic use shortly after treatment success.

IBD is a chronic idiopathic GI disorder that is diagnosed based on (1) the presence of chronic GI signs (duration of usually >3 weeks); (2) histopathologic evidence of mucosal inflammation; (3) the inability to identify other causes of GI inflammation; (4) an inadequate response to dietary, antibiotic, and anthelmintic treatment; and (5) a clinical response to anti-inflammatory or immunosuppressive agents (Washabau et al., 2010). The cause of IBD is currently unidentified, but the pathogenesis of IBD is believed to involve a dysregulation of an interaction between the intestinal immune system and luminal intestinal bacteria or dietary antigens that may lead to an improper response to intestinal bacteria or dietary antigens (Allenspach, 2011; Allenspach et al., 2010).

The histological findings in dogs with IBD vary because different inflammatory cells are found within the lesions, and the inflammatory cell infiltration can either be of single-cell or mixed-cell type. Lymphocytic-plasmacytic enteritis (LPE) is the most common form of canine IBD (German, 2013). Eosinophilic gastroenteritis (EGE) is less common than LPE, while granulomatous enteritis and histiocytic ulcerative colitis (HUC) are rare (Washabau, 2013). In dogs with IBD, the small intestine is affected more commonly than the large intestine (Allenspach et al., 2007).

The canine chronic enteropathy activity index (CCECAI) was established to evaluate disease severity and treatment response in dogs with CE (Allenspach et al., 2007). It is used in conjunction with the three-step diagnostic-treatment method described earlier. The CCECAI was adapted from the canine IBD activity index (CIBDAI), which is a clinical scoring system for dogs with IBD (Jergens, 2004; Jergens et al., 2003). The CCECAI scoring system provides quantifiable and repeatable measures of canine CE. The CCECAI uses nine parameters (attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss, albumin levels, ascites and peripheral edema, and pruritus) to evaluate the disease severity as mild, moderate, severe, or very severe. The scoring system evaluates the disease severity by assigning a score to each parameter and adding the total number of scores (Allenspach et al., 2007). While dogs with FRD have been mostly reported to be moderate, the CCECAI scores for dogs with IBD have been reported as very severe (Allenspach et al., 2007). This scoring system could potentially be combined with new minimally-invasive markers of GI inflammation to better determine the disease severity of CE in clinical patients.

### **Eosinophilic Gastroenteritis (EGE)**

The recruitment of eosinophils into GI tissue is a complex process induced by systemic diseases or primary GI disorders. Eosinophils tend to be located in the tissue rather than the peripheral blood. In fact, eosinophils persist in the circulation for less than 1 hour in dogs and 6 to 8 hours in humans and then migrate quickly into tissues (Tizard, 2009; Young and Meadows, 2010). The GI tract has been described as the major site of



eosinophilic migration (Lamouse-Smith and Furuta, 2006; Tizard, 2009). Human GI eosinophilia falls into three categories: primary eosinophilic GI disorders (EGIDs); hypereosinophilic syndrome (HES), which can induce GI eosinophilia; and GI eosinophilia due to known causes (Zuo and Rothenberg, 2007). In humans, the term inflammatory bowel disease (IBD) refers to Crohn's disease and ulcerative colitis (Xavier and Podolsky, 2007). Consequently, human EGE is not classified as IBD, even though inflammation of the bowel is present and corticosteroids are routinely utilized for the treatment. Human EGE is marked by the presence of GI symptoms and eosinophilic infiltration from the esophagus to the colon with no evidence of parasitic or extraintestinal disease (Blackshaw and Levison, 1986; Talley et al., 1990). The diagnosis of EGE in humans also requires the identification of eosinophilic infiltration in the GI tract.

Canine EGE can be seen in dogs of all ages and breeds, but it is most commonly found in Boxers, Doberman pinschers, German shepherds, Rottweilers, and Shar-Peis (Dossin, 2008; Hall and German, 2008). EGE and other forms of GI eosinophilia in dogs are still poorly understood. In general, an immune response to parasites or to diet is considered to be the main cause of eosinophilic infiltration of the GI tract in dogs (Kleinschmidt et al., 2007). Eosinophils have long been recognized as inflammatory leukocytes that are commonly involved in a response to parasites. However, recent studies have shown infiltration of the GI tract with eosinophils unrelated to parasitic infestation (Mazzei et al., 2009; McTavish, 2002).

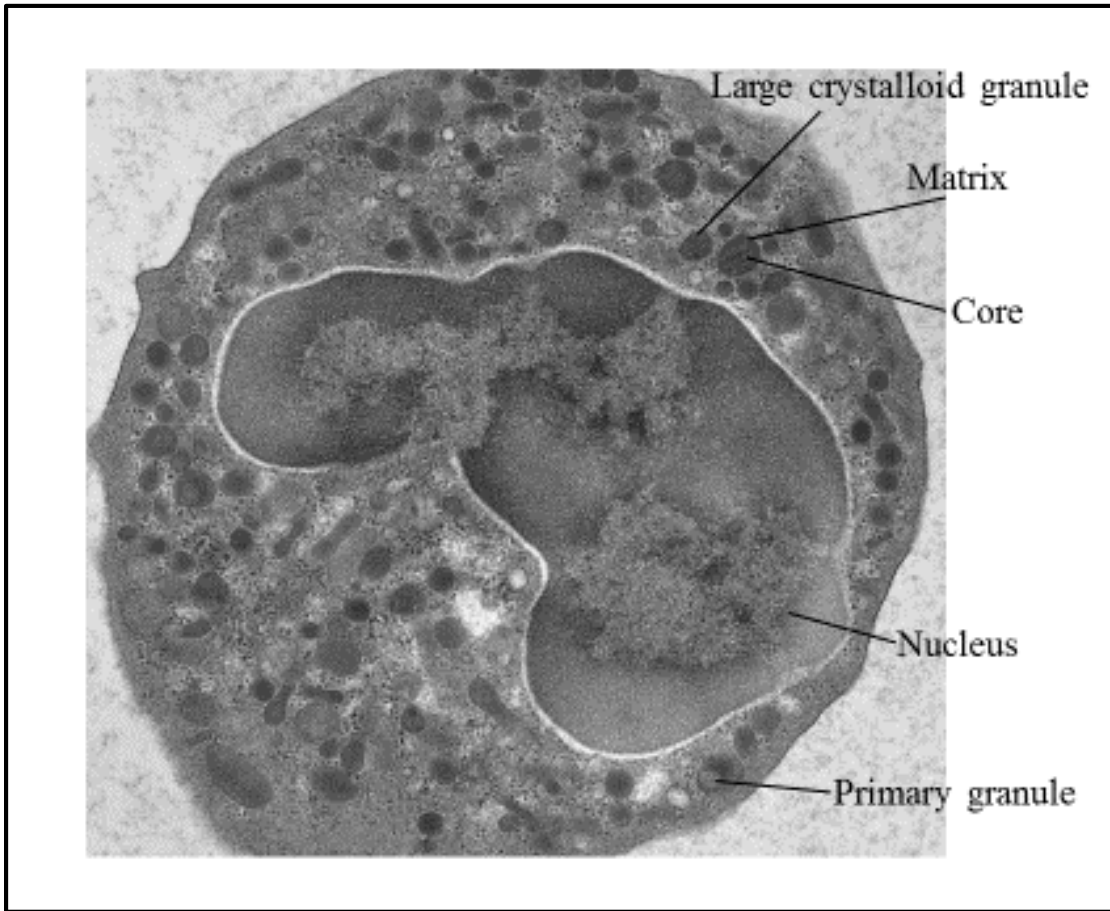
Canine EGE can only be diagnosed by using an invasive diagnostic modality, GI biopsies. The diagnosis requires identification of an eosinophil infiltration in the GI tract

as well as ruling out known causes of such eosinophilic infiltration, such as GI parasitic infestation or food allergies. In contrast to the human form of the disease, canine EGE is considered an inflammatory bowel disease.

### ***Eosinophils: morphology, activation, degranulation, and mediators***

#### ***Morphology of eosinophils***

Eosinophils are polymorphonuclear white blood cells that play a role in innate, acquired, and adaptive immunity, as well as in tissue remodeling. Eosinophils are characterized by the fact that their cytoplasm can be stained intensively with anionic dyes, such as eosin (Khan, 2005; Young and Meadows, 2010). Eosinophils have small primary granules that contain acrylsulfatase, peroxidase, and acid phosphatase as well as large crystalloid granules that contain major basic proteins (MBPs), eosinophil cationic proteins (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (Tizard, 2009) (Table 1, Figure 1). MBPs, which are located in the core of large granules, have activities that affect smooth muscle contraction and peripheral nerve plasticity (Rothenberg and Hogan, 2006). ECP, EPO, and EDN are found in the cellular matrix of the large granules. ECP is a ribonuclease A (RNase A) that has both cytotoxic and non-cytotoxic activities. ECP has anti-viral activity and can also suppress the proliferation of T cells and immunoglobulin synthesis by B cells (Rothenberg and Hogan, 2006). EPO is more potent than myeloperoxidase (MPO), which is secreted by neutrophils to kill infectious organisms. EPO, in contrast to MPO, forms a highly reactive oxygen species (hypobromous acid) in the presence of superoxide (Weiss et al., 1986). EDN, which is an RNase A and a cytotoxic



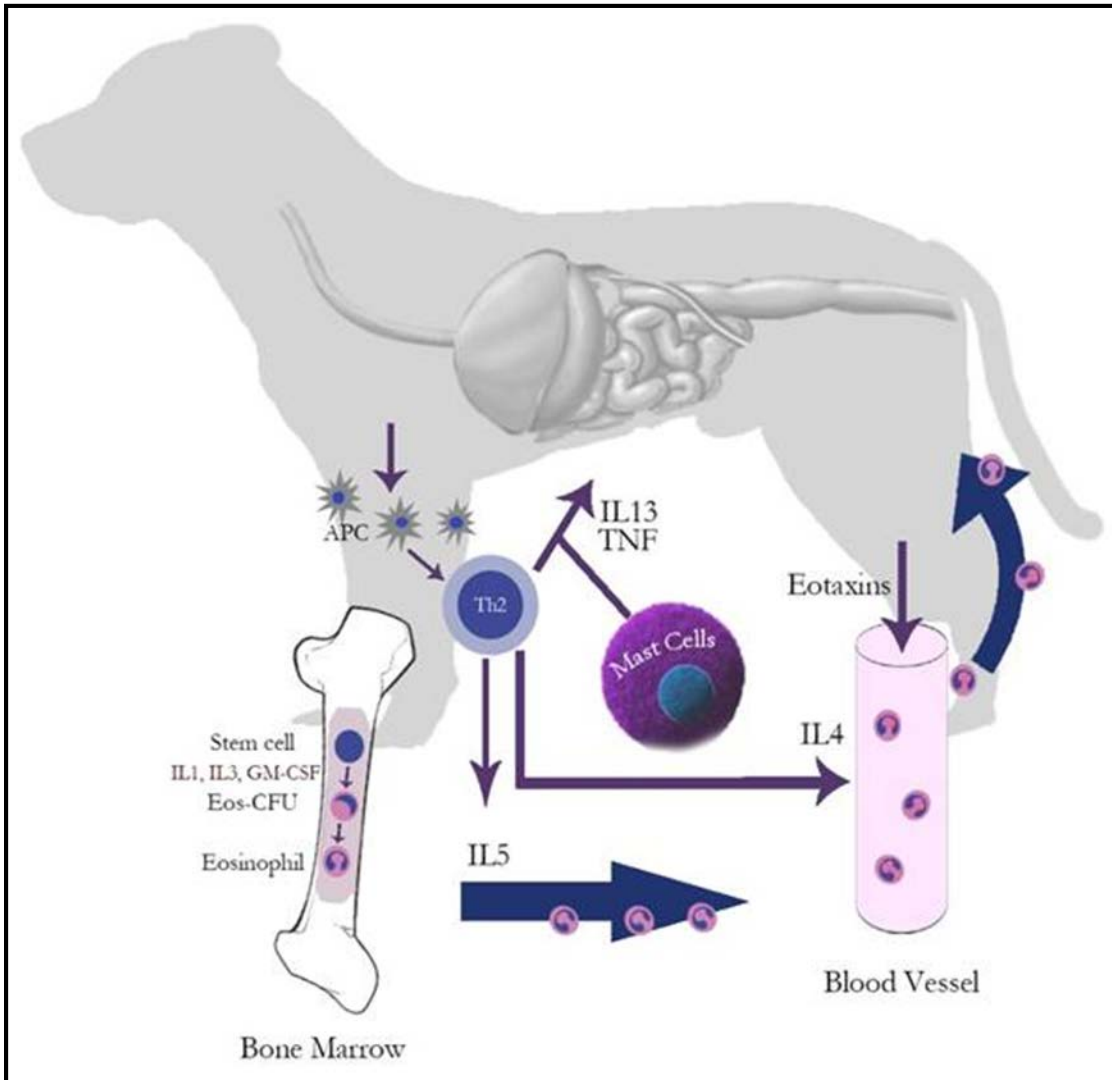
**Figure 1.** The intracellular structure of an eosinophil (*Courtesy of Dr. Craig A Thompson, Purdue University*).

**Table 1. The location and activity of major proteins in large crystalloid granules of eosinophils.**

<b>Major protein</b>	<b>Location</b>	<b>Activity</b>
Major basic protein (MBP)	Large crystalloid granule core	Cytotoxic to microorganisms, epithelial cells, and tumor cells Neutralizes heparin Activates platelets and white blood cells (basophils, mast cells, neutrophils) Induces bronchospasm Increases smooth muscle contraction Regulates peripheral nerve plasticity
Eosinophil peroxidase (EPO)	Large crystalloid granule matrix	In the presence of superoxide, produces brominating oxidizing agent, which is toxic to microorganisms In the absence of superoxide, EPO serves as cationic toxin Toxic to the host epithelium Promotes the histamine release from mast cells Inactivates leukotrienes
Eosinophil cationic protein (ECP)	Large crystalloid granule matrix	Ribonuclease A (RNase A) activity Toxic to microorganisms Degranulates mast cells Neurotoxic Neutralizes heparin Promotes degranulation of mast cells Antiviral activity
Eosinophil-derived neurotoxin (EDN, protein X, EPX)	Large crystalloid granule matrix	Ribonuclease A (RNase A) activity Toxic to myelinated nerve fibers Antiviral activity

agent, is associated with host defense against viruses and can be secreted by other inflammatory cells, such as mononuclear cells and neutrophils (Rothenberg and Hogan, 2006; Young and Meadows, 2010).

Eosinophils are considered late-phase cells involved in the host's immunity (Rothenberg and Hogan, 2006). They originate and develop from pluripotent stem cells in the bone marrow. Eosinophil expansion is regulated by eosinophilopoietins, IL-1, IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rothenberg and Hogan, 2006; Yang et al., 2001). Primitive stem cells become eosinophilic precursor myeloblasts in the presence of IL-1, IL-3, and GM-CSF (Yan and Shaffer, 2009). IL-1, IL-3, and GM-CSF play a major role in eosinophil development, whereas IL-5, which is produced by mast cells, is the major cytokine that regulates the migration of eosinophils from bone marrow to blood circulation (Latimer and Prasse, 2003). Eosinophils cross the endothelium into target tissues by the regulation of chemokine eotaxin-1, eosinophil adhesion molecules, and adhesion receptors on the endothelium (Figure 2). Because eosinophils are predominantly tissue-dwelling cells, only a small number of eosinophils can be found in the circulation. The major target organ of eosinophils in dogs is the GI tract (Khan, 2005; Rothenberg and Hogan, 2006; Tizard, 2009; Young and Meadows, 2010).



**Figure 2. The development, migration, and activation of eosinophils. IL-1, IL-3 and GM-CSF stimulate the generation of eosinophil colony-stimulating units from stem cells in the bone marrow. Antigen-presenting cells present the antigen to Th2 cells. Th2 cells release IL-5, which stimulates eosinophil differentiation and proliferation. IL-4 from Th2 cells promotes the accumulation of eosinophils and IgE production from B cells. Th2 cells and activated mast cells produce IL-13 and TNF to promote local inflammation.**

### *Activation of eosinophils*

During eosinophil activation, various changes in cell morphology, cell surface characteristics, and functional activities occur. Eosinophilic mobilization is orchestrated by T helper 2 (Th2) cells, mast cells, and eotaxins. Once antigen-presenting cells present the antigen to Th2 cells, the activated Th2 cells produce IL-4, IL-5, and tumor necrosis factor (TNF). IL-4 stimulates eosinophilic accumulation and an immunoglobulin E (IgE) response from B cells. IL-5 is important for the termination of differentiation and proliferation of eosinophils. In addition, mast cells release IL-13 and TNF, which play a role in promoting the local inflammation (Yan and Shaffer, 2009) (Figure 2).

Eotaxins are chemokines that act as eosinophilic chemoattractants to the mast cell degranulation site. CCR3 is a receptor on eosinophils for eotaxins. Gurish, et al. (2002) found that a low abundance of CCR3 was associated with a decreased response of eosinophil recruitment in mice during parasitic infestation. In addition, activated eosinophils can express MHC class II and immunosuppressive enzymes. The mobilization of eosinophils to the site of mast cell degranulation and the activation of eosinophils increase their ability to kill and respond to the inflammation of other eosinophils (Young and Meadows, 2010).

### *Eosinophil degranulation and mediators*

Eosinophils can destroy small particles through exocytosis and large particles through extracellular destruction. Exocytosis is regulated by the formation of a docking complex that consists of the soluble N-ethylmaleimide-sensitive factor attachment protein

(SNAP) and its receptor (SNARE) on the vesicle and the target membrane (Blackshaw and Levison, 1986). Eosinophil sombrero vesicle (EoSV), a unique vesicular compartment of eosinophils, migrates to the docking site on the plasma membrane to release protein mediators during eosinophil activation. This piecemeal degranulation plays a role in the elimination of large parasitic infections. Eosinophils degranulate in response to IgE, chemokines, C5a, and platelet-activating factor (PAF) (Rothenberg and Hogan, 2006; Young and Meadows, 2010). Lipid mediators, such as prostaglandins, leukotrienes, and thromboxane A<sub>2</sub>, are produced and released from eosinophils to play a role in the host defense mechanism; however, prostaglandins may also down regulate eosinophil functions.

Eosinophils also release inflammatory and toxic mediators, such as ECP, MBP, and EOP. Eosinophils also release an array of cytokines: IL-1, IL-2, TNF $\alpha$ , TNF $\beta$ , IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18, TGF $\alpha/\beta$ , and CCL5 (Blackshaw and Levison, 1986). The release of granule proteins, lipid mediators, and cytokines during eosinophil degranulation can not only eliminate bacterial and parasitic infections, but can also harm the cell or surrounding tissues. Mast cells and eosinophils coordinate with each other during allergic reactions. In contrast, the uncontrolled or excessive release of inflammatory mediators from mast cells and eosinophils leads to type I hypersensitivity (Tizard, 2009).



## ***Eosinophilic disorders***

### *Eosinophilic esophagitis*

Eosinophilic esophagitis, also called idiopathic eosinophilic esophagitis, is the most common eosinophil-associated GI tract disorder in human patients (Blanchard and Rothenberg, 2008). Eosinophilic esophagitis manifests itself as a chronic immune-mediated disease characterized by esophageal dysfunction with a predominantly eosinophilic inflammation. Young male adults appear predisposed to eosinophilic esophagitis, which is also commonly associated with allergic diseases (Liacouras et al., 2011). Eosinophilic esophagitis is characterized by an infiltration of eosinophils into the esophagus. Because eosinophils are normally not present in the mucosa of the esophagus, the presence of eosinophils in the esophageal mucosa is diagnostic for eosinophilic esophagitis (Rothenberg et al., 2001). In humans, eosinophilic esophagitis can be diagnosed by the exclusion of other causes of esophageal disease, such as parasites or neoplasia, and by the presence of  $\geq 15$  eosinophils per high power field (hpf) in mucosal biopsy specimens (Hogan, 2009; Liacouras et al., 2011).

Canine eosinophilic esophagitis was first reported in 2009. The dog in the report had a history of allergic skin disease. The underlying cause of eosinophilic esophagitis in this instance was believed to be food allergy (Mazzei et al., 2009). The dog had clinical signs of esophageal disease, such as regurgitation, coughing, and dysphagia. The dog was unresponsive to anti-reflux therapy, but responded well to corticosteroid therapy and an elimination diet. In this case, the diagnosis of eosinophilic esophagitis was based on clinical signs, endoscopic and histopathologic findings, failure of prokinetic and gastric protectant

drug therapy, and response to corticosteroid administration and allergen restriction (Mazzei et al., 2009). Other diseases associated with eosinophilic infiltration, such as spirocercosis, must also be ruled out before eosinophilic esophagitis can be diagnosed (Van der Merwe et al., 2008).

### *Eosinophilic gastritis*

Gastritis and duodenitis often occur concurrently, which may be related to the close anatomical and physiological relationship between the stomach and small intestine (Lidbury et al., 2009). Lymphocytic-plasmacytic gastritis is the most common form of canine gastritis (Lidbury et al., 2009; Simpson, 2013). Eosinophilic gastritis has also been reported, but occurs less frequently (Lidbury et al., 2009). The eosinophilic infiltration is mainly limited to the gastric mucosa and rarely extends into the muscularis or serosa of the stomach wall. The infiltration can cause hypertrophy of the rugal folds as well as an ulcerated mucosa (Neiger, 2008). In severe cases, eosinophilia may also be present in the blood. Eosinophilic gastritis is more common in dogs under five years of age (Van der Gaag, 1988b). Eosinophilic gastritis can be associated with urticaria and allergic skin lesions (Neiger, 2008). The cause of eosinophilic gastritis remains unknown; however, several factors, such as genetic predisposition and diet, are likely involved in its pathogenesis.

Unlike the esophagus, eosinophils can normally be found in the stomach and intestines. Therefore, the diagnosis of eosinophilic gastritis is more complicated than that of eosinophilic esophagitis. In human medicine, there is no gold standard for diagnosis of

eosinophilic gastritis, but combinations of clinical and histopathological findings are usually used to diagnose the disease. Gastric biopsies that are characterized by the infiltration of eosinophils into the gastric glands combined with the exclusion of other causes of eosinophilia, such as infection, support a diagnosis of eosinophilic gastritis (Rothenberg, 2004).

In dogs, a diagnosis of eosinophilic gastritis requires the exclusion of other causes of eosinophilic infiltration into the gastric mucosa. These other causes include parasitic infections of the stomach caused by *Physaloptera* spp., *Gnathostoma* spp., and *Spirocerca* spp., all of which can also cause an infiltration of a dog's stomach wall by eosinophils (Neiger, 2008; Simpson, 2010). The major clinical sign of eosinophilic gastritis is chronic, persistent or intermittent vomiting. Delayed gastric emptying, anorexia, and weight loss can also be found in dogs with chronic gastritis (Neiger, 2008). Because the clinical signs of eosinophilic gastritis cannot be distinguished from other forms of gastritis, a gastric biopsy is the only diagnostic tool to identify eosinophilic gastritis. The International GI Standardization Group of the World Small Animal Veterinary Association (WSAVA) has provided guidelines for the normal histology of the stomach (Washabau et al., 2010). The mucosa of the normal gastric body and gastric antrum may have 0–2 (mean: 0.5) and 0–6 (mean: 2.7) eosinophils per 10,000  $\mu\text{m}^2$ , respectively (Figure 3). Eosinophil counts above these levels would suggest eosinophilic gastritis.



**Figure 3. Histopathological appearance of the canine stomach infiltrated with eosinophils (arrow). Gastric antrum: mild eosinophilic gastritis. (Stain: H&E)**

### *Eosinophilic enteritis*

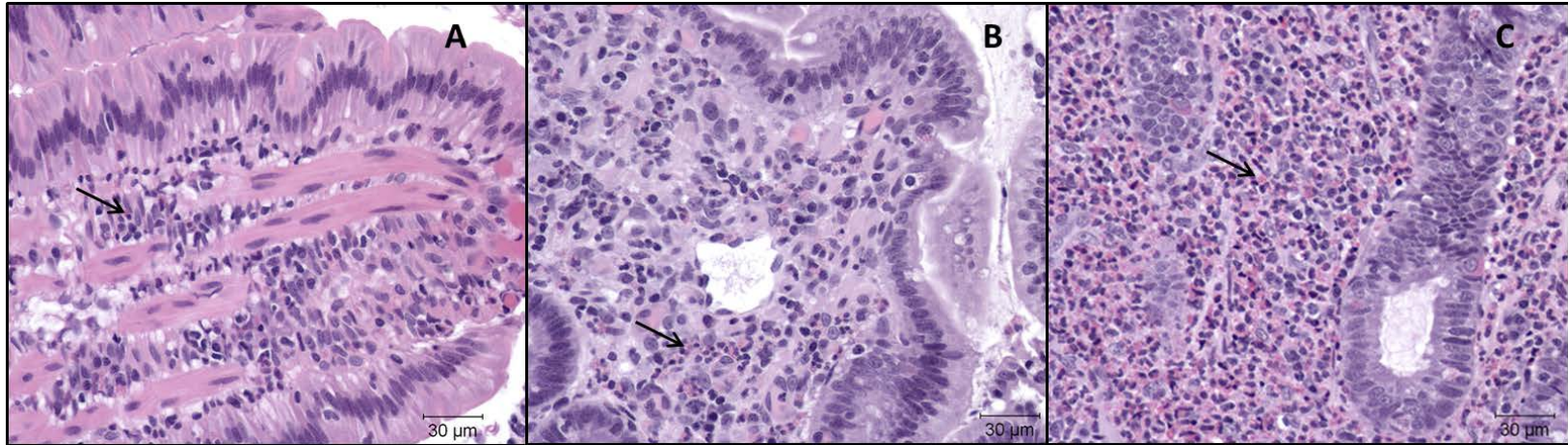
Eosinophilic enteritis includes eosinophilic duodenitis, eosinophilic jejunitis, and eosinophilic ileitis. In human patients, the causes of IBD and eosinophilic enteritis remain unknown. However, it is widely hypothesized that these disorders are due to a loss of tolerance to luminal antigens. Disruption of the mucosal barrier, dysregulation of the immune system, disturbances in the intestinal microbiota, or a combination of these factors is thought to cause the loss of tolerance seen in this condition (Elson et al., 1998). Eosinophilic enteritis is an uncommon idiopathic disease, which is found more frequently in men than in women (1.4:1) (Edelman, 1998). Histopathological findings associated with eosinophilic enteritis include increased numbers of eosinophils, hyperplastic crypts, villous atrophy, and epithelial cell necrosis (Collins, 2009). Peripheral eosinophilia is reported in only 75% of patients with eosinophilic enteritis; thus, peripheral eosinophilia is not a reliable indicator of the disease (Talley et al., 1990).

*Ancylostoma caninum*, a canine hookworm, can cause eosinophilic enteritis and peripheral eosinophilia in dogs and, on rare occasions, in people (Walker et al., 1995). Primary eosinophilic enteritis has been reported to cause small intestinal obstruction in humans without peripheral eosinophilia (Uenishi et al., 2003; Yun et al., 2007). Eosinophils infiltrate the muscularis layer of the small intestinal mucosa. This infiltration leads to thickening of the intestinal wall and obstruction of the intestinal lumen (Yun et al., 2007).

In dogs, eosinophilic enteritis is the second most commonly diagnosed form of IBD, while LPE is the most common (Hall and German, 2008). Parasitic infection and food allergy must be excluded before making a diagnosis of eosinophilic IBD. Eosinophilic

enteritis may be associated with hypereosinophilic syndrome. Diarrhea, weight loss, and abdominal pain are the most common clinical signs of eosinophilic enteritis. Peripheral eosinophilia can also be found in patients with eosinophilic enteritis (Quigley and Henry, 1981). Chronic and often bloody diarrhea is commonly found in dogs with eosinophilic enteritis (O'Brien, 1989), as well as mucosal erosion and ulceration (Van Der Gaag et al., 1983). Hypoalbuminemia may also be associated with chronic eosinophilic enteritis due to protein-losing enteropathy.

Eosinophilia is not pathognomonic of eosinophilic enteritis, so intestinal biopsies are needed for diagnosis (Yan and Shaffer, 2009; Yang et al., 2001). Biopsies of both the duodenum and ileum should be evaluated for a diagnosis of small intestinal inflammation (Dossin et al., 2007). Because intestinal mucosa may contain a small number of eosinophils physiologically, it is important to perform the intestinal histopathological interpretation based on the WSAVA standardization guidelines (Washabau et al., 2010). In an adult dog, the normal eosinophilic count in the cryptal lamina propria, villous lamina propria, and tip of the villi are  $9.8 \pm 7.5$ ,  $3.7 \pm 3.5$ , and  $3.8 \pm 6.1$  per 10,000  $\mu\text{m}^2$ , respectively (Washabau et al., 2010) (Figure 4A, 4B and 4C).



**Figure 4. Histopathological appearance of the canine small intestines infiltrated with eosinophils (arrows). (A) Duodenum: moderate eosinophilic duodenitis. (B) Ileum: moderate eosinophilic ileitis. (C) Ileum: Severe eosinophilic enteritis. (Stain: H&E)**

### *Eosinophilic colitis*

Eosinophilic colitis is the least common of the human eosinophilic GI diseases (Alfadda et al., 2011; Yen and Pardi, 2012). The cause of primary eosinophilic colitis, which affects mainly infants and young children, is unknown (Yen and Pardi, 2012). Secondary eosinophilic colitis may result from an IgE-mediated food allergy, drug-induced eosinophilic colitis, or IBD (Tortora et al., 2012; Yen and Pardi, 2012). Non-IgE associated eosinophilic colitis is found mainly in adults (Yen and Pardi, 2012). The clinical signs of eosinophilic colitis include abdominal pain, bloody or non-bloody diarrhea, and weight loss. The diagnosis of eosinophilic colitis in human patients is based on increased numbers of mucosal eosinophils and other abnormalities, such as architectural changes in crypts, Paneth cell metaplasia in the distal colon, basal lymphoid aggregates, diffuse plasmacytosis, and eosinophils in the muscularis mucosa (Collins, 2009).

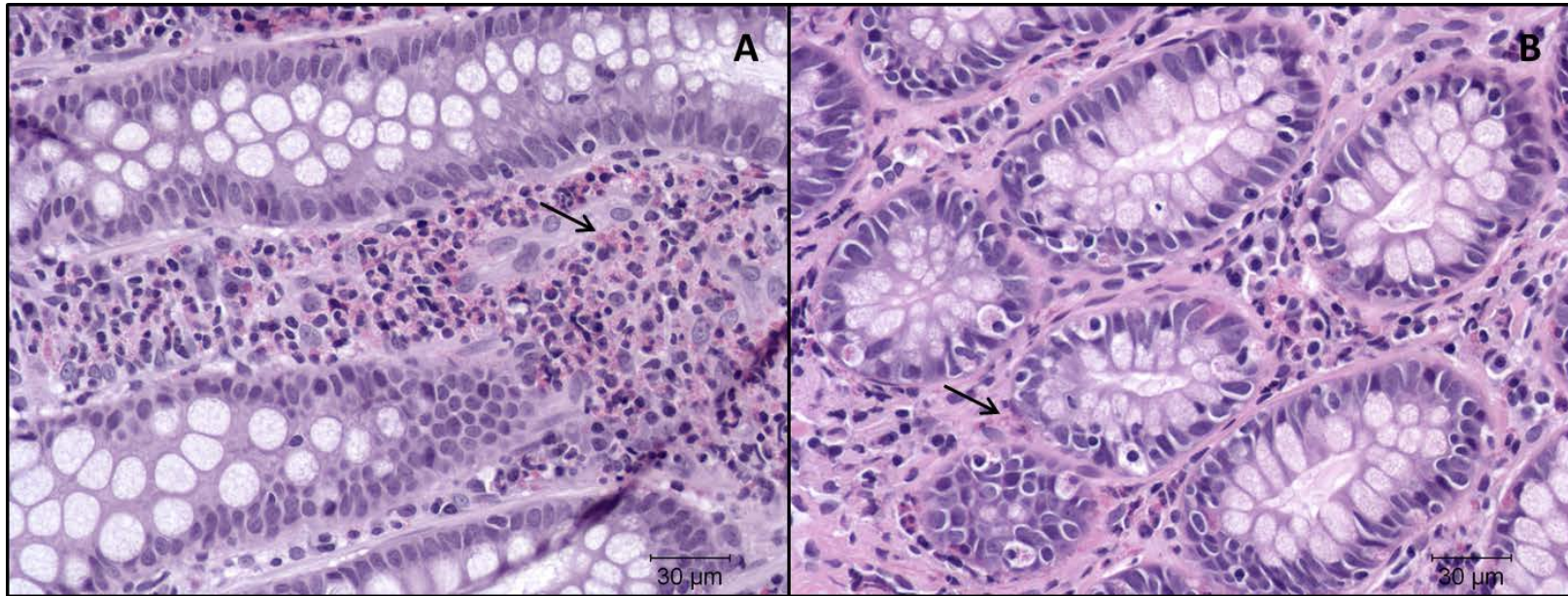
In dogs, eosinophilic colitis (eosinophilic ulcerative colitis) is considered to be a form of IBD that is characterized by eosinophilic infiltration of the colon (Leib, 2008; Van der Gaag et al., 1990). Eosinophilic colitis is rare in dogs, while atrophic colitis, diffuse colitis, and canine histiocytic ulcerative colitis are more common (Van der Gaag, 1988a). The cause of eosinophilic colitis is still unclear, but the average age of dogs diagnosed was 3.9 years (Van der Gaag and Van der Linde-Sipman, 1987). Van der Gaag and Van der Linde-Sipman (1987) reported a case of eosinophilic ulcerative colitis in a three-year-old dog. The dog presented with hemorrhagic diarrhea, anorexia, weight loss, hypoalbuminemia, and anemia. However, eosinophilia was not present. Before diagnosing eosinophilic colitis, one should eliminate other causes of eosinophilia in the colon, such as



endoparasites, autoimmune disease, and hypersensitivity. A colonoscopy and biopsy should be performed to diagnose eosinophilic colitis (Van der Gaag et al., 1990). The WSAVA guidelines suggest that the physiologic count of eosinophils in the lamina propria between the basal crypts of the colon is  $3.8 \pm 3.7$  cells per  $10,000\mu\text{m}^2$  (Washabau et al., 2010) (Figure 5A and 5B).

#### *Eosinophilic gastroenteritis (EGE) and eosinophilic gastroenterocolitis*

Eosinophilia in more than one segment of the GI tract of dogs has been reported in several publications (Brellou et al., 2006; Fonseca-Alves et al., 2012; McTavish, 2002; Rodriguez et al., 1995; Van Der Gaag et al., 1983). Eosinophilia in the stomach and small intestinal segments of the GI tract, which is called eosinophilic gastroenteritis (EGE), has been reported in a German shepherd, a Basset hound, a Siberian husky, and a mixed-breed dog (Brellou et al., 2006; Fonseca-Alves et al., 2012; McTavish, 2002; Rodriguez et al., 1995; Van Der Gaag et al., 1983). Several studies have attempted to identify the cause of EGE and eosinophilic gastroenterocolitis in dogs. Kleinschmidt, et al. (2007) found an increased abundance of mast cells in the area of the eosinophilic gastroenterocolitis and concluded that a type I hypersensitivity reaction was involved in eosinophilic gastroenterocolitis. Mast cell tumors and lymphomas also release cytokines that secrete eosinophil polymorphonuclear leukocyte chemotaxis factors (Marchetti et al., 2005; Ozaki et al., 2006; Tomiyasu et al., 2010). The factors responsible for this stimulation include IL-5, IL-3, GM-CSF, and eotaxin. These tumors result in paraneoplastic eosinophilia and eosinophilic infiltrates in the GI tract.



**Figure 5. Histopathological appearance of the canine colon infiltrated with eosinophils (arrows). (A) Colon: severe eosinophilic colitis. (B) Colon: moderate eosinophilic colitis. (Stain: H&E)**

The clinical signs of eosinophilic gastroenterocolitis are chronic GI signs, such as vomiting, hematemesis, inappetence, weight loss, abdominal pain, melena, and bloody diarrhea (Brellou et al., 2006; Fonseca-Alves et al., 2012; Mazzei et al., 2009; McTavish, 2002; Rodriguez et al., 1995; Van Der Gaag et al., 1983). Hematemesis, hematochezia, melena, weight loss, and regenerative anemia are thought to be due to GI ulceration (McTavish, 2002). However, peripheral eosinophilia is not always present in these cases (Fonseca-Alves et al., 2012). Thickening of the gastric and intestinal walls can also be found in eosinophilic gastroenterocolitis (Fonseca-Alves et al., 2012; Van Der Gaag et al., 1983). The diagnosis of EGE and gastroenterocolitis in dogs is based on clinical signs along with histopathological findings in the GI tract (McTavish, 2002).

The diagnosis of EGE in both humans and canines requires the identification of eosinophilic infiltration in the GI tract. Several studies have attempted to develop non-invasive diagnostic techniques for human EGE, but unfortunately, non-invasive biomarkers have not yet been identified for human or canine EGE. Furthermore, patients with EGE may have a normal or elevated eosinophil count in their peripheral blood. The development of such a non-invasive biomarker for the diagnosis of gastrointestinal eosinophilia would significantly advance the diagnostic capabilities for EGE, as well as other eosinophilic diseases.

### *Treatments*

Management of eosinophilic GI disorders in dogs involves the use of immunosuppressive drugs and allergen restriction along with symptomatic therapy,

including anti-emetics, antacids, anthelmintics, and antibiotics (Mazzei et al., 2009). A combination of immunosuppressive drugs, such as glucocorticoids with symptomatic therapy, is crucial to improve clinical signs. Food elimination trials may also aid in treating these conditions (Talley et al., 1990).

The treatment of eosinophilic esophagitis requires a combination of allergen restriction and corticosteroid administration. Relief of clinical signs comes mainly from the combination of corticosteroids and a food-elimination trial (Sellon and Willard, 2003). Sellon and Willard (2003) reported on a case in which a small oral dose of prednisone and intra-lesion triamcinolone given to a dog with eosinophilic esophagitis led to clinical improvement. The minimization of exposure of the esophageal mucosa to gastric acid also helps alleviate esophagitis (Sellon and Willard, 2003). H<sub>2</sub>-receptor antagonists, such as famotidine, but more importantly proton pump inhibitors (PPIs), such as omeprazole, are very helpful in the reduction of gastric acid secretion (Sellon and Willard, 2003). However, therapy with H<sub>2</sub>-receptor antagonists and/or PPIs alone is not effective in dogs with eosinophilic esophagitis. Unsurprisingly, the exclusive use of prokinetic medications (such as metoclopramide) and anti-emetic medications (such as ondansetron or maropitant) was unable to resolve the clinical signs in one report (Mazzei et al., 2009). The use of immunosuppressive agents, such as anti-IL-5, anti-IL-3, and anti-eotaxin, has been recommended for people with eosinophilic esophagitis, but the beneficial effects of these treatments are still under scrutiny (Liacouras et al., 2011).

The treatment of chronic gastritis in dogs should begin by treatment of the underlying causes. However, because the underlying cause of eosinophilic gastritis is often

unidentified, the treatment of eosinophilic gastritis is complicated. In human patients, identification of food allergies is part of the initial approach. If a specific type of food cannot be identified or restricted, immunosuppressive drugs are the choice of treatment (Rothenberg, 2004). Monteleukast, a leukotriene receptor blocker, has a similar successful treatment outcome as corticosteroids in human patients (Jawairia et al., 2012). Recommended treatments of this disorder in dogs include dietary management, immunosuppressive therapy, and inhibition of gastric acid secretion (Neiger, 2008; Simpson, 2010, 2013). The neutralization of gastric acid by H<sub>2</sub>-receptor antagonists or, more importantly, PPIs, is also effective in alleviating the clinical signs of gastritis and promoting the healing of the gastric mucosa (Rothenberg, 2004; Simpson, 2010). The aim of dietary management is to avoid allergens that can activate the body's immune response. A single novel protein and single-source carbohydrate diet or a hydrolyzed protein diet are ideal for a feeding trial in such patients (Guilford et al., 2001; Neiger, 2008). Most animals show improvement two weeks after the treatment is started (Guilford et al., 2001; Neiger, 2008). Immunosuppressive drugs, such as corticosteroids, azathioprine, or cyclophosphamide, are also recommended for the treatment of canine eosinophilic gastritis (Simpson, 2013).

The treatment of eosinophilic enteritis in dogs centers first on eliminating known causes of eosinophilic infiltration of the intestines (Neiger, 2008). For example, anthelmintic and antiprotozoal drugs should be given to eliminate possible infections. Antigen-restricted or protein hydrolysate-based diets should be given if there is no response to anthelmintic and antiprotozoal trials. The use of immunosuppressive drugs is considered

the last choice of treatment for eosinophilic enteritis (Simpson and Jergens, 2011). Hypoalbuminemia due to excessive intestinal protein loss should be monitored to prevent unexpected complications. Eosinophilic enteritis in dogs commonly recurs (Neiger, 2008). In human medicine, the treatment of eosinophilic enteritis usually relies on corticosteroid therapy, which results in a 90% successful response rate within two weeks (Rothenberg, 2004; Tortora et al., 2012). However, for human patients, surgery may be needed in the case of obstruction or perforation of the small intestine (Uenishi et al., 2003; Yun et al., 2007).

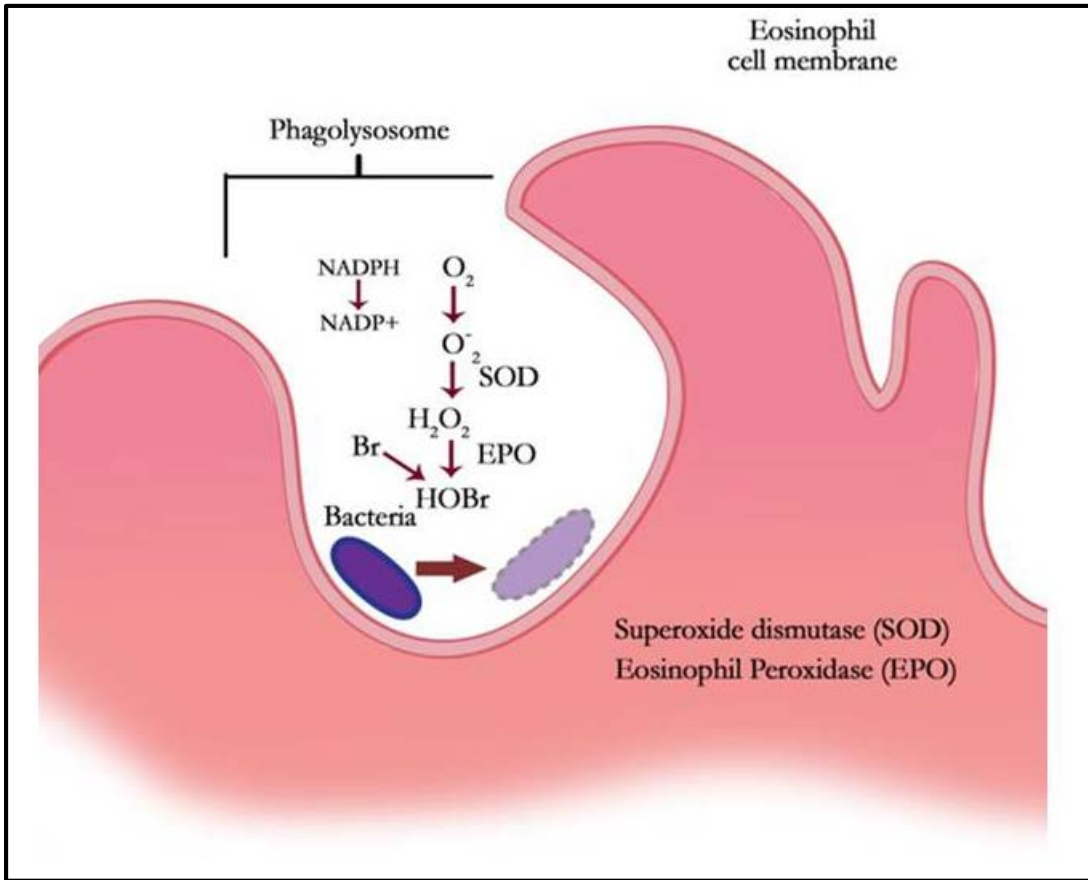
In dogs, the treatment of eosinophilic colitis resembles that of other eosinophilic conditions, with an elimination diet being an important component of the treatment (Hall and German, 2008). Patients tend to respond well to corticosteroid therapy but may relapse when the medication is discontinued or tapered. In humans, eosinophilic colitis is a non-IgE related disease (Rothenberg, 2004). Therefore, the use of IgE-modulating drugs, such as cromoglycate and histamine receptor antagonists, is not effective in treating eosinophilic colitis (Zuo and Rothenberg, 2007). Immunosuppressive therapy using corticosteroids is the main treatment option for human patients with eosinophilic colitis and usually leads to a successful response (Rothenberg, 2004; Tortora et al., 2012; Yen and Pardi, 2012).

Future development of immunomodulator regimens aiming to inhibit chemokine eotaxins might help reduce the number of eosinophils infiltrating the affected tissue. Furthermore, the development of novel therapeutic agents that can inhibit activation or degranulation of eosinophils may transform the treatment of EGE.

### **3-Bromotyrosine: A Marker for Eosinophil Activation**

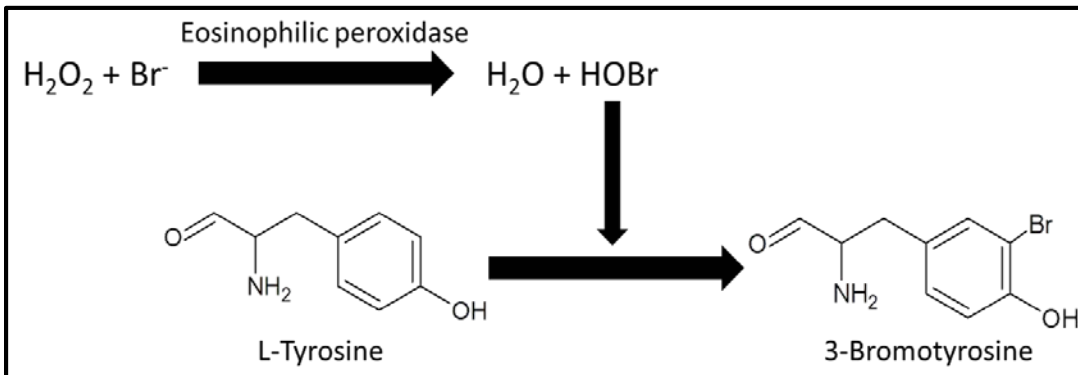
As noted previously, eosinophils play a pivotal role in the response to various inflammatory and infectious diseases, such as asthma, parasitic infestation, allergic dermatitis, food allergies, and idiopathic IBDs (McEwen, 1992; Woolley et al., 1995). Because eosinophils predominantly dwell in tissues instead of circulating in the blood, infiltration of eosinophils into the intestinal mucosa is not always associated with increased counts of eosinophil in the blood (Zuo and Rothenberg, 2007). Activation of eosinophils leads to a release of the lysosomal protein known as heme-enzyme EPO (Weiss et al., 1986; Wu et al., 1999). EPO is more potent than myeloperoxidase (MPO), a heme-enzyme that is secreted by neutrophils to kill infectious organisms.

EPO is the most abundant cytoplasmic protein within eosinophils. Superoxide ( $\cdot\text{O}_2$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), the reactive oxygen intermediates, are released by stimulated eosinophils. Eosinophil peroxidase in the presence of a halide and  $\text{H}_2\text{O}_2$  can produce a halogenated oxidant through the EPO- $\text{H}_2\text{O}_2$ -halide system (Spalteholz et al., 2006; Wu et al., 1999) (Figure 6). Interestingly, EPO selectively generates reactive brominating reagents ( $\text{OBr}^-$ ) at physiological concentrations of halides, where chloride concentrations are far higher than bromide concentrations (100  $\text{mMCl}^-$ , 20-150  $\mu\text{M Br}^-$ , and 0.1-0.6  $\mu\text{M I}^-$ ) (Shen et al., 2001). Hypobromous acid ( $\text{HOBr}$ ) is a highly potent, non-stable oxidizing agent that is generated by eosinophils (Mayeno et al., 1989; Van Dalen and Kettle, 2001).  $\text{HOBr}$  has activities against various microorganisms, such as viruses, bacteria, fungi, and parasites (Mayeno et al., 1989). This acid is a non-stable product that



**Figure 6. Generation of an EPO-H<sub>2</sub>O<sub>2</sub>-halide system during an eosinophil respiratory burst. Superoxide is formed during the respiratory burst of eosinophils and generates hydrogen peroxide in the presence of superoxide dismutase. Hydrogen peroxide then reacts with EPO to produce HOBr through the EPO-H<sub>2</sub>O<sub>2</sub>-halide system. HOBr is toxic to microorganisms, which will be destroyed.**





**Figure 7. The generation of 3-bromotyrosine from EPO-H<sub>2</sub>O<sub>2</sub>-halide. HOBr, a product of EPO and superoxide, reacts with L-tyrosine. The stable byproduct of the HOBr and tyrosine reaction is 3-BrY.**

will react with various biological materials in blood and tissue (Van Dalen and Kettle, 2001; Weiss et al., 1986). The reaction of HOBr and tyrosine then generates 3-bromotyrosine (3-BrY), which is a stable cytotoxic product (Figure 7). The presence of 3-BrY indicates activation of eosinophils (Van Dalen and Kettle, 2001; Weiss et al., 1986). This halogenated amino acid serves as a molecular fingerprint of eosinophil peroxidase-catalyzed oxidation reactions, identifying sites where damage from EPO occurs (Wu et al., 1999).

Concentrations of 3-BrY have been measured in humans by using electron capture-negative chemical ionization gas chromatography/mass spectrometry (EC-NCI GC/MS). In human asthmatic patients, 3-BrY has been detected in biological samples of plasma, urine, and bronchoalveolar lavage fluid. 3-BrY concentrations are higher in asthmatic patients than in healthy humans because of eosinophilic activation in those with asthma (Mita et al., 2004; Wu et al., 2000). However, this method of measuring 3-BrY concentrations has not been previously used in small animals.

The aim of the proposed study is to develop and analytically validate a method for measuring 3-BrY concentrations in canine serum with electron ionization gas chromatography/mass spectrometry (EI-GC/MS). This study also aim to assess the clinical usefulness of measuring 3-BrY concentrations in diagnosing canine EGE. The development and analytical validation of such a non-invasive method would significantly improve the diagnosis of canine CE.

## **Hypothesis and Objectives**

The hypothesis of this study is serum 3-BrY concentrations in dogs with CE can serve as a minimally invasive or non-invasive marker of the activation of eosinophils in the GI tissues.

The objectives to prove or disprove the aforementioned hypotheses are:

1. To develop and analytically validate a method to measure 3-BrY concentrations in canine serum samples and to establish a reference interval.
2. To evaluate 3-BrY concentrations in serum samples of both healthy dogs and dogs with CE.
3. To determine the stability of 3-BrY concentrations in serum samples.
4. To evaluate the clinical usefulness of measuring 3-BrY concentrations of serum from dogs with GI diseases.

CHAPTER II  
DEVELOPMENT AND ANALYTICAL VALIDATION OF AN ELECTRON  
IONIZATION GAS CHROMATOGRAPHY/MASS SPECTROMETRY (EI-GC/MS)  
METHOD FOR THE MEASUREMENT OF 3-BROMOTYROSINE IN CANINE  
SERUM

**Summary**

The activation of eosinophils generates the release of eosinophil peroxidase and subsequent production of a specific and stable byproduct, 3-bromotyrosine (3-BrY). In humans, 3-BrY is used as a biomarker for eosinophil activation. Measurement of 3-BrY in biological samples from dogs, however, has not previously been described. Therefore, the objective of this study was to develop and analytically validate an electron ionization gas chromatography/mass spectrometry (EI-GC/MS) method for the measurement of 3-BrY in canine serum samples. Pooled canine serum samples were used to validate the assay (i.e., limit of blank (LOB), limit of detection (LOD), precision, reproducibility, linearity, and accuracy). Serum samples from 41 healthy pet dogs were used to establish a reference interval for 3-BrY in the dog serum. A stable isotope of 3-BrY made in-house was added to the canine serum samples and then extracted by solid-phase extraction before being separated and detected by EI-GC/MS. The LOB and LOD were 0.33 and 0.63  $\mu\text{mol/L}$ , respectively, and the coefficients of variation for precision and reproducibility for the 3-BrY were <13.9% and <11.0%, respectively. The observed-to-expected ratios for linearity and accuracy ranged from 84.0% to 134.4% (mean  $\pm$ SD: 109.6  $\pm$ 17.2%) and 79.1% to

126.7% (mean  $\pm$ SD: 98.7  $\pm$ 11.3%), respectively. The reference interval was  $\leq$ 1.12  $\mu$ mol/L. The EI-GC/MS assay for the measurement of 3-BrY described here was precise, reproducible, linear, and accurate for the measurement of 3-BrY in canine serum samples. Further studies are underway to investigate the clinical usefulness of this biomarker for diagnosing and monitoring canine patients with chronic enteropathy.

## **Introduction**

Eosinophils play an important role in various inflammatory and infectious diseases, such as asthma, allergic dermatitis, food allergies, parasitic infestations, and idiopathic inflammatory bowel diseases (Kleinschmidt et al., 2007; McEwen, 1992; Woolley et al., 1995). Eosinophilic granules contain various proteins, including eosinophil peroxidase (EPO), which is released during the activation of eosinophils (Tizard, 2009; Young and Meadows, 2010). EPO, a heme protein, is the most abundant cytoplasmic granule protein in eosinophils (Mita et al., 2004; Wu et al., 1999). Another product of the activation of eosinophils is superoxide ( $\cdot\text{O}_2^-$ ), which is reduced to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) by superoxide dismutase. EPO utilizes  $\text{H}_2\text{O}_2$  to catalyze the peroxidation of halides, especially bromide ( $\text{Br}^-$ ) (Mita et al., 2004). Although chloride is the most abundant halide under normal physiological conditions, EPO preferentially reacts with  $\text{Br}^-$  (Mayeno et al., 1989; Mita et al., 2004; Senthilmohan and Kettle, 2006; Van Dalen and Kettle, 2001; Weiss et al., 1986; Wu et al., 1999). The products of this reaction are hypobromous acid (HOBr) and hypobromide ( $\text{OBr}^-$ ), both of which are highly cytotoxic for microorganisms. These products also have the potential to damage the host tissue and cause inflammation at the

site of eosinophil activation (Mita et al., 2004; Senthilmohan and Kettle, 2006; Wu et al., 1999). After its formation, HOBr quickly reacts with primary amines to form bromoamines (Wu et al., 1999; Wu et al., 2000). Tyrosine is a target amino acid in tissue for oxidative agents (Kato et al., 2009). The final stable product of the reaction between HOBr and tyrosine is 3-bromotyrosine (3-BrY) (Gaut et al., 2002; Kato et al., 2009; Mayeno et al., 1989; Mita et al., 2004; Senthilmohan and Kettle, 2006; Wu et al., 1999; Wu et al., 2000). Therefore, 3-BrY is considered a molecular marker for eosinophil activation (Wu et al., 1999).

Biopsies and histopathologic evaluations are typically required to diagnose and evaluate the severity of eosinophil-related diseases, but these methods are invasive. In addition, eosinophil counts in tissues do not always correlate with peripheral eosinophil counts. As a noninvasive alternative, 3-BrY has been used in humans as a biomarker for eosinophil-related diseases such as asthma, both for clinical and research purposes (Gaut et al., 2001; Kato et al., 2009; Mita et al., 2004). It can be detected in various biological samples of humans, such as bronchoalveolar lavage fluid (BALF), plasma, or urine (Gaut et al., 2001; Kato et al., 2009; Mita et al., 2004). An increased concentration of 3-BrY in these samples indicates increased eosinophil activation.

Concentration of 3-BrY in biological samples from humans has been measured using electron capture-negative chemical ionization gas chromatography/mass spectrometry (EC-NCI GC/MS) and electrospray ionization tandem mass spectrometry (Gaut et al., 2002; Mita et al., 2004; Wedes et al., 2009). In dogs, however, measurement of serum 3-BrY concentrations has not previously been documented. Therefore, the aims

of this study were (1) to develop and analytically validate a method to measure the concentration of 3-BrY in canine serum samples by using electron impact ionization gas chromatography/mass spectrometry (EI-GC/MS), (2) to establish a reference interval for serum 3-BrY concentrations in healthy pet dogs, and (3) to determine the relationship between serum 3-BrY concentration and eosinophil counts in healthy dogs.

## **Materials and Methods**

### ***Preparation of internal standard***

D<sub>3</sub>-bromotyrosine (D<sub>3</sub>-BrY) was prepared using a previously published protocol that was slightly modified (Gaut et al., 2002; Hazen et al., 1997; Wu et al., 1999). The reaction mixture consisted of 10 mmol/L of N-bromosuccinimide (Sigma-Aldrich, St. Louis, MO) and 10 mmol/L of d<sub>4</sub>-L-tyrosine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) in water. The reaction was performed under constant stirring at 37°C for 1 h. In order to terminate the reaction, methionine (Sigma-Aldrich, St. Louis, MO) was added to a final concentration of 3 mmol/L. The reaction mix was immediately acidified by addition of trifluoroacetic acid (TFA; Sigma-Aldrich, St. Louis, MO) in a final concentration of 0.1%. The acidified reaction mix was purified by reversed-phase high performance liquid chromatography (HPLC). The reaction mix was separated with a Gemini NX50 C18 column (4.6 x 250 mm; Phenomenex, Torrance, CA) at a flow rate of 1 mL/min. The UV absorbance was set at 276 nm to monitor the elution of D<sub>3</sub>-BrY. Solvents A and B were 0.1% TFA in water, pH 2.5 and 0.1% TFA in methanol, pH 2.5, respectively. The gradient setting was started with an isocratic 5% solvent B for 1 min,

followed by an increase in solvent B from 5% to 35% over 10 min, isocratic solvent B at 35% for 10 min, another increase in solvent B from 35% to 100% over 5 min, and an isocratic elution at 100% of solvent B for 2 min. D<sub>3</sub>-BrY was eluted during isocratic elution with 65% of solvent A and 35% solvent B. Next, D<sub>3</sub>-BrY fractions were collected, combined, and concentrated by drying under a constant stream of nitrogen gas at room temperature. The dried D<sub>3</sub>-BrY was dissolved in HPLC-grade water. The concentration and purity of D<sub>3</sub>-BrY were determined by reversed-phase HPLC analysis. The areas under the curve of 3-BrY standards were used to quantify the D<sub>3</sub>-BrY concentration. The D<sub>3</sub>-BrY was stored at -80°C under helium until use.

### ***Sample collection and preparation***

For assay validation, left-over canine serum samples from submissions to the Gastrointestinal Laboratory at Texas A&M University and Kasetsart Veterinary Teaching Hospital in Thailand were collected over a period of three months (July to August 2013). All samples were stored at -80°C at the Gastrointestinal Laboratory until analysis.

To establish the reference interval of 3-BrY, 41 serum samples were collected from the jugular vein of healthy dogs with their owner's permissions. The sample collection procedure was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC; #2012-101). All dogs were regularly treated with anthelmintics, had received recommended vaccinations, and did not have any clinical signs of disease at the time of sampling. Signalments were recorded for each dog (Table 2).



**Table 2. Signalment for 41 healthy control dogs enrolled.**

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Age (years)	
Median (range)	4.00 (0.96–10.00 )
Sex	
Intact Spayed female	1
Spayed female	17
Intact male	1
Castrated male	22
Breed	
Australian shepherd	6
Basset hound	1
Beagle	1
Boston terrier	1
Chihuahua	4
Dachshund	1
English cocker spaniel	1
German shepherd	1
Labrador retriever	5
Miniature schnauzer	1
Mixed breed	12
Poodle	1
Redbone coonhound	1
Shih tzu	2
Siberian husky	1
St. Bernard	1
Weimaraner	1

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The sample preparation protocol was modified from previous publications (Frost et al., 2000; Gaut et al., 2002; Mita et al., 2004). Briefly, the sample mixture consisted of 8 nanomoles (16  $\mu\text{mol/L}$ ) of D<sub>3</sub>-BrY, 250  $\mu\text{L}$  of water, and 250  $\mu\text{L}$  of 3-BrY standard or serum sample. Five hundred microliters of this mixture were volume adjusted to 2 mL with 0.1% TFA, pH 5.0 (the pH was adjusted by addition of a small amount of NaOH) and centrifuged at 4°C for 10 min at 16,000  $\times$  g. The supernatants were applied to solid-phase extraction columns (Supelclean<sup>TM</sup> ENVI-18; Sigma-Aldrich Company, St. Louis, MO) (Frost et al., 2000; Heinecke et al., 1999) that had been activated with 2.5 mL of 100% methanol (Sigma-Aldrich Company, St. Louis, MO) and washed with 8 mL of 0.1% TFA, pH 5.0. Two mL of water were added to wash out any non-binding substances. Then, 3-BrY and its internal standard were eluted by 1.5 mL of 25% methanol in water. The eluates were completely dried in a rotary vacuum device (Eppendorf, Hauppauge, NY) at 45°C and stored at -80°C until further processing.

### ***Measurement of 3-BrY concentrations***

#### *Derivatization*

The derivatization protocol was modified from previous publications (Frost et al., 2000; Gaut et al., 2002; Mita et al., 2004). Acetonitrile (100  $\mu\text{L}$ ) (Thermo Fisher Scientific, Inc., Pittsburgh, PA) and diisopropylethylamine (40  $\mu\text{L}$ ) (Sigma-Aldrich, St. Louis, MO) were added to the completely dried samples and followed by incubating the mixtures on ice for 5 min. After adding 40  $\mu\text{L}$  of ethyl heptafluorobutyrate (Sigma-Aldrich, St. Louis, MO), the samples were incubated on ice for 30 min. Then, the samples were sonicated in

a water bath for 1 h at room temperature. After sonication, samples were immediately dried under nitrogen at room temperature. Thirty microliters of N-methyl-N-(t-butyltrimethylsilyl)-trifluoroacetamide (MtBSTFA; Thermo Fisher Scientific, Inc., Pittsburgh, PA) were added to the samples. After 30 min of incubation at room temperature, the samples were completely dried under a nitrogen stream. The samples were reconstituted in 50  $\mu$ L of 25% MtBSTFA in undecane (Sigma-Aldrich, St. Louis, MO) and centrifuged at  $14,000 \times g$  for 15 min. The clear supernatants were then analyzed by EI-GC/MS.

*Electron ionization gas chromatography/mass spectrometry (EI-GC/MS) analysis*

The 3-BrY concentration was analyzed using an Agilent 7890A gas chromatograph and 5975C mass selective detector (Agilent Technologies, Santa Clara, CA) equipped with a VF-17 ms capillary column (30 ms, 0.25 mm internal diameter, 0.25  $\mu$ m film thickness; Agilent Technologies, Santa Clara, CA). The samples were injected by an Agilent 7693 auto sampler injector (Agilent Technologies, Santa Clara, CA). Helium was used as a carrier gas. The injector, transfer line, and source temperature were set at 180°C, 300°C, and 250°C, respectively. The oven was initially held at 180°C for 1 min, and then the temperature was increased at a rate of 40°C/min up to 310°C. The temperature was held at 310°C for 5 min at the end of the run. 3-BrY and its isotope ( $D_3$ -BrY) were quantified using the ions at  $m/z$  257 and 259, respectively.

### ***Analytical assay validation***

The analytical validation of the 3-BrY assay involved the determination of a 3-BrY standard working range, the limit of blank (LOB), the limit of detection (LOD), precision, reproducibility, linearity, and accuracy. The 3-BrY standard working range was determined by the recovery rates of nine 3-BrY standards (0.5, 1, 2.5, 5, 10, 20, 30, 40, and 50  $\mu\text{mol/L}$ ). Pure 3-BrY (BOC Science Company, Shirley, NY) was added to serum samples at various known 3-BrY concentrations within the 3-BrY working range prior to the solid-phase extraction and derivatization steps. LOB of the assay was assessed by measuring the six replicates of blank samples that contained only D<sub>3</sub>-BrY and by calculating the mean result and standard deviation ( $\text{mean}_{\text{blank}} + 2(\text{SD}_{\text{blank}})$ ) (Armbruster and Pry, 2008). LOD is the lowest analyte concentration that the assay can detect, which was calculated by  $\text{LOB} + 2(\text{SD}_{\text{low-concentration sample}})$  (Armbruster and Pry, 2008).

Precision was determined by calculating intra-assay coefficients of variation (%CV) of four different serum samples six times within the same assay run. In addition to one individual (unpooled) serum sample, three pooled serum samples were prepared with three different volumes of known 3-BrY concentrations, where the volumes of added known 3-BrY did not exceed 10% in any of them. Reproducibility was evaluated by calculating the inter-assay %CV of four different serum samples each analyzed in six consecutive assays on six different days. The samples were prepared in the same manner as they were for precision.

Linearity was determined by calculating observed-to-expected (O/E) ratios for three different serum samples serially diluted 1/2, 1/4, 1/8 and 1/16. In addition to one

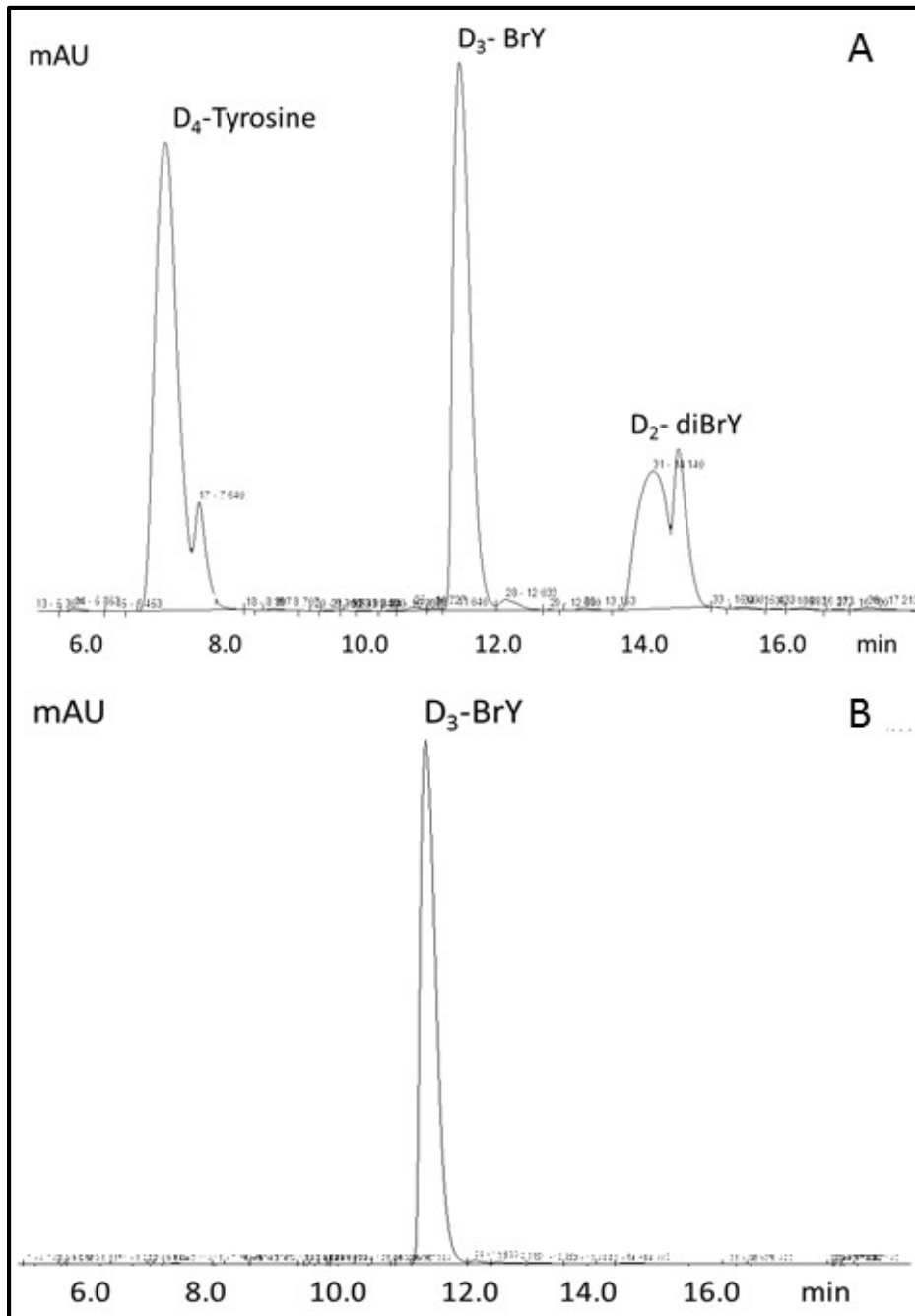
individual (unpooled) serum sample, two pooled serum samples were prepared with two different volumes of known 3-BrY concentrations, where the volumes of added known 3-BrY did not exceed 10% in any of them. Accuracy was evaluated by calculating the O/E ratios for three different serum samples that were spiked with four different 3-BrY concentrations (2.5, 5, 10, and 20  $\mu\text{mol/L}$ ; equivalent to assay standards). The samples were prepared in the same manner as they were for linearity.

### ***Statistical analyses***

Commercially available statistical software packages (JMP Pro 10; SAS Institute Inc., Cary, NC and GraphPad PRISM version 5.0; GraphPad Software, Inc., La Jolla, CA), were used for the data analysis. A Shapiro-Wilk test was used to assess the normality of serum 3-BrY concentrations in healthy dogs. A central 95<sup>th</sup> percentile of serum 3-BrY concentration in 41 healthy dogs was calculated using Microsoft Excel. The correlation between eosinophil counts and serum 3-BrY concentrations was also determined.

### **Results**

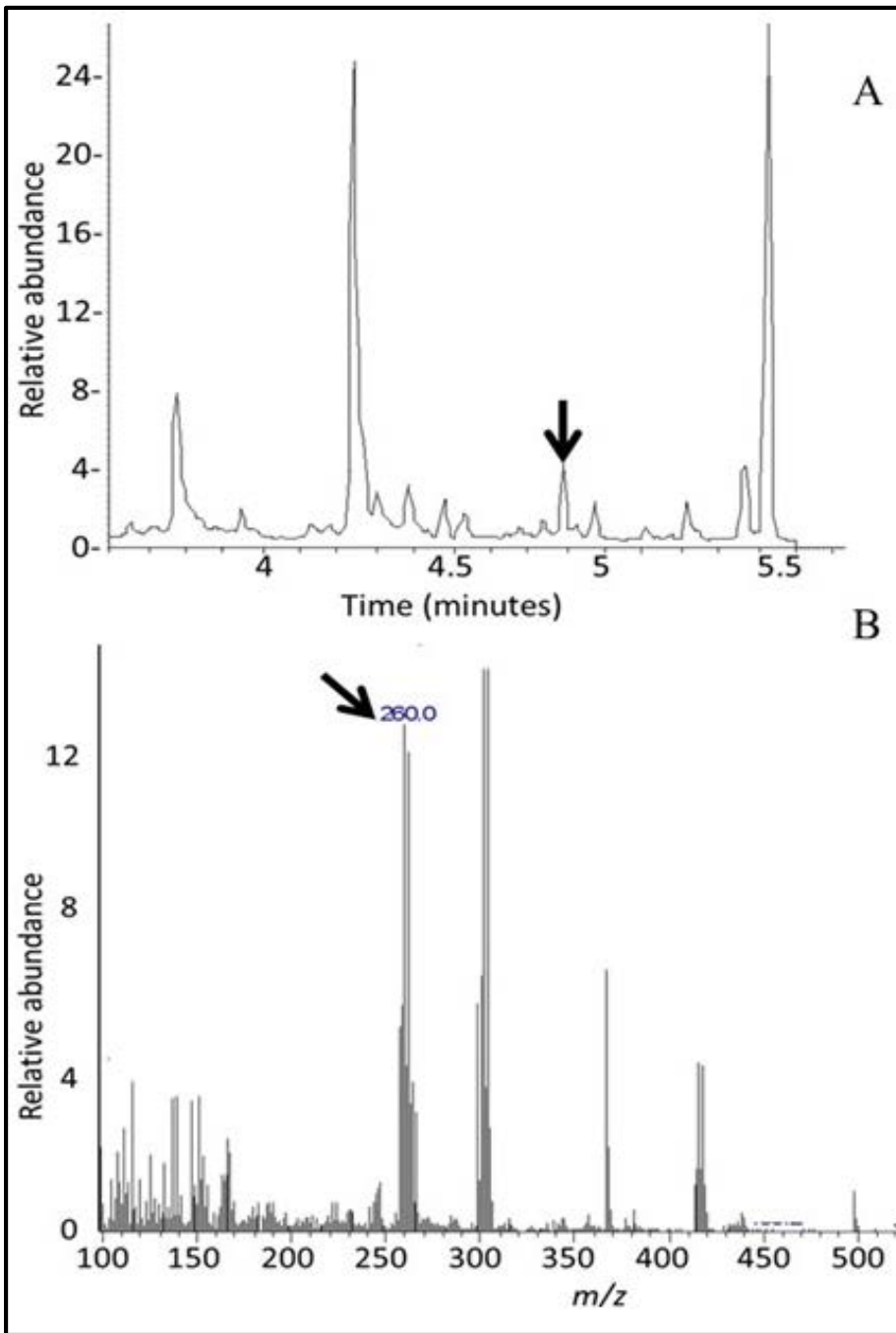
The generated D<sub>3</sub>-BrY was identified and purified by reversed-phase HPLC (Figure 8). The reaction between D<sub>4</sub>-L-tyrosine and N-bromosuccinimide at 37°C for 60 min was



**Figure 8. Tracings of reversed-phase HPLC showing the purification of D<sub>3</sub>-BrY. Panel A: reversed-phase HPLC separation of the bromination reaction product between D<sub>4</sub>-L-tyrosine and N-bromosuccinimide. The tyrosine oxidative product, D<sub>3</sub>-BrY, eluted after D<sub>4</sub>-L-tyrosine, but before D<sub>2</sub>-dibromotyrosine. Panel B: all D<sub>3</sub>-BrY fractions were collected, combined, and concentrated under a nitrogen stream. The concentrated product was analyzed for concentration and purity.**

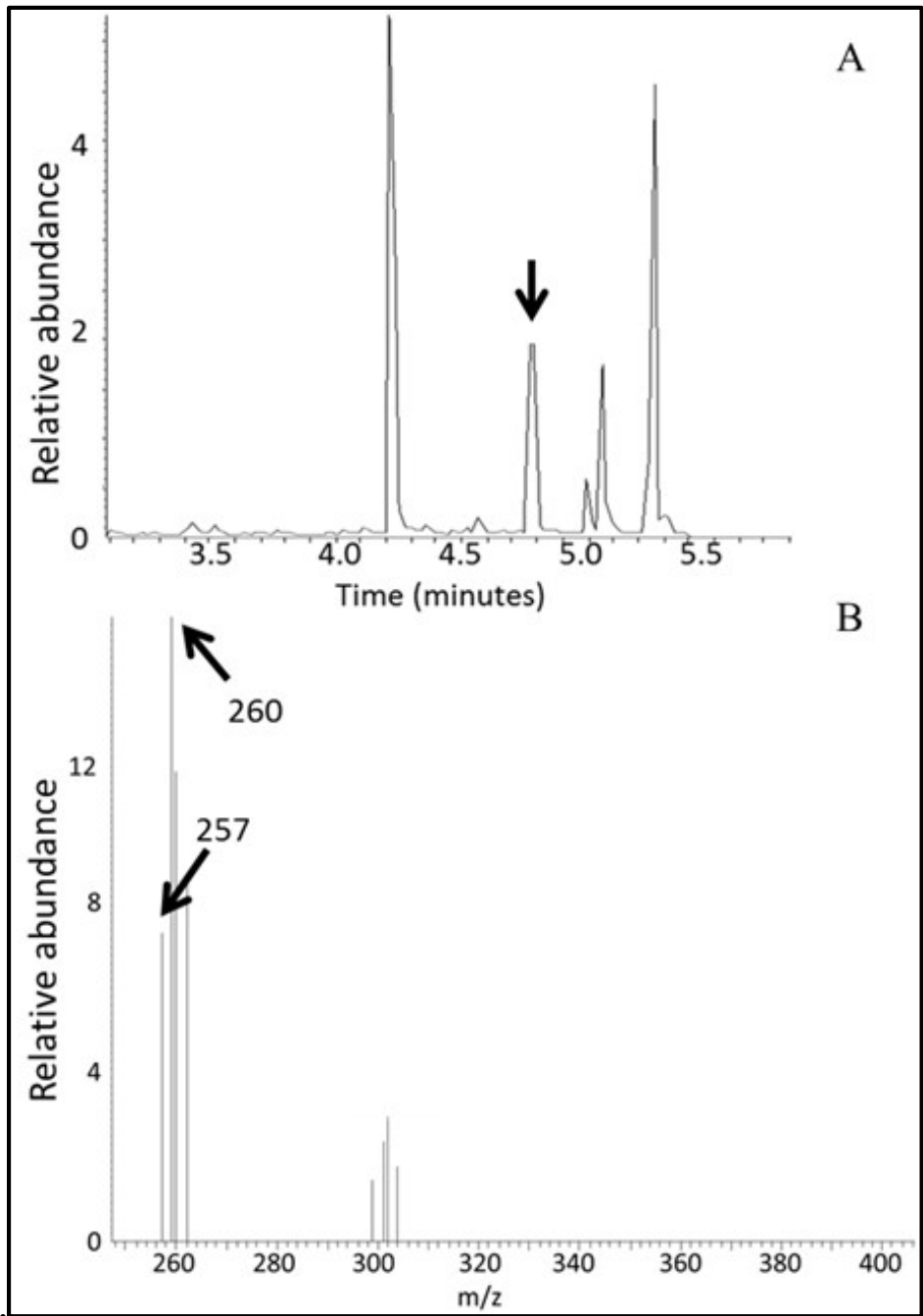
sufficient to generate D<sub>3</sub>-BrY. D<sub>4</sub>-L-tyrosine, D<sub>3</sub>-BrY, and D<sub>2</sub>-dibromotyrosine were separated according to their hydrophobic properties. Incomplete D<sub>4</sub>-L-tyrosine eluted earlier than D<sub>3</sub>-BrY, which eluted during the early isocratic elution with 35% solvent B. The purity of the bromination product is shown in Figure 8. In addition, D<sub>3</sub>-BrY was stable when stored under helium gas at -80°C during the four-month period that the assay method was analytically validated.

3-BrY and D<sub>3</sub>-BrY eluted during EI-GC/MS at 4.77 min in both full-scan and selected ion monitoring EI-GC/MS (Figures 9 and 10). The full-scan EI-GC/MS showed various fractionated ions. After multiple runs of pure 3-BrY and D<sub>3</sub>-BrY in the EI-GC/MS mode, the selected ion monitoring EI-GC/MS of *m/z* ratios of 257/260, 259/262, 299/302, and 301/304 revealed the highest consistency and accuracy of the ratio between pure 3-BrY and D<sub>3</sub>-BrY at an *m/z* of 257/260. Therefore, we used 257 and 260 as representative ions for the quantification of 3-BrY and D<sub>3</sub>-BrY, respectively. The standard curves were generated by the ratio of the peak areas of 3-BrY and D<sub>3</sub>-BrY at *m/z* of 257/260 and the concentrations of 3-BrY standards that were added to a constant quantity of D<sub>3</sub>-BrY. A representative standard curve from 0.5 to 50 µmol/L is shown in Figure 11.

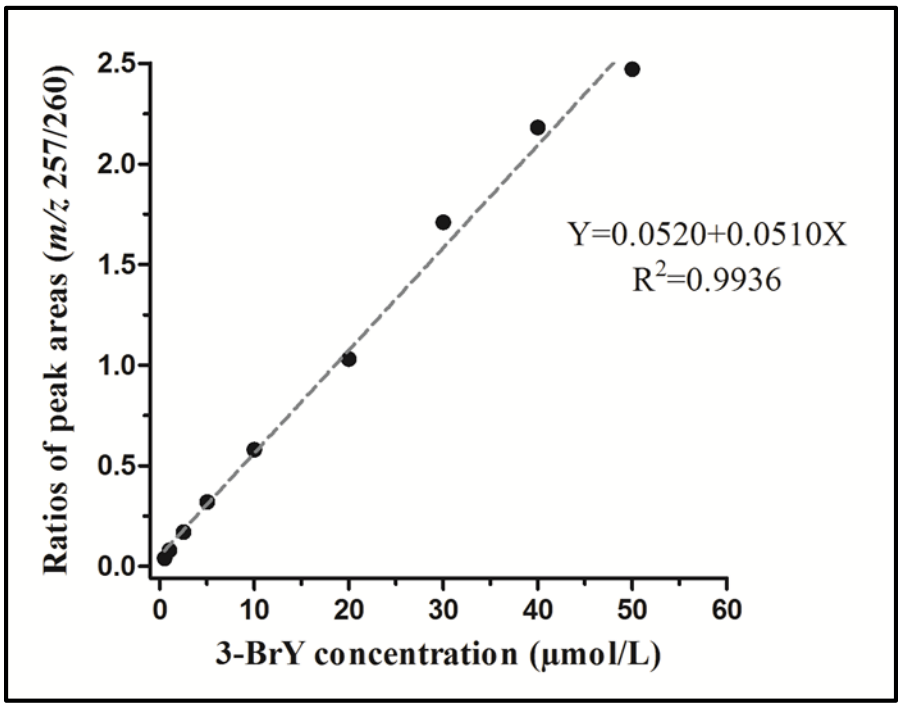


**Figure 9. Representative full-scan chromatogram ( $m/z$  ratio range 100–650) of EI-GC/MS analysis for 3-BrY and D3-BrY. Panel A: 3-BrY and D3-BrY eluted at 4.7 min (arrow). Panel B: the electron ionization fragment pattern shows the  $m/z$  at 257 and 260, which represents 3-BrY and D3-BrY, respectively.**





**Figure 10. Representative selected ion monitoring trace chromatogram for canine 3-BrY in serum. Panel A: 3-BrY and D3-BrY eluted at 4.7 min (arrow). Panel B: m/z 257 represents 3-BrY and m/z 260 represents D3-BrY.**



**Figure 11. Representative standard curve (0.5, 1, 2.5, 5, 10, 20, 30, 40, and 50 µmol/L). Nine plots were generated using the ratios of peak areas of m/z 257/260 and the concentrations of 3-BrY standards that were added to a constant quantity of D3-BrY.**

The LOB for the detection of 3-BrY was 0.33  $\mu\text{mol/L}$ , and the LOD for the detection of 3-BrY in canine serum samples was 0.63  $\mu\text{mol/L}$ . An assay working range for 3-BrY was established as 0.63–50  $\mu\text{mol/L}$ . Intra-assay %CVs for the four different serum samples were 7.3, 13.9, 4.7, and 13.8%, and inter-assay %CVs for the four different serum samples were 5.7, 5.6, 8.1, and 11.0% (Table 3). Assay linearity was determined by preparing serial dilutions of three serum samples, and O/E ratios ranged from 84.0 to 134.4% (Table 4). Assay accuracy was determined by spiking recovery with different concentrations of purified 3-BrY. The O/E ratios ranged from 79.1 to 126.7% (Table 5).

The normality test revealed that the 3-BrY concentrations from healthy dogs were not normally distributed ( $P < 0.0001$ ). Because the majority of dogs had an undetectable 3-BrY concentration below the LOD ( $\leq 0.63 \mu\text{mol/L}$ ), the reference interval was calculated by the 97.5<sup>th</sup> percentile and determined as  $\leq 1.12 \mu\text{mol/L}$  (Figure 12). The mean ( $\pm\text{SD}$ ) eosinophil count was 473.24 ( $\pm 260.68$ ) cell/ $\mu\text{L}$ . A Spearman's rank correlation test revealed no correlation between serum 3-BrY concentration and peripheral eosinophil counts ( $\rho = 0.2681$ ,  $P = 0.0901$ ; Figure 13).

**Table 3. Precision and reproducibility of the EI-GC/MS assay for 3-BrY in canine serum.**

<b>Sample</b>	<b>Mean (<math>\mu\text{mol/L}</math>)</b>	<b>Standard deviation (<math>\mu\text{mol/L}</math>)</b>	<b>CV(%)</b>
<i>Intra-assay variability</i>			
Serum I	1.0	0.1	13.8
Serum II <sup>a</sup>	2.4	0.3	13.9
Serum III <sup>a</sup>	5.7	0.3	4.7
Serum IV <sup>a</sup>	17.7	1.3	7.3
<i>Inter-assay variability</i>			
Serum V	1.2	0.1	11.0
Serum VI <sup>a</sup>	5.9	0.5	8.1
Serum VII <sup>a</sup>	18.6	1.1	5.7
Serum VIII <sup>a</sup>	37.5	2.1	5.6

<sup>a</sup>Canine serum samples II, III, IV, VI, VII, and VIII contained added known concentrations of 3-BrY.

**Table 4. Assay linearity of the EI-GC/MS assay for 3-BrY in canine serum.**

Sample	Dilution	Observed ( $\mu\text{mol/L}$ )	Expected ( $\mu\text{mol/L}$ )	Observed-to- Expected (%)
Serum I	1/2	2.1		
Serum I	1/4	0.9	1.06	84.0
Serum II <sup>a</sup>	1/2	17.7		
Serum II <sup>a</sup>	1/4	10.0	8.8	112.7
Serum II <sup>a</sup>	1/8	5.4	4.4	121.5
Serum II <sup>a</sup>	1/16	3.0	2.2	134.4
Serum III <sup>a</sup>	1/2	40.6		
Serum III <sup>a</sup>	1/4	20.5	20.3	100.7
Serum III <sup>a</sup>	1/8	11.9	10.2	117.2
Serum III <sup>a</sup>	1/16	6.0	5.1	117.6
Serum III <sup>a</sup>	1/32	3.2	2.5	127.2

<sup>a</sup>Canine serum samples II and III contained added known concentrations of 3-BrY.

**Table 5. Assay accuracy of the EI-GC/MS assay for 3-BrY in canine serum.**

<b>Sample</b>	<b>Concentration added in <math>\mu\text{mol/L}</math></b>	<b>Observed (<math>\mu\text{mol/L}</math>)</b>	<b>Expected (<math>\mu\text{mol/L}</math>)</b>	<b>Observed-to- expected (%)</b>
Serum I	0.0	0.7		
Serum I	2.5	2.5	3.2	79.1
Serum I	5.0	6.0	5.7	105.7
Serum I	10.0	12.7	10.7	119.3
Serum I	20.0	18.6	20.7	89.9
Serum I	30.0	27.0	30.7	90.0
Serum II <sup>a</sup>	0.0	4.6		
Serum II <sup>a</sup>	2.5	5.7	7.1	80.4
Serum II <sup>a</sup>	5.0	12.2	9.6	126.7
Serum II <sup>a</sup>	10.0	16.8	14.6	114.7
Serum II <sup>a</sup>	20.0	23.0	24.6	93.2
Serum II <sup>a</sup>	30.0	37.0	34.6	106.8
Serum III <sup>a</sup>	0.0	20.7		
Serum III <sup>a</sup>	2.5	24.3	23.2	104.7
Serum III <sup>a</sup>	5.0	26.8	25.7	104.3
Serum III <sup>a</sup>	10.0	30.1	30.7	98.3
Serum III <sup>a</sup>	20.0	38.3	40.7	94.1
Serum III <sup>a</sup>	30.0	47.0	50.7	92.6

<sup>a</sup>Canine serum samples II and III contained added known concentrations of 3-BrY.

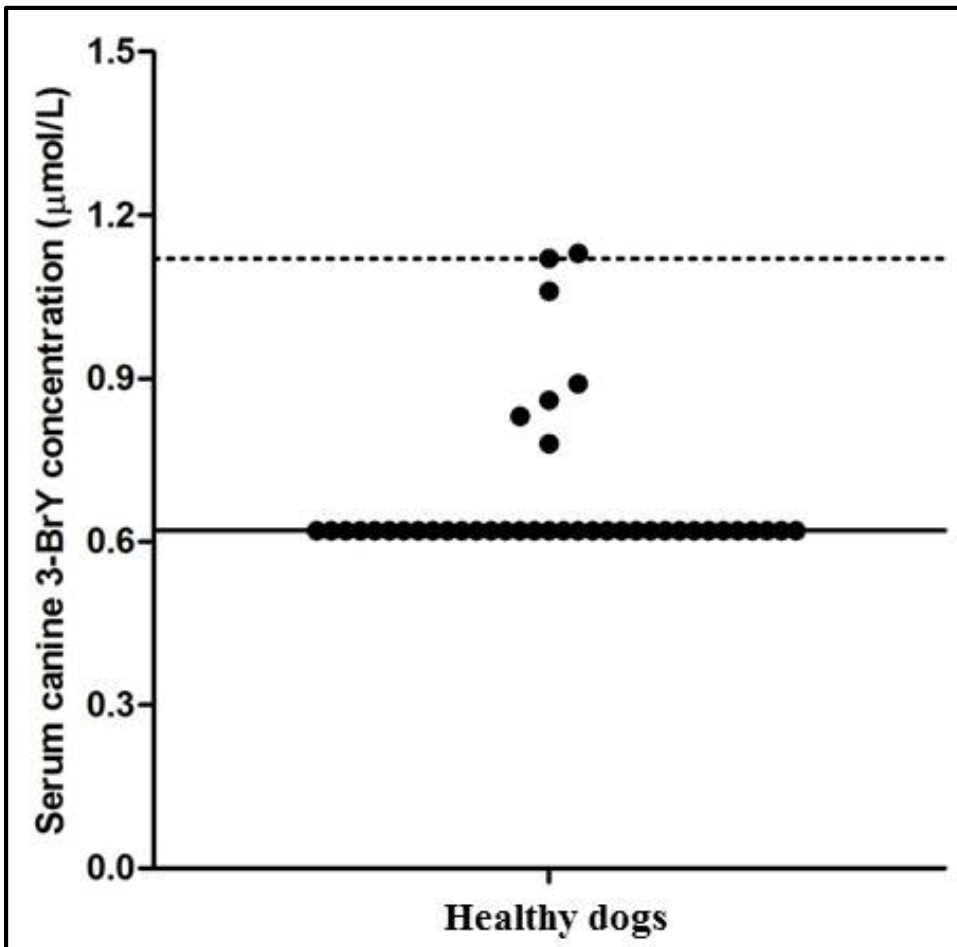


Figure 12. Scatter plot showing serum canine 3-BrY concentrations in 41 healthy control dogs. The median ( $\leq 0.63$  µmol/L; bold horizontal line) and 97.5<sup>th</sup> percentile of the reference interval ( $\leq 1.12$  µmol/L; dashed horizontal line) are also shown.

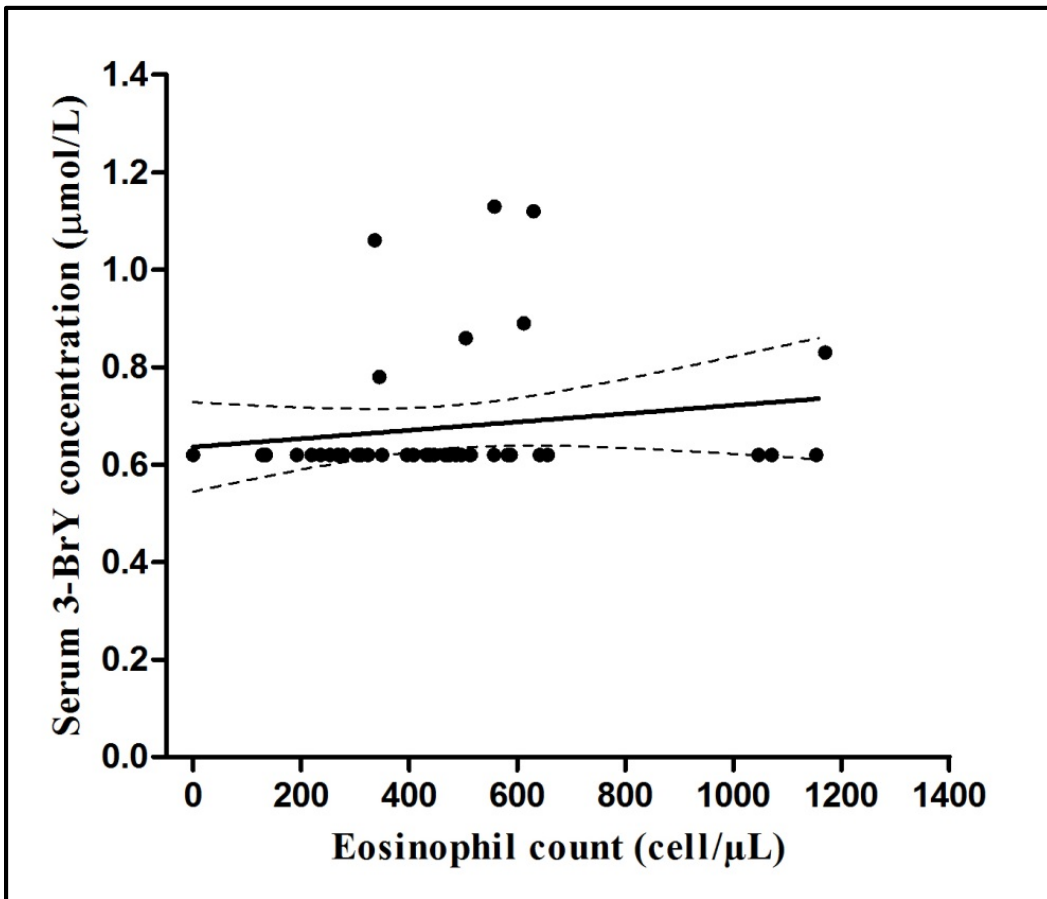


Figure 13. Scatter plot presenting the relationship between serum 3-BrY concentration ( $\mu\text{mol/L}$ ) and peripheral eosinophil count in 41 healthy control dogs. The Spearman rank sum correlation test showed no correlation between 3-BrY concentration and peripheral eosinophil counts ( $\rho=0.2681$ ,  $P=0.0901$ ). Dotted lines represent the 95% confidence interval for predicted values (95% CI: -0.05 to 0.54).



## Discussion

In this study, HPLC and EI-GC/MS were used to purify D<sub>3</sub>-BrY and quantify 3-BrY concentrations in dog serum samples. D<sub>3</sub>-BrY was stable under helium gas for at least 4 months at -80°C. This result is similar to that of a previous study in which D<sub>3</sub>-BrY was stable under argon gas for up to 6 months (Gaut et al., 2002). The storage of D<sub>3</sub>-BrY under argon gas was not performed in our study due to its cost.

Quantification of 3-BrY concentrations in human biological samples has been described using EC-NCI GC/MS (Gaut et al., 2002). However, to our knowledge, the analytical validation of a method to quantify 3-BrY concentrations in dog serum has not previously been published. In the present study, we successfully developed an EI-GC/MS assay to measure 3-BrY concentration in canine serum. In humans, the EC-NCI GC/MS method reportedly requires a 1 mL sample of plasma to quantify 3-BrY concentrations, but the method used in this study requires only a 250 µL sample of dog serum. The smaller sample volume required for EI-GC/MS may make it more clinically applicable than the EC-NCI GC/MS method. Although EI-GC/MS can measure 3-BrY in canine serum samples, its sensitivity is still lower than that of the EC-NCI GC/MS method developed for human plasma. The sensitivity of the EC-NCI GC/MS assay used for human serum was 0.19 fmol (Gaut et al., 2002), whereas the sensitivity of the canine assay was 2.5 pmol. Sensitivity was calculated by the lowest working standard (0.5 µmol/L; data not shown).

One limitation of this study is that all collected canine serum samples had a low 3-BrY concentration (1 to 4 µmol/L). Therefore, adding known concentrations of 3-BrY to

some serum samples to yield 3-BrY concentrations within the assay's working range (0.63 to 50  $\mu\text{mol/L}$ ) was necessary for analytical validation.

Precision and reproducibility of the assay were evaluated by intra-assay and inter-assay coefficients of variation. Ideally, coefficients of variation (%CV) should be <15% (Bansal and DeStefano, 2007; FDA, 2001). The mean %CV across all serum samples for intra-assay and inter-assay variation was <9.9%. The %CV varied by the concentration of the analyte. This variation may be explained by the higher %CV found at the lower concentration, which was close to the LOD (0.63  $\mu\text{mol/L}$ ).

Linearity of the assay was assessed by dilutional parallelism. The recoveries for dilutional parallelism of canine serum samples had a mean  $\pm$ SD of  $109.6 \pm 17.2\%$ . Ideally, recovery for O/E of down dilution should be from 80 to 120% (Bansal and DeStefano, 2007; FDA, 2001). Our results showed a slight increase in recovery with the lower dilutions. This increase may be explained by the concentrations that were measured at the higher dilutions, which had concentrations close to the LOD of the assay.

Accuracy of the assay was evaluated by using spiking recovery. The recoveries for spiking recovery of the canine serum samples had a mean  $\pm$ SD of  $98.7 \pm 11.2\%$ . The recovery for O/E of spiking recovery should be from 80 to 120% (Bansal and DeStefano, 2007; FDA, 2001). The slight decrease in recovery for our samples with higher concentrations may be explained by the fact that the concentrations that were measured were nearly over the maximum concentration of the assay working range.

The distribution of 3-BrY concentrations from 41 healthy dogs was skewed to the right because almost all of the healthy dogs had 3-BrY concentrations below the LOD of

the assay. This may be due to the lack of EPO activation in healthy dogs or the low analytical sensitivity of the EI-GC/MS. However, since the assay would be expected to be utilized for detection of increased 3-BrY concentrations this should not limit its clinical utility.

Since the peripheral eosinophil count does not necessarily represent an activation of eosinophils, the relationship between serum 3-BrY concentration and eosinophil count was evaluated by Spearman's rank correlation test. No correlation between serum 3-BrY concentration and peripheral eosinophil count was found. This suggests that serum 3-BrY concentration might be more useful for identification of eosinophilic diseases in dogs than peripheral eosinophil counts or it may also suggest that 3-BrY is not a useful marker for eosinophilic disease. Clinical studies are warranted to answer this question.

## **Conclusions**

We can conclude that EI-GC/MS was sufficiently repeatable, reproducible, linear, and accurate for the quantification of 3-BrY in canine serum. The availability of this assay may provide a new non-invasive method to help diagnose and monitor eosinophilic diseases in small animals such as asthma, sepsis, and eosinophilic gastroenteritis. The evaluation of the clinical usefulness of this assay is warranted and underway.

## CHAPTER III

### STABILITY AND CONCENTRATIONS OF SERUM 3-BROMOTYROSINE IN DOGS WITH EOSINOPHILIA AND VARIOUS GASTROINTESTINAL DISEASES

#### **Summary**

3-Bromotyrosine (3-BrY) is a stable product of eosinophil peroxidase (EPO) and may serve as a marker of eosinophil activation. A method to measure serum 3-BrY concentrations in dogs has recently been established and analytically validated. The aims of this study were to determine the short-term stability of serum 3-BrY concentrations and assess the clinical usefulness of its measurement. Serum samples were collected from healthy dogs, dogs with eosinophilia, eosinophilic gastroenteritis (EGE), lymphocytic-plasmacytic enteritis (LPE), exocrine pancreatic insufficiency, and pancreatitis. Serum 3-BrY concentrations were measured by the previously validated gas chromatography/mass spectrometry method. Serum 3-BrY concentrations were stable for up to 8, 30, and 180 days at 4°C, -20°C, and -80°C, respectively. Serum 3-BrY concentrations were significantly higher in dogs with eosinophilia (median= 1.81 µmol/L) than in healthy dogs (median= ≤0.63 µmol/L;  $P<0.0001$ ). Also, Serum 3-BrY concentrations were significantly higher in dogs with EGE (median = 5.04 µmol/L), LPE (median = 3.60 µmol/L), and pancreatitis (median = 1.49 µmol/L) than in healthy control dogs (median = ≤0.63 µmol/L;  $P<0.0001$ ), whereas concentrations in dogs with EPI (median = 0.73 µmol/L) were not different compared to healthy control dogs. In conclusion, the present study revealed that 3-BrY concentrations were stable in serum when refrigerated and frozen. In addition,

serum 3-BrY concentrations were increased in dogs with EGE, but also in dogs with LPE and pancreatitis. Further studies are needed to determine whether measurement of 3-BrY concentrations in serum may be useful to assess patients with suspected or confirmed EGE or LPE.

## **Introduction**

Chronic enteropathy (CE) is characterized by recurrent or persistent intestinal signs for more than 3 weeks (Allenspach et al., 2007; Procoli et al., 2013). The diagnostic process for patients with suspected CE requires exclusion of gastrointestinal (GI) parasites and other extra GI diseases (e.g., pancreatitis, exocrine pancreatic insufficiency (EPI)). CE is classified by a patient's response to a given treatment trial, namely, food-responsive diarrhea, antibiotic-responsive diarrhea, and steroid-responsive diarrhea. The histological findings in dogs with steroid-responsive diarrhea (idiopathic inflammatory bowel disease; IBD) differ due to various inflammatory cells within the intestinal mucosa. IBD in dogs can thus be subclassified into eosinophilic gastroenteritis (EGE), lymphocytic-plasmacytic enteritis (LPE), granulomatous enteritis, and histiocytic ulcerative colitis (Hall and German, 2008; Washabau et al., 2010). The histological findings in dogs with IBD differ due to various inflammatory cells within the affected tissue. IBD can be subclassified into eosinophilic gastroenteritis (EGE), lymphocytic-plasmacytic enteritis (LPE), granulomatous enteritis, and histiocytic ulcerative colitis (German, 2013; Washabau, 2013). In dogs with IBD, the small intestine is affected more commonly than the large intestine (Allenspach et al., 2007). Clinical scoring systems including the canine chronic

enteropathy clinical activity index (CCECAI) (Allenspach et al., 2007) and the canine IBD activity index (CIBDAI) (Jergens et al., 2003), which can be used to evaluate the disease severity and treatment response in dogs with IBD. Nevertheless, no biomarker for eosinophils has been identified for dogs with GI diseases.

The defining feature of EGE is infiltration of eosinophils in the GI tract. EGE can be caused by parasite infestation, hypereosinophilic syndrome, neoplasia, allergy, or IBD. Although previous studies have reported that the histopathological findings of GI biopsies would suggest that EGE is secondary to LPE in canine IBD (Craven et al., 2004; Hall and German, 2008), eosinophils were shown to play crucial roles in stimulating inflammation and motility, leading to clinical signs such as diarrhea, inflammation, tissue destruction, fibrosis formation, and/or strictures (Al-Haddad and Riddell, 2005; Lampinen et al., 2005). Therefore, a marker of eosinophil activation may provide a useful tool for evaluating the contribution of eosinophils in patients with CE.

Eosinophil peroxidase (EPO) is a potent granular cytotoxic heme-protein released during the activation of eosinophils (Mita et al., 2004; Weiss et al., 1986; Wu et al., 1999). Under physiological conditions, EPO utilizes bromide to yield hypobromous acid (HOBr) (Shen et al., 2001). Bromination of tyrosine by HOBr in surrounding tissues and blood occurs rapidly and results in the production of 3-bromotyrosine (3-BrY) (Mita et al., 2004; Wu et al., 1999). Structurally and physiologically, 3-BrY is a stable product. Therefore, it can be used as a noninvasive marker of eosinophil-catalyzed protein oxidation. A method to measure 3-BrY in dog serum using electron ionization gas chromatography/mass spectrometry (EI-GC/MS) has recently been developed and analytically validated

(Sattasathuchana et al., 2014). The hypothesis is that activation of EPO plays an important role in the pathogenesis of EGE, and thus the measurement of serum concentrations of 3-BrY as a specific biomarker for EGE may have diagnostic potential. The assay may be particularly useful to differentiate between patients with EGE and LPE, as well as dogs with other GI diseases. The objectives of this study were (1) to assess the stability of serum 3-BrY concentrations after storage at 4°C, -20°C, and -80°C, (2) to compare serum 3-BrY concentrations in dogs with peripheral eosinophilia and healthy dogs, and (3) to compare serum 3-BrY concentrations between healthy control dogs and dogs with various GI diseases, such as EGE, LPE, EPI, and pancreatitis.

## **Materials and Methods**

### ***Determination of stability for 3-BrY in serum samples***

Excess serum samples that were submitted to the Gastrointestinal Laboratory (GI Lab) at Texas A&M University for diagnostic purposes were pooled. Known quantities of pure 3-BrY (BOC Science Company, Shirley, NY) were mixed to pool serum samples to obtain 10 different 3-BrY concentrations within the working range of the 3-BrY assay (0-50 µmol/L). On day 0, samples were prepared, divided into aliquots, and stored at 4°C, -20°C, or -80°C until analysis. Serum 3-BrY concentrations were determined on days 0, 2, 8, 16, 30, 60, and 180. On day 0, samples were analyzed immediately after they were prepared. Serum samples stored at 4°C were analyzed on days 2 and 8. Serum samples stored at -20°C were analyzed on days 8, 16, 30, and 60. Finally, serum samples stored at -80°C were analyzed on days 16, 30, 60, and 180.

***Comparison of serum 3-BrY concentrations in dogs with peripheral eosinophilia and healthy dogs***

*Sample from healthy control dogs*

Serum samples from 41 healthy dogs were collected between July 2013 and September 2013. The sample collection protocol was approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC; #2012-101), and informed owner consent was obtained for all dogs. None of the healthy dogs manifested any clinical or laboratory abnormalities, and they received regular vaccinations and deworming.

*Dogs with peripheral eosinophilia*

Surplus serum samples from 21 dogs with peripheral eosinophilia (eosinophil count >750 cells/ $\mu$ L) were collected at Kasetsart Veterinary Teaching Hospital in Bangkok, Thailand.

***Comparison of serum 3-BrY concentrations between healthy control dogs and dogs with various gastrointestinal diseases***

*Samples from healthy control dogs*

Serum samples from 52 healthy control dogs were collected. The sample collection protocol was approved by the Texas A&M University Institutional Animal Care and Use Committee (#2012-101), and informed owner consent was obtained for all dogs. None of



the healthy dogs did manifest any clinical or laboratory abnormalities and received regular vaccinations and deworming.

*Samples from dogs with EGE*

Surplus canine serum samples from submissions to the GI Lab at Texas A&M University from 27 dogs with EGE were used for this study. The diagnosis was based on the presence of eosinophilic infiltrates in GI biopsy specimens. All patients had serum cPLI and cTLI concentrations within the reference interval.

*Samples from dogs with LPE*

Surplus canine serum samples from submissions to the GI Lab at Texas A&M University from 25 dogs with LPE were used. The diagnosis of LPE was based on the presence of lymphocytes and plasma cells with an absence of eosinophils in the GI mucosa during histological evaluation of GI biopsies. All patients had serum cPLI and cTLI concentrations within the reference interval.

*Samples from dogs with EPI*

Surplus canine serum samples from submissions to the GI Lab at Texas A&M University from 26 dogs with a serum canine trypsin like immunoreactivity (cTLI) concentration  $\leq 2.5$   $\mu\text{g/L}$  were used. All of these samples showed a normal serum canine pancreatic lipase immunoreactivity (cPLI) concentration and an undetectable serum cobalamin concentration  $\leq 149$   $\text{ng/L}$ .

### *Samples from dogs with pancreatitis*

Surplus canine serum samples from submissions to the GI Lab at Texas A&M University from 27 dogs with a serum cPLI concentration  $\geq 1001$   $\mu\text{g/L}$  were used. It should be noted that the suggested diagnostic cut-off value of serum cPLI concentration for a diagnosis of pancreatitis is  $>400$   $\mu\text{g/L}$ . Also, all patients had serum cobalamin and cTLI concentrations within the respective reference interval.

All serum samples were stored at  $-80^{\circ}\text{C}$  for up to 6 months until analysis. Standard questionnaires were sent out to the primary care veterinarian to obtain histories, clinical signs at the time of sample collection, and the final diagnosis. Questionnaires for 25 (92.6%) dogs with EGE, 23 (92.0%) dogs with LPE, 14 (53.8%) dogs with EPI, and 15 (55.6%) dogs with pancreatitis were completed by the primary care veterinarian. The clinical diagnosis for each dog was based on the result of the histological evaluation of GI biopsies or clinical laboratory data such as cTLI and cPLI.

### ***Measurement of serum 3-BrY***

#### *Preparation of internal standards*

D<sub>3</sub>-bromotyrosine (D<sub>3</sub>-BrY) was used as an internal standard and prepared by reacting d<sub>4</sub>-L-tyrosine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) with N-bromosuccinimide (Sigma-Aldrich, St. Louis, MO) in water (Gaut et al., 2002; Hazen et al., 1997; Wu et al., 1999). D<sub>3</sub>-BrY was isolated by reverse-phase high performance liquid chromatography (HPLC) using a C18 HPLC column (Phenomenex, Torrance, CA). Purified D<sub>3</sub>-BrY fractions were collected and stored at  $-80^{\circ}\text{C}$  under helium until use.

### *Analysis of 3-BrY in canine serum*

The sample preparation protocol was adapted from previous publications (Frost et al., 2000; Gaut et al., 2002; Mita et al., 2004). Briefly, eight nanomoles (16  $\mu\text{mol/L}$ ) of D<sub>3</sub>-BrY was added in a 1 to 2 dilution mixture of water (250  $\mu\text{l}$ ) and serum sample (250  $\mu\text{l}$ ). The volume of the mixture was adjusted to 2 mL with 0.1% TFA (Sigma-Aldrich, St. Louis, MO), pH5.0 and centrifuged at 4°C for 10 min at 16,000 x g. After centrifugation, the mixture was passed through a C18 solid phase extraction column (Sigma-Aldrich Company, St. Louis, MO). 3-BrY was eluted from the column with 25% methanol (Sigma-Aldrich, St. Louis, MO) in water. The eluent was immediately dried in a rotary vacuum device (Eppendorf, Hauppauge, NY) at 45°C and stored at -80°C until further analysis.

The derivatization protocol was modified from protocols described previously (Frost et al., 2000; Gaut et al., 2002; Mita et al., 2004). Briefly, the previously dried sample was mixed with 100  $\mu\text{L}$  of acetonitrile (Thermo Fisher Scientific, Inc., Pittsburgh, PA) and 40  $\mu\text{L}$  of diisopropylethylamine (Sigma-Aldrich, St. Louis, MO). The sample was incubated on ice for 5 min. Ethyl heptafluorobutyrate (Sigma-Aldrich, St. Louis, MO) was added into the sample and the sample underwent an incubation period for 30 min on ice. Then, the sample was sonicated in a water bath for 1 h at room temperature. Excess reagents were evaporated under a nitrogen stream at room temperature. Thirty  $\mu\text{L}$  of N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MtBSTFA) (Thermo Fisher Scientific, Inc., Pittsburgh, PA) was added to the sample followed by 30 min incubation at room temperature. The sample was completely dried under a nitrogen stream and redissolved in

50  $\mu$ L of undecane (Sigma-Aldrich, St. Louis, MO) containing 25% (v/v) MtBSTFA. The clear supernatants were analyzed immediately by EI-GC/MS.

An Agilent 7890A gas chromatography (Agilent Technologies, Santa Clara, CA) and a 5975C mass detector (Agilent Technologies, Santa Clara, CA) were used to measure 3-BrY concentrations. A capillary column (Agilent Technologies, Santa Clara, CA) was used to separate the analytes using helium as a carrier gas. The injector, transfer line, and source temperature were initially set at 180, 300, and 250°C, respectively. The oven temperature gradient was increased at a rate of 40°C/min from 180°C to 310°C. Ions were monitored at  $m/z$  257 and 259 for 3-BrY and D<sub>3</sub>-BrY, respectively.

### ***Statistical methods***

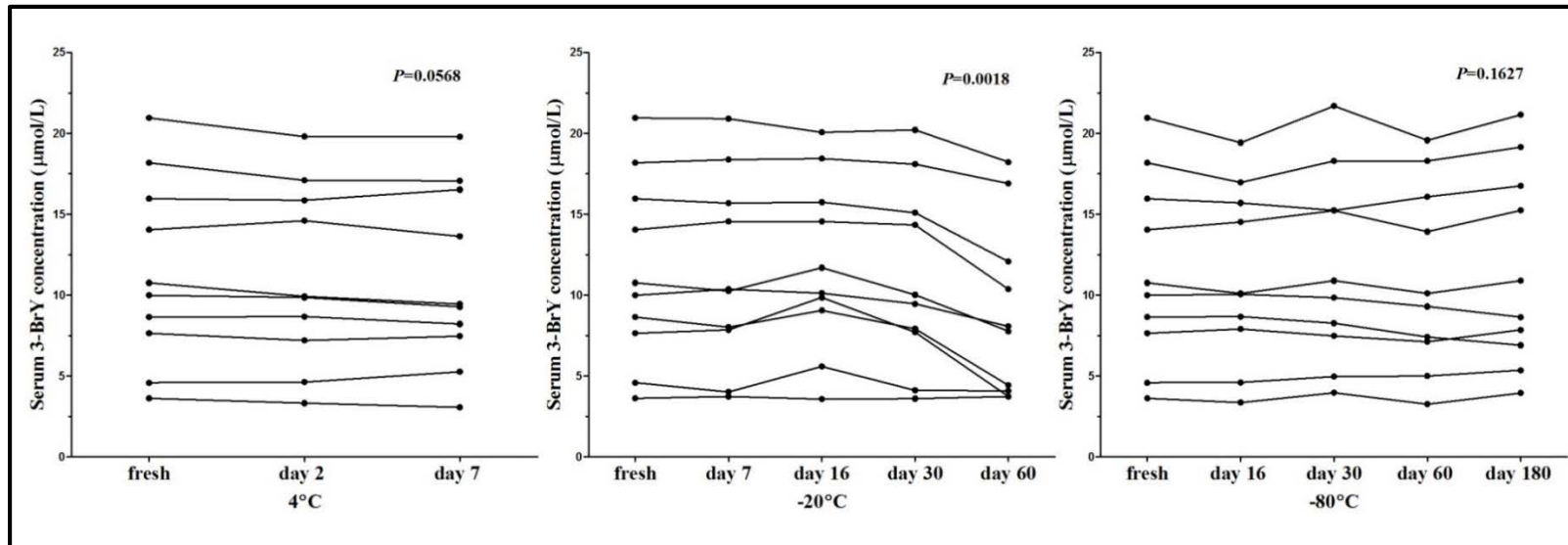
All statistical analyses were performed with commercial software packages, JMPPro 10 (SAS Institute Inc., Cary, NC) and GraphPad PRISM5.0 (GraphPad software, Inc. La Jolla, CA). Each data set was tested for normality using a Shapiro-Wilk's test. To evaluate stability, serum 3-BrY concentrations for fresh samples and samples at each of the 3 temperatures (4°C, -20°C, and -80°C) were compared through a repeated-measurement ANOVA. The Dunn's post-test was used to determine the differences in serum 3-BrY concentrations at different time points under the same storage temperature. In addition, the coefficient of variation (%CV = [SD/mean] x 100) was used to determine the variability of serum 3-BrY concentration under each storage condition. The difference between 3-BrY concentrations in serum samples of healthy dogs and dogs with peripheral eosinophilia was evaluated by using a Man-Whitney *U* test. The correlation between

eosinophil count and serum 3-BrY concentrations in healthy dogs and dogs with peripheral eosinophilia was determined by Spearman's rank sum test. A Kruskal-Wallis test was used to evaluate the difference of serum 3-BrY concentrations between healthy dogs and dogs with various GI diseases. The Dunn's post-test was applied to determine differences between groups. For all analytical tests, significance was set at  $P < 0.05$ .

## Results

No statistically significant differences were found between the mean serum 3-BrY concentrations for the 10 individual samples stored at 4°C, -20°C, or -80°C for ≤8 days, 30 days, or 180 days, respectively (Figure 14). However, the Dunn's post-test showed that 3-BrY was not stable after 60 days at -20°C ( $P < 0.0001$ ). The mean and %CV for each sample in each storage condition are displayed in Table 6.

Population demographics for comparison of serum 3-BrY concentrations in dogs with peripheral eosinophilia and healthy dogs are shown in Table 7. The age of peripheral eosinophilia dogs was significantly higher than healthy dogs. There was no discrepancy of sex, or breed between each study group. In healthy dogs, the median serum 3-BrY concentration was ≤0.63 μmol/L and ranged from ≤0.63 to 1.13 μmol/L. The median serum 3-BrY concentration in dogs with peripheral eosinophilia was 1.81 μmol/L and ranged from ≤0.63 to 26.26 μmol/L. Median serum 3-BrY concentrations were significantly higher in dogs with peripheral eosinophilia compared to healthy dogs ( $P < 0.0001$ ; Figure 15). The



**Figure 14. Stability of serum 3-BrY concentrations for various storage temperatures. When compared to fresh samples, 3-BrY concentrations were stable at 4°C for 7 days (%CV≤8.47, P=0.0568) and -80°C for 180 days (%CV≤9.88, P=0.1627). However, 3-BrY concentrations was not stable at -20°C for 60 days (%CV≤30.31, P=0.0018).**

**Table 6. Mean and %CV of 3-BrY concentrations for each pooled canine serum sample.**

Sample	Mean 3-BrY $\pm$ SD ( $\mu\text{mol/L}$ )	%CV		
		Fresh and 4°C	Fresh and -20°C	Fresh and -80°C
1	3.92 $\pm$ 1.27	8.47	1.96	9.08
2	4.66 $\pm$ 0.60	7.93	14.84	6.60
3	7.43 $\pm$ 1.36	2.96	30.31	4.18
4	7.62 $\pm$ 1.43	2.93	24.23	9.88
5	9.72 $\pm$ 0.92	3.95	9.62	6.28
6	10.28 $\pm$ 1.04	6.59	14.40	3.90
7	13.86 $\pm$ 1.49	3.46	13.28	4.28
8	15.47 $\pm$ 1.19	2.21	10.87	3.45
9	17.81 $\pm$ 0.77	3.62	3.47	4.30
10	19.77 $\pm$ 1.64	3.31	5.54	4.91

Mean and %CV of 3-BrY concentrations for each pooled canine serum samples (n=10) when measured fresh or stored under different storage temperatures (4°C for 7 days, -20°C for 60 days, and -80°C for 180 days).

**Table 7. Population demographics for comparison of serum 3-BrY concentration in dogs with peripheral eosinophilia and healthy dogs.**

	Healthy dogs	Peripheral eosinophilia
<b>Number of dogs</b>	41	34
<b>Age*</b> (years)	4.0 (1-10)	6.0 (<1-14)
<b>Sex</b>		
Male	56.1% (n=23)	55.9% (n=19)
Female	43.9% (n=18)	44.1% (n=15)
<b>Breed</b>		
Small (<10 kg)	24.4% (n=10)	32.4% (n=11)
Medium (10-20 kg)	48.8% (n=20)	32.4% (n=11)
Large (>20 kg)	26.8% (n=11)	35.2% (n=12)

\*Median (minimum-maximum range)



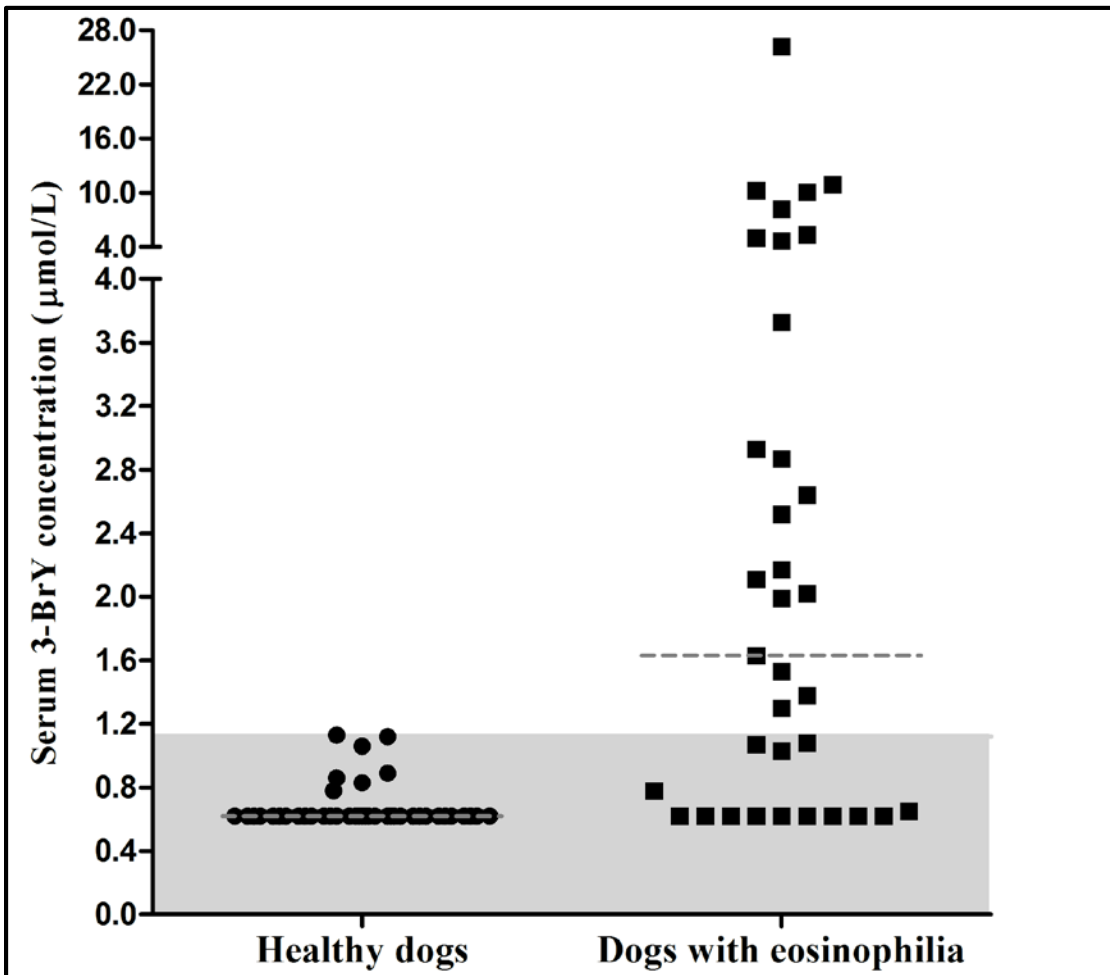
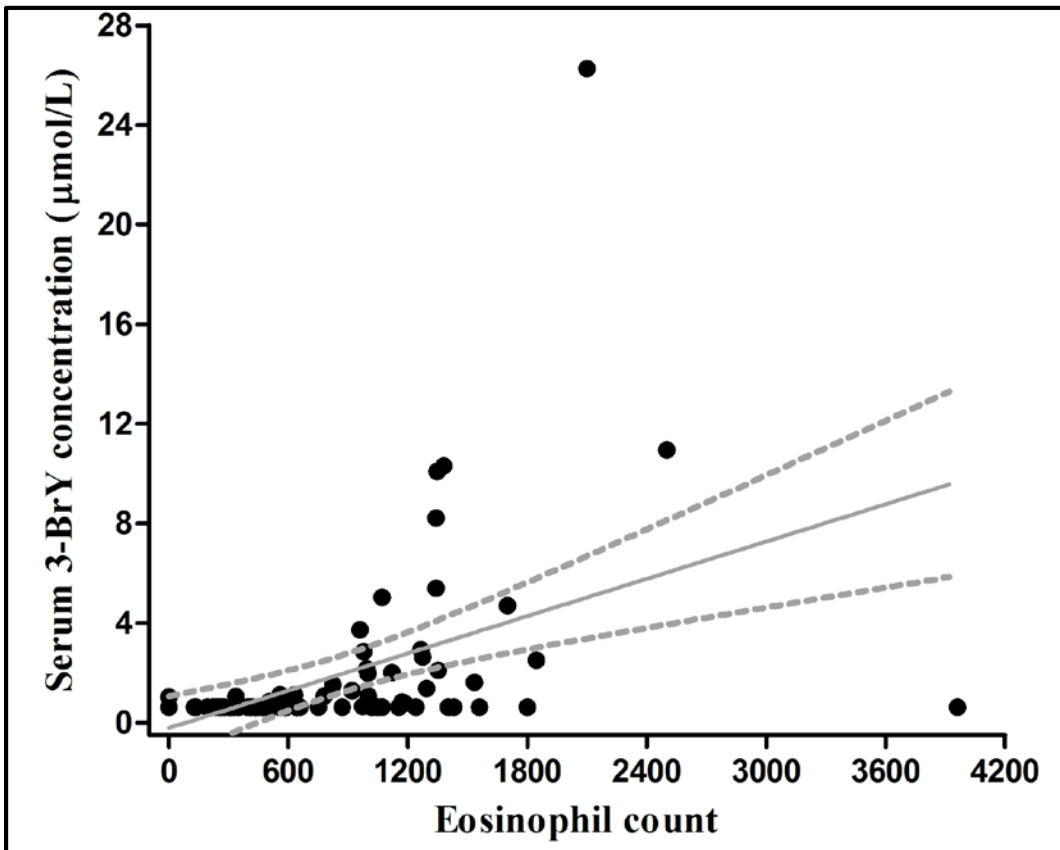


Figure 15. Serum 3-BrY concentrations for 41 healthy control dogs and 35 dogs with peripheral eosinophilia. Serum 3-BrY concentrations were significantly higher in dogs with peripheral eosinophilia compared to healthy dogs ( $p < 0.0001$ ). The medians for each dog group are indicated (dashed line).

Spearman's rank sum test revealed a moderate correlation between the peripheral eosinophil count and serum 3-BrY concentrations for healthy dogs and dogs with peripheral eosinophilia ( $\rho = 0.5639$ ,  $P < 0.0001$ ; Figure 16).

Population demographics for comparison of serum 3-BrY concentrations in healthy dogs and dogs with various GI diseases are shown in Table 8. The age of dogs with pancreatitis was significantly higher than healthy dogs ( $P < 0.0001$ ). There was no discrepancy of sex and breed between dog groups.

In healthy dogs, the median serum 3-BrY concentration was  $\leq 0.63$   $\mu\text{mol/L}$  with a range of  $\leq 0.63$  to  $1.79$   $\mu\text{mol/L}$ . The median [range] of serum 3-BrY concentrations in dogs with EGE, LPE, EPI, and pancreatitis were  $5.04$  [ $\leq 0.63$ - $26.26$ ],  $3.60$  [ $\leq 0.63$ - $15.67$ ],  $0.73$  [ $\leq 0.63$ - $4.59$ ], and  $1.49$  [ $\leq 0.63$ - $4.46$ ]  $\mu\text{mol/L}$ , respectively. There was a statistically significant difference in serum 3-BrY concentrations between dogs with EPI, pancreatitis, LPE, EGE, and healthy dogs ( $P < 0.0001$ ; Figure 17). The Dunn's post-test analysis revealed differences of serum 3-BrY concentrations between healthy control dogs and dogs with either EGE, LPE, or pancreatitis. Serum concentrations of 3-BrY were significantly higher in dogs with EGE than in healthy dogs ( $P < 0.0001$ ) or dogs with EPI ( $P = 0.0072$ ). Also, serum 3-BrY concentrations were significantly higher in dogs with LPE than those in healthy dogs ( $P < 0.0001$ ) or dogs with EPI ( $P = 0.0039$ ). Serum 3-BrY concentrations were significantly higher in dogs with pancreatitis than in healthy dogs ( $P < 0.0135$ ).



**Figure 16.** Correlation of peripheral eosinophil counts and serum 3-BrY concentrations for healthy control dogs and dogs with eosinophilia. The Spearman's rank correlation coefficient showed a moderate relationship between serum 3-BrY concentrations and peripheral eosinophil counts ( $\rho = 0.60$ ,  $P < 0.0001$ ). Dashed lines represent the 95% confidence interval for the predicted values (95% CI: 0.40 to 0.74).

**Table 8. Population demographics for comparison of serum 3-BrY concentration in dogs with various GI diseases and healthy dogs.**

	Healthy dogs	EGE	LPE	EPI	Pancreatitis
Number of dogs	52	27	25	26	27
Age* (years)	4.0 (1-10)	5.0 (<1-12)	8.5 (2-12)	5.8 (1-16)	11.0 (<1-16)
Sex					
Male	61.5% (n=32)	44.4% (n=12)	52.0% (n=13)	42.3% (n=11)	48.1% (n=13)
Female	39.2% (n=20)	55.6% (n=15)	48.0% (n=12)	57.7%(n=15)	52.9% (n=14)
Breed					
Small (<10 kg)	23.1% (n=12)	18.6% (n=5)	56.0% (n=14)	23.1% (n=6)	59.3% (n=16)
Medium (10-20 kg)	42.3% (n=22)	33.3% (n=9)	20.0% (n=5)	23.1% (n=6)	7.4% (n=2)
Large (> 20 kg)	34.6% (n=18)	48.1% (n=13%)	24.0% (n=6)	53.8% (n=14)	33.3% (n=9)

\*Median (minimum to maximum range)

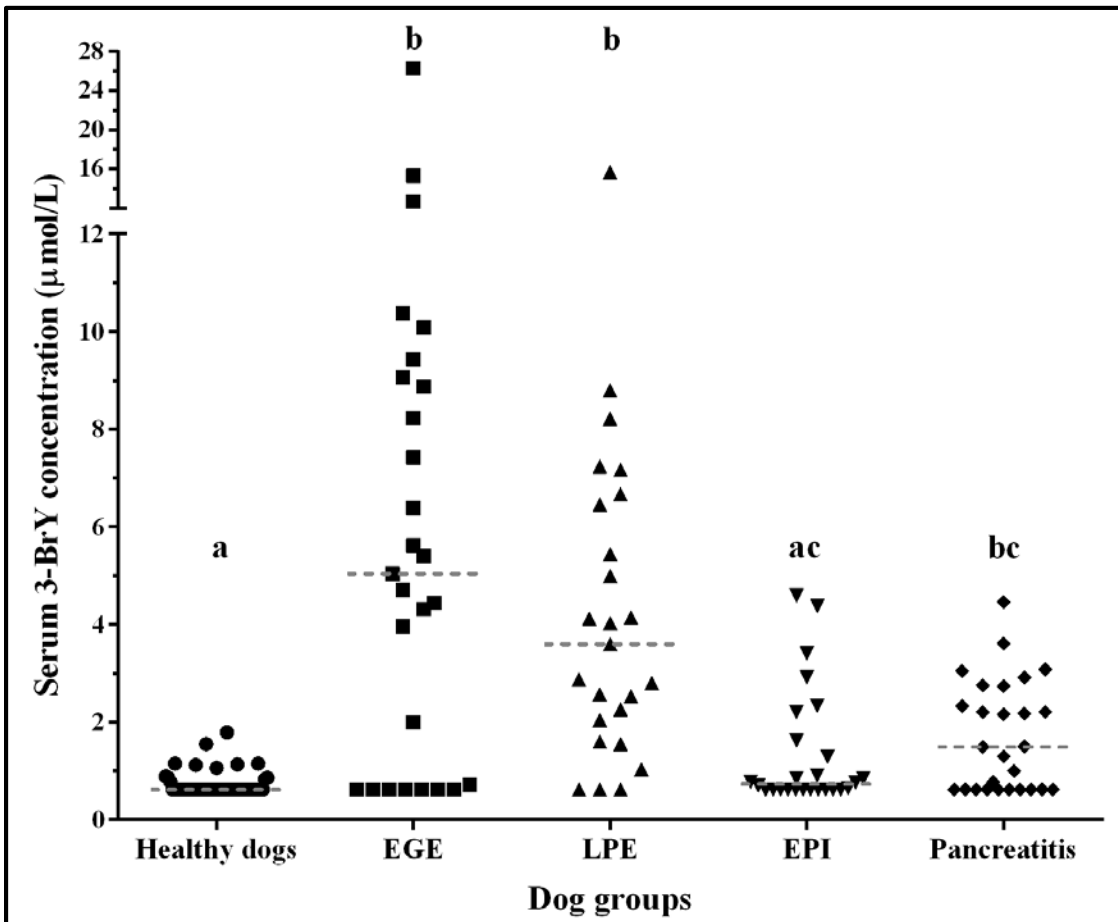


Figure 17. Serum 3-BrY concentrations in healthy dogs (n=41), dogs with EGE (n=27), LPE (n=25), EPI (n=26), or pancreatitis (n=27). The medians of serum 3-BrY concentrations are shown in dashed lines. Columns not sharing a common superscript are significantly different (P<0.05).

## **Discussion**

The stability of 3-BrY concentrations in serum was evaluated in the present study because serum 3-BrY concentration may be affected by storage conditions. The results from this study suggest that 3-BrY serum concentrations were stable when stored at 4°C for  $\leq 8$  days, -20°C for  $\leq 30$  days, and -80°C for  $\leq 180$  days. Therefore, shipping and storage conditions require careful consideration.

Serum 3-BrY concentrations were slightly altered on day 8 when stored at 4°C and day 180 when stored at -80°C; however, this difference was not statistically significant. This finding may have been caused by inter-assay variation. The %CV for inter-assay variation of serum 3-BrY concentration was previously reported as  $\leq 11.0\%$ . (Sattasathuchana et al., 2014), whereas the %CVs for the storage conditions at 4°C and -20°C were  $\leq 8.47$  and  $9.88\%$ , respectively. Thus, we concluded that the alterations of serum 3-BrY concentrations in samples stored at 4°C and -80°C were most likely an effect of inter-assay variation.

The limitation of this study may be that serum samples were pooled, and a known concentration of 3-BrY had been added. However, at least 3 ml of serum were required to measure the stability for each sample, and obtaining this amount of serum from diseased patients was not possible. In addition, very low concentrations of 3-BrY were found in the serum samples. Therefore, a known concentration of 3-BrY was added to each serum sample to reach the quantification limit at which 3-BrY can be detected in serum.

This study provides the first clinical evaluation of measuring 3-BrY concentrations in serum from dogs with GI diseases. The results showed that not all dogs with peripheral

eosinophilia have detectable serum 3-BrY concentrations, which suggests that not all circulating eosinophils have been activated during the disease process. Because circulating eosinophils have a short transit time from marrow to tissue, (Tizard, 2009) peripheral eosinophilia may not always represent the eosinophil activation at the affected tissue. The moderate association between 3-BrY concentrations and eosinophil counts suggest that peripheral eosinophilia partially contributed to increased 3-BrY concentrations. However, more importantly, eosinophilic infiltration of tissues, such as the infiltration observed in dogs with EGE, may also contribute to increased serum 3-BrY concentrations.

3-BrY has been shown to be a specific eosinophil activation byproduct (Mita et al., 2004; Van Dalen et al., 2009; Van Dalen and Kettle, 2001; Weiss et al., 1986; Wu et al., 1999; Wu et al., 2000). In the present study, concentrations of 3-BrY in serum from dogs with peripheral eosinophilia were significantly increased compared to serum of healthy dogs. Furthermore, our findings revealed a significant increase in serum 3-BrY in dogs with LPE and EGE, suggesting an important pathophysiological role of eosinophil activation in dogs with GI disease.

Lymphocytes and neutrophils have been reported as the predominant inflammatory cells present in dogs with pancreatitis (Newman et al., 2006). In this study, serum 3-BrY concentrations were significantly increased in dogs with pancreatitis compared to healthy dogs. This was an unexpected finding, suggesting eosinophil activation in dogs with pancreatitis. This may suggest that pancreatic proteases are released and activate the inflammatory pathway in dogs with pancreatitis, which may lead to eosinophil activation in other organs such as the GI tract.

Our findings revealed a significant increase of serum 3-BrY in dogs with LPE and EGE, suggesting an important pathophysiological role of eosinophil activation in dogs with these two forms of CE. Serum 3-BrY concentrations for both dogs with EGE or LPE were statistically significantly different from healthy dogs. Although the median 3-BrY concentration in dogs with EGE was higher than in dogs with LPE, there was no statistically significant difference between these two groups. These findings suggest the presence of eosinophil activation in the GI tract of dogs with LPE or EGE. However, a larger sample set may have been needed to determine a possible difference in serum 3-BrY concentrations between dogs with EGE and those with LPE.

The presence of eosinophilic infiltration in the GI tract in dogs with EGE supports the hypothesis that 3-BrY can serve as a potential biomarker for eosinophil activation. Our findings are the first to demonstrate that 3-BrY, a stable product of eosinophilic peroxidase, can be detected in serum samples from dogs with chronic GI disease. Other studies in humans and mice have revealed the important pathophysiological role of EPO in affected GI tissues (Forbes et al., 2004; Carlson et al., 1999). The lack of CCECAI and CIBDAI scores due to the use of left-over serum samples precluded us to identify the relationship between the severity of clinical signs and serum 3-BrY concentrations. Therefore, further investigations of the relationship between serum 3-BrY concentration and the severity of clinical signs are needed and underway.

The increased serum 3-BrY concentration in dogs with LPE was an unexpected finding in the present study. Lymphocytes and plasma cells play a central role in dogs with LPE. The increased concentration of serum 3-BrY in LPE may be the result of eosinophil



activation stimulated by T-lymphocytes in dogs with chronic GI disease. It is known that T-lymphocytes secrete IL-5, which is a key mediator to moderate the maturation, migration, and activation of eosinophils (Takatsu et al., 1980; Yan et al., 2009). Moreover, eosinophil infiltration may be overlooked during the assessment in the different compartments (i.e., duodenum, jejunum, ileum, or colon) of the GI tract and this may lead to the failure to detect the eosinophil infiltration in those tissues. In addition, the use of hematoxylin and eosin (H&E) stain may have led to failure to detect eosinophil activation (Gomes et al., 2013; Protheroe et al., 2009) when compared to other methods such as immunohistochemical staining (e.g., EPO antibody). However, measurements of 3-BrY concentration were performed in serum samples for this study, which may not accurately characterize inflammation in the GI tract. Therefore, the development and analytical validation of this assay for the measurement of 3-BrY concentration in fecal samples is warranted and underway.

## **Conclusions**

This is the first study that measured serum 3-BrY concentrations in dogs with various GI diseases. Our results suggest that eosinophil activation occurs in dogs with EGE, LPE, or pancreatitis. The present study also suggests that measurement of serum 3-BrY concentration may serve as a potential diagnostic marker for dogs with CE after the exclusion of pancreatitis.

## CHAPTER IV

### CONCLUSIONS

Gastrointestinal (GI) endoscopy with the collection of GI biopsies has been considered the most useful tool for the investigation of dogs with chronic enteropathy (CE). These procedures, however, require expensive equipment and expertise. In addition, these procedures carry the risks associated with general anesthesia. Therefore, non-invasive diagnostic biomarkers for further investigating patients with CE would be desirable, but such biomarkers have not yet been identified.

The type of inflammatory cells that predominate an infiltration of the GI tract in patients with CE varies. Lymphocytes and plasma cells are the cell types of inflammatory cells that are found in the GI tract of dogs with CE. However, eosinophils are the second most common inflammatory cell type in dogs with CE. However, peripheral eosinophil counts are not a representative marker for eosinophil activation in the GI tract. Thus, the development of a marker that accurately reflects eosinophil activation in the GI tract is needed.

Eosinophil peroxidase (EPO) is a specific enzyme of eosinophils that is released during their activation. Moreover, the reaction that generates 3-bromotyrosine (3-BrY) is unique to EPO. Thus, 3-BrY could serve as a specific biomarker for eosinophil activation in the GI tract of dogs.

This study was designed to have two phases. The purpose of the first phase was to develop and analytically validate a method to measure 3-BrY concentrations in canine

serum samples. The goal of the second phase was to determine the stability of serum 3-BrY concentrations and to evaluate the clinical usefulness for measuring serum 3-BrY concentrations in dogs with peripheral eosinophilia and dogs with CE.

In this study, a method to measure 3-BrY concentrations in canine serum samples was developed and established to identify the activation of eosinophils in the GI tract. The analytical validation of this method showed that this newly developed assay was precise, reproducible, linear, and accurate for the measurement of 3-BrY concentrations in canine serum samples. The stability of this biomarker in serum samples was also evaluated. The results revealed that the concentration of 3-BrY was stable enough to be measured in serum samples from dogs with clinical diseases such as CE.

The concentration of 3-BrY was also measured in serum from healthy pet dogs. The results showed that almost all of the 41 healthy dogs had undetectable 3-BrY concentrations in their serum. These low concentrations could be interpreted as a lack of EPO activation in healthy dogs or the low analytical sensitivity of this method. The correlation of peripheral eosinophil count and serum 3-BrY concentration was also evaluated, but no correlation was found. These findings suggest that measuring 3-BrY concentrations in canine serum may be a better indicator for the identification of eosinophil activation in dogs than performing peripheral eosinophil counts

The hypothesis for the second part of the study was that 3-BrY may be a diagnostic marker for dogs with eosinophilic gastroenteritis (EGE) and that this assay may be useful to differentiate between dogs with EGE and other forms of CE, such as lymphocytic-plasmacytic enteritis (LPE). 3-BrY concentrations in serum samples from dogs with

peripheral eosinophilia and healthy dogs were compared, and serum 3-BrY concentrations were higher in dogs with peripheral eosinophilia than in healthy dogs. Additionally, concentrations of 3-BrY were measured in serum from dogs with EGE, LPE, exocrine pancreatic insufficiency (EPI), and pancreatitis, and the results indicated that dogs with EGE, LPE, and pancreatitis have significantly higher 3-BrY concentrations in serum than the dogs belonging to the other groups. These findings suggest that eosinophil activation occurs in dogs with peripheral eosinophilia, EGE, LPE, and pancreatitis. And, it can be speculated that in dogs with EGE and LPE this eosinophil activation occurs in the intestinal tract. The findings further suggest that measurement of 3-BrY concentrations in canine serum may serve as a potential diagnostic marker for dogs with CE after the exclusion of pancreatitis.

In conclusion, this research offers an important clinical application relating to the diagnosis of canine CE: Elevated concentrations of 3-BrY in canine serum may suggest inflammation in the GI tract, especially in dogs with EGE and LPE. Therefore, measurement of 3-BrY in canine serum has the potential to serve as a non-invasive diagnostic marker for EGE and LPE in dogs. Future studies are required to evaluate the clinical usefulness of 3-BrY as a biomarker for the diagnosis of CE in dogs.

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