DISSERTATION

CLINICAL, CLINICOPATHOLOGIC, HISTOPATHOLOGIC, AND IMMUNOHISTOCHEMICAL FEATURES OF DOGS WITH CHRONIC ENTEROPATHY WITH AND WITHOUT PROTEIN-LOSING ENTEROPATHY: FOCUS ON THE INTESTINAL LYMPHATIC VASCULATURE

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ABSTRACT

CLINICAL, CLINICOPATHOLOGIC, HISTOPATHOLOGIC, AND IMMUNOHISTOCHEMICAL FEATURES OF DOGS WITH CHRONIC ENTEROPATHY WITH AND WITHOUT PROTEIN-LOSING ENTEROPATHY: FOCUS ON THE INTESTINAL LYMPHATIC VASCULATURE

Chronic enteropathy (CE) is a term used to describe a group of chronic intestinal conditions in the dog that may respond to a variety of therapies including food, antibiotics, glucocorticoids, and immunosuppressives. Idiopathic CE is diagnosed after exclusion of extra-intestinal, infectious, parasitic, neoplastic and mechanical causes of gastrointestinal signs. Chronic enteropathies are often associated with histologic evidence of inflammation in the small intestine. In up to 20% of cases of CE with histologic evidence of inflammation in the small intestine, intestinal protein-loss occurs, a condition termed protein-losing enteropathy (PLE). Although several previous studies have identified the development of protein loss as a negative prognostic indicator in dogs with CE, the exact reasons why some dogs with CE develop concurrent PLE while others do not is not well understood. The goal of this work was to examine clinical, clinicopathologic, histopathologic, and immunohistochemical features of dogs with CE with and without PLE, with a special focus on the role of the intestinal lymphatic vasculature in cases of CE.

In the first part of this work, we retrospectively examined histologic findings in a group of dogs with CE, and compared those findings between hypoalbuminemic and normoalbuminemic dogs. We found that many histopathologic features of idiopathic CE differed

between dogs that were hypoalbuminemic versus those that were normoalbuminemic. Specifically, villous stunting, epithelial injury, crypt distension, lacteal dilation, intraepithelial lymphocytes, and lamina propria neutrophils were more common in hypoalbuminemic dogs with CE when compared to normoalbuminemic dogs with CE. We concluded that histopathologic features differ between hypoalbuminemic and normoalbuminemic dogs with CE and suggested that additional work is necessary to elucidate the clinical relevance of these differences. In particular, we noted that the high prevalence of lacteal dilation in hypoalbuminemic dogs with a primary diagnosis of inflammatory CE may be important and warranted further investigation.

Next, we set out to prospectively evaluate whether a novel dietary approach could result in clinical improvement in dogs with CE and PLE who were not responding well to traditional therapy with glucocorticoids. We found that 8/10 dogs in the study achieved a complete clinical remission following a novel therapeutic dietary approach, with 7/8 dogs remaining in remission up to 3.5 years following the initial dietary change. Because 7/8 dogs that achieved a complete remission had been switched to a more fat-restricted diet than what they had previously been fed, we hypothesized that dietary fat-restriction may have played a role in their improvement. Because most of these dogs had not been diagnosed with lymphangiectasia, we speculated that the presence or extent of lymphatic abnormalities could have been underappreciated on routine histopathologic exam in this group of dogs.

Lymphatic endothelial cell (LEC) immunohistochemical markers have identified intestinal lymphatic vasculature abnormalities in humans with inflammatory bowel disease (IBD)**.** In the next chapter of our work, we utilized LEC markers and immunohistochemistry to evaluate the intestinal lymphatic vasculature in a group of dogs with CE. We uncovered significant mucosal lymphatic distension in some dogs with CE, most striking of which was in

the ileum of dogs with CE and concurrent PLE. Several of these dogs did not have significant villous lymphangiectasia, therefore we concluded that routine histopathologic examination likely underestimated their lymphatic abnormalities. We also found that various abnormalities of the lymphatic vasculature were correlated with serum albumin levels.

Finally, we aimed to improve our understanding of the pathogenesis of low serum vitamin D in dogs with CE. We did this by evaluating a variety of variables we felt could be associated with the mechanisms of low serum vitamin D in dogs with CE. We found that higher canine chronic enteropathy clinical activity (CCECAI) scores and serum CRP concentrations, and lower serum Vitamin E, cholesterol, and albumin concentrations were more likely in dogs with CE and low serum 25- hydroxyvitamin D (25[OH]D) concentrations compared to dogs with CE and normal serum 25(OH)D concentrations. In addition, histopathologic morphologic scores as well as overall WSAVA scores were correlated with serum 25(OH)D levels. Of particular relevance to this body of work were the findings of lower serum cholesterol and Vitamin E concentrations in dogs with low serum 25(OH)D and CE compared to dogs with normal 25(OH)D levels and CE. We concluded that the mechanism of low serum 25(OH)D in dogs with CE is likely multifactorial but fat malabsorption due to a variety of causes, including lymphatic dysfunction, could play a major role in the development of low 25(OH)D in this population.

The work described in this dissertation has increased our understanding of protein-losing enteropathy as a consequence of canine CE, and in particular has highlighted the role of the intestinal lymphatic vasculature in these cases. This work can help to improve the lives of dogs suffering from CE and PLE and provides a solid foundation for further research in this arena.

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1.1.1 Pathophysiology and Etiology of Chronic Enteropathy

Chronic enteropathies (CE) in dogs comprise a group of disorders characterized by persistent or relapsing gastrointestinal signs including diarrhea, weight loss, decreased appetite, abdominal pain, borborygmus, and vomiting.[1-3] Diagnosis of CE requires exclusion of extra-gastrointestinal or other gastrointestinal disorders. Based on response to therapy, CE is typically further classified in dogs as food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (ARE), or idiopathic inflammatory bowel disease (IBD), which may also be termed steroid/immunosuppressant-responsive enteropathy (SRE/IRE).[1-3] A diagnosis of idiopathic IBD is applied to dogs with histologic confirmation of intestinal inflammation that have had an inadequate response to appropriate elimination diet trials and symptomatic therapies.[4,5]

Histopathologic analysis does not reliably distinguish between the various causes of CE.[1,6] When intestinal inflammation is present, lymphoplasmacytic infiltrates are most common, but granulomatous, suppurative, and eosinophilic infiltrates also occur.[7-10] The pathogenesis of chronic enteropathy is not fully understood. Among dogs with CE, FRE is reported as the most common type, followed by idiopathic IBD, and lastly ARE.[1,3,8] The majority of cases of FRE are suspected to be caused by an immune-response to dietary antigens, but studies evaluating the pathogenesis are limited.[11,12] Bacterial invasion of the intestinal wall and improvement of clinical disease with antimicrobial therapies has been well documented in boxers and French bulldogs with granulomatous colitis.[13,14] The role of antibiotics in other cases of CE is less clear. Possible

effects that have been suggested include modulation of the immune system and alteration of the intestinal microbiota.[2]

Of the causes of CE in dogs, idiopathic IBD is likely the most similar to IBD in humans, which include Crohn's disease (CD) and ulcerative colitis (UC). In brief, CD is characterized by idiopathic inflammation and the formation of granulomas that commonly involve the entire intestinal wall and can be localized to the ileum or diffuse throughout the small intestine, and UC is an inflammatory and ulcerative disease typically confined to the superficial layers of the colon.[15]

In humans with CD and UC, genetic predispositions have been recognized, and mutations in specific genes have been identified and investigated. For example, in individuals with CD a defect in the innate immune receptor NOD2/CARD15 has been shown to result in increased mucosal cytokine production in the presence of enteric microflora. This is thought to promote and perpetuate intestinal inflammation and lead to impaired bacterial killing.[15] A variety of other genes have been identified, including those that contribute to disease susceptibility through their effects on innate and/or adaptive immunity and barrier function.[5] While a variety of genes that contribute to disease susceptibility have been identified, genetically susceptible individuals do not always experience clinical disease. In addition, an increasing incidence in IBD has been observed in the last several decades, correlating with changes in lifestyle habits, environmental conditions, and diet.[16] Thus, the etiopathogenesis of IBD in humans is suspected to involve a complex interplay between environmental triggers, dietary factors, abnormal immune responses and altered enteric microbiotia in a genetically susceptible individual. Additionally, although the etiologic and pathogenic role is not completely defined, alterations of the intestinal lymphatic network are long-recognized and well-described features of human IBD.[17]

While the clinical manifestation in dogs can be quite different from the human correlates, we suspect that a similar interplay between genetics, the immune system, environmental triggers, and an altered intestinal microenvironment is responsible for the development of idiopathic IBD in dogs.[5] Apparent breed predispositions (e.g. German shepherd dog, basenji, Boxer dog) support the role of host genetics in the pathogenesis of IBD.[4] Further, mutations in toll-like receptors (TLR), namely TLR4 and TLR5, have recently been identified in dogs and found to be significantly associated with an increased risk for developing IBD.[18] An immune-mediated component to the disease is suspected based on the response to immunomodulatory therapies. Further, an immune-mediated pathogenesis is supported by findings of increased IgE positive cells in the duodenum,[19] upregulated TLRs in the duodenum,[20] higher presence of activation of NFkB in intestinal biopsies,[21] and alterations of enteric immune cell populations[10] in dogs with IBD when compared to healthy controls. Importantly, alterations of the intestinal microbiota have been well described in both humans and dogs with IBD.[22] In general, the intestinal microbiota in dogs with IBD has significantly lower alpha diversity when compared to healthy controls, and the presence or absence of particular bacterial phyla has been associated with IBD in various studies.[22-24] The functional consequences of these alterations remain poorly understood, however it is possible that the dysbiosis is responsible for driving the inflammatory process in IBD.[24] Investigation into the contribution of the intestinal lymphatic network to the etiopathogenesis of canine IBD is lacking.

1.1.2 Pathophysiology and Etiology of Protein-Losing Enteropathy

Protein-losing enteropathy (PLE) is a syndrome of excessive intestinal protein loss. While the exact mechanisms resulting in the protein loss have not been well described in

veterinary medicine, the general assumption is that it occurs as a result of altered intestinal permeability, direct mucosal erosion or ulceration, and/or in association with altered lymphatic function and direct loss of protein-rich lymph.[25] Potential causes of canine PLE are numerous including chronic enteropathies, intestinal lymphangiectasia (IL), parasitic enteritis, fungal enteritis, intestinal obstruction (due to intussusception or foreign body) neoplasia (such as alimentary lymphoma), intestinal crypt disease, regional enteritis, and intestinal erosions/ulceration, among others.[1,25,26]

Approximately 20% of dogs with CE and histologic evidence of intestinal inflammation are reported to have concurrent PLE characterized by hypoalbuminemia with or without hypoglobulinemia.[1,8] Several previous studies have identified hypoalbuminemia as a negative prognostic indictor in dogs with chronic enteropathy or idiopathic IBD.[1 4,7,8,27] However, the reasons why some dogs with CE develop concurrent PLE while others maintain normal protein levels are not well understood. A genetic predisposition is suggested by the fact that some breeds of dogs are considerably more likely to develop PLE than other breeds of dogs, such as Yorkshire terriers, Rottweilers, Shar-Peis, Maltese and Norwegian Lundehunds. A familial PLE has also been described in soft-coated Wheaton terriers.[25]

In humans with PLE, mechanisms responsible for increased intestinal protein loss include mucosal injury resulting in increased mucosal permeability and lymphatic system abnormalities resulting in leakage of protein-rich lymph.[28,29] Enteric albumin loss can be up to 60% of the total albumin pool, and in turn albumin synthesis is typically increased. Despite the increase in its synthesis, albumin remains low due to excessive loses. Albumin is the most affected protein, which is thought to be due to its slow turnover.[30] Causes of PLE in humans are numerous and include non-erosive gastrointestinal disorders, erosive gastrointestinal disorders, and disorders

resulting in increased central venous pressure or lymphatic obstruction or dysfunction.[29] Specific examples of non-erosive disease include amyloidosis, Celiac disease, and Whipple's disease. Intestinal lymphoma, IBD, and sarcoidosis are examples of erosive diseases leading to PLE, and congestive heart failure, constrictive pericarditis, portal hypertensive gastropathy, and intestinal lymphangiectasia (IL) are examples of diseases leading to increased interstitial pressure and subsequent PLE.[28,29]

The mechanism of protein loss secondary to CD and UC is generally thought to be a consequence of leakage of protein-rich fluids across severely denuded intestinal mucosa. However, CD and UC can also result in secondary lymphangiectasia, which can occur due to inflammation, obstruction of the vessels or elevated lymph pressure, and result in loss of protein-rich lymph through the intestine. In patients with CD and refractory PLE, surgery is often considered and in some cases lymphadenopathy and dilated intestinal and mesenteric lymphatics are encountered.[31]

1.1.3 Therapeutic Management and Prognosis of Chronic Enteropathy and Protein-Losing Enteropathy

In both human and veterinary medicine the main challenges in the treatment of CE and IBD stem from an incomplete understanding of the etiopathogenesis of these diseases[15] and the varied responses of individuals to routine therapies. Therapeutic decisions in the treatment of canine CE are often based on the apparent severity of disease as determined by clinical signs and biochemical abnormalities, histopathologic findings, and/or on whether there is suspicion of a breed- related disorder.[4] Controlled clinical trials in the treatment of CE are lacking. Therapies

are largely empiric and the reasons for response or lack of response in an individual dog are not well understood.

Goals of therapy include the reduction of inflammation, counteraction of dysbiosis, and the correction of nutritional deficiencies. As FRE is the most common type of CE, the initial treatment approach in dogs with CE typically involves some type of dietary modification.[1,3,8] In more clinically severe cases, glucocorticoids may be employed as an initial therapy. Various immunosuppressive medications are also used in combination with glucocorticoids, or when glucocorticoids fail to show a beneficial effect. It is often necessary to consider symptomatic and supportive therapies as well, including antiemetics, appetite support medications, and in some cases, gastroprotectants.[4]

The approach to treatment in humans with IBD is similar to that of dogs with a few noteworthy differences. Dietary modification has not been central to the management of the majority of cases of idiopathic IBD in humans, and immunosuppressive medications are generally employed earlier in the course of management when compared to dogs.[2] More recently, monoclonal antibodies (e.g. Infliximab [TNF-α inhibitor]) are increasingly prescribed to modulate the immune response pathways in patients with IBD.[2] Additionally, amongst human patients with IBD, surgery is a common treatment. Forty to fifty percent of human patients with IBD will have surgical treatment within 10 years of their initial diagnosis.[32] In comparison, the need for surgery is extremely rare in dogs. Treatments targeting the lymphatic alterations of IBD have thus far only been performed experimentally in mouse-models of IBD, but have shown encouraging results.[17]

Following diagnostic confirmation of PLE, the underlying cause must be identified to optimize treatment. In dogs with CE complicated by PLE, initial therapeutic management is

typically more aggressive when compared to normoalbuminemic dogs with IBD. Due to the more guarded prognosis associated with PLE, the use of glucocorticoid and other immunosuppressive agents are typically employed immediately after diagnosis. Dietary modification is also considered key, although due to the lack of controlled clinical trials, information regarding the preferred dietary approach in specific cases of PLE is unknown. Therefore, various dietary approaches are used. Complications of PLE are common and are often addressed therapeutically. These include effusions and edema, hypocobalaminemia, hypercoagulability and thrombosis, hypocalcemia, hypomagnesemia, and hypovitaminosis D, among others.[2,4,7,25]

Similarly, in humans with PLE, treatment is directed at the underlying condition responsible for PLE. Treatment of underlying inflammatory disease is similar to treatment of normoalbuminemic patients with CD or UC,[28,29] however surgery may be necessary in refractory cases, particularly when disease is limited to isolated parts of the bowel.[31] A few publications have suggested that treatment with octreotide, a somatostatin analogue, has resulted in improvement in enteric protein loss and clinical signs in some patients with PLE.[29] However, the mechanism of action responsible for this improvement is not well understood. Diet is also considered critical to the management of PLE in humans, and will be discussed further in the next section of this chapter.

In veterinary medicine, evidence of the benefits of specific therapeutic protocols for dogs with CE and PLE are lacking. The controlled studies evaluating nutritional therapies will be discussed in the next chapter. Some antibiotic-responsive cases have been reported,[3,33] but controlled studies do not exist and response to antibiotics is anecdotally reported to be short-lived in most cases. A recent study showed a beneficial effect of the probiotic strain VSL #3

(now known as Visbiome™) in dogs with idiopathic IBD.[34] Response rates to glucocorticoids for treatment of idiopathic IBD and PLE have been reported in a few studies. Short-term response to prednisolone or budesonide in dogs with idiopathic IBD have ranged from 69-88% with follow up times ranging from 21-42 days.[35,36] In Yorkshire terriers with PLE, response to prednisolone in one study was 53%, with follow-up times ranging from 4 to 80 months.[37] The use of prednisone versus budesonide in the treatment of idiopathic IBD was compared in a double-blinded randomized controlled trial and there was no difference in remission rates or incidence of side effects between the two groups of dogs, with a small number of dogs in this study reported to be hypoproteinemic.[38] In one study of dogs with glucocorticoid-refractory idiopathic IBD, cyclosporine treatment (5 mg/kg [2.3 mg/lb], po, q24 h) induced clinical remission in 12/14 dogs. Ten of these dogs had PLE, and 7 had long-term survival.[39] However, in another group of dogs with glucocorticoid-refractory CE, only 2/8 responded to cyclosporine.[1] Another study evaluated dogs with CE and PLE receiving prednisolone and azathioprine versus prednisolone and chlorambucil (4-6 mg/m2, po, q24 h for 7-14 days initially). This retrospective review found that dogs receiving the prednisolone and chlorambucil combination had significant improvement in serum albumin and weight, as well as a survival advantage when compared to dogs receiving prednisolone and azathioprine.[40] Interestingly, in two separate studies involving dogs with PLE, a survival benefit has been seen in dogs treated with lower doses of glucocorticoids[40] or treated without the use of glucocorticoids[41], though neither study set out to evaluate this.

In general, the prognosis in cases of canine CE is good, with a reported response rate of 82-87%.[1,8] However, spontaneous exacerbation of disease is common. In dogs with CE complicated by PLE, the prognosis has been consistently reported as worse compared to dogs with CE maintaining normal albumin levels.[1,8] Specific prognostic factors that have been

identified in dogs with PLE include a higher initial canine inflammatory bowel disease clinical activity index (CIBDAI) score[42] (Figure 1.1),[43] vomiting, severity of hypoalbuminemia, and monocytosis.[37] The type or severity of intestinal inflammatory infiltrate in cases of PLE have not been correlated with serum albumin level.[1,8,43] One recent study found that lacteal height, width, and height/width ratio were inversely correlated with serum albumin concentration in a group of dogs with PLE.[44]

Further investigation is needed to determine the etiopathogenesis associated with the development of hypoalbuminemia in cases of CE. An enhanced understanding of the reasons some dogs with CE develop hypoalbuminemia will likely lead to improved therapeutic protocols and outcomes in this population of dogs.

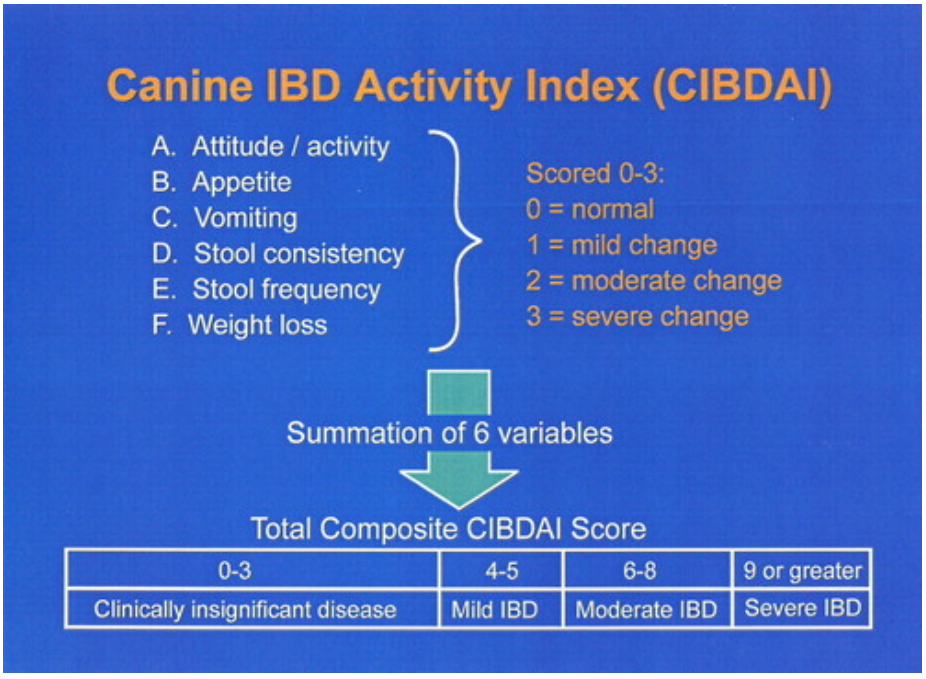

Figure 1.1 Criteria for determination of the canine inflammatory bowel disease (IBD) activity index (CIBDAI). From: Jergens AE,Schrreiner CA, Frank DE, et al. A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med 2003;17:291-297. Used with permission.

1.2 Histopathologic Findings in Chronic Enteropathy and Protein-Losing Enteropathy

In the last several decades, the widespread availability of flexible endoscopy has lead to intestinal biopsies becoming more practical and available to a larger group of clients and veterinarians. However, significant concerns have been raised about the value of histopathologic exam of intestinal tissue in determining the cause of the patient's clinical disease. First, despite the development of specific guidelines for interpretation[45], histopathologic evaluation of intestinal tissue remains largely subjective. In fact, in one study of 14 slides of intestinal tissue, nonuniformity of opinion among pathologists was detected in 7 of the slides. In this study interobserver variation was high, but also unpredictable.[46] Additionally, dogs expected to have normal intestinal tissue were receiving a histopathologic diagnosis of lymphoplasmacytic or eosinophilic enteritis, despite no clinical correlates to explain these findings.[47] Lack of agreement on what constitutes normal histology of the canine and feline intestinal tract is arguably the reason for the inaccurate diagnosis of intestinal inflammation in veterinary patients.[47] Finally, another concern with the availability of endoscopy and the subsequent diagnosis of idiopathic IBD in veterinary medicine is the tendency of practitioners to rely too heavily on histopathology to determine the cause of gastrointestinal (GI) illness in patients. In this scenario, it is possible that appropriate clinical trials such as dietary modification, deworming, and/or antimicrobial therapies are ignored in favor of intestinal biopsy. There is no clear distinction in the histopathologic changes found in dogs with FRE versus dogs with idiopathic IBD, so where appropriate, non-invasive therapeutic trials should always be performed prior to intestinal biopsy.

Furthermore, the quality of the endoscopic sample affects the histopathologic interpretation. Sample quality is affected by many things, including, but not limited to operator

experience for the detection of mucosal lesions and tissue and slide processing following the collection of the sample.[48]

Over the past 20 years multiple grading schemes have been developed for defining gastrointestinal inflammation in endoscopic samples.[7,9,49,50] These grading schemes largely emphasize the importance of the type and degree of laminar propria cellular infiltrate in the diagnosis of chronic gastrointestinal disease in the dog and cat. In comparison, the morphologic changes of the intestine have been relatively undervalued despite the fact that morphologic changes have been shown to correlate with clinicopathologic markers and clinical severity.[51] Based on the nonuniformity of the various grading schemes as well as the observation that gastrointestinal histopathologic interpretation varies between pathologists[46], the World Small Animal Veterinary Association (WSAVA) International Gastrointestinal Standardization Group published guidelines for the endoscopic, biopsy, and histopathologic definitions of gastrointestinal inflammation in 2010. These guidelines include scoring metrics for morphologic and inflammatory changes typical of the duodenal mucosa. Morphologic criteria that are scored include villus stunting, epithelial injury, crypt distension, lacteal dilation and mucosal fibrosis. Inflammatory criteria include intraepithelial lymphocytes, lamina propria lymphocytes/plasma cells, lamina propria eosinophils, and lamina propria neutrophils.[45] Despite these consensus guidelines the grading criteria for defining intestinal inflammation remain highly controversial.[51]

Another limitation of histopathologic evaluation of intestinal tissue for the diagnosis of CE is the consistent lack of correlation to clinical findings. Several different researchers at different institutions have attempted to correlate histopathologic findings, including type and degree of inflammatory infiltrate, to various clinical findings. Collectively, these studies have failed to find an association between histologic findings and clinical signs, clinicopathologic

biomarkers, response to various treatments, and outcomes.[1,6,8,52,53] Notably, despite the fact that the development of PLE secondary to CE is widely thought to indicate a more severe phenotype of CE, hypoalbuminemia has not been correlated with the type or severity of inflammatory infiltrate.[1,4,8] A large-scale study comparing the histologic lesions of dogs with CE with and without PLE has not been performed. Jergens and colleagues were able to find an association between histopathology and severity of a clinical activity index they developed for dogs idiopathic IBD,[42] however others were not able to repeat this observation.[54]

In addition to this, most investigators have been unable to find improvement in intestinal histopathology of dogs that have clinically responded to treatment for chronic GI signs.[1,52] It is possible that the inadequate quality of samples, lack of agreement on grading schemes, and inconsistency between pathologists is the reason histopathologic lesions have been unable to be correlated to clinical findings. However, it is perhaps more likely that the lack of correlation reflects our incomplete understanding of the disease processes resulting in chronic disease in the dog. Despite all the attempts at improvement and standardization, the current state of histopathologic evaluation of intestinal biopsies likely remains deficient in part due to the historic emphasis on the importance of the lamina propria cellular infiltrate.

Recent studies have highlighted the importance of morphologic changes to the intestine in dogs with CE. A group of dogs with FRE had improvement in the ultrastructural lesions in their intestine that correlated with clinical improvement despite a lack of significant improvement in the cellular infiltrates in their intestine.[55] A series of 58 dogs with CE found that dogs with crypt lesions were more likely to have severe protein loss and shorter survival times than dogs with no crypt lesions.[56] A recent study evaluated 94 dogs with lymphoplasmacytic enteritis (LPE) found that lacteal height, width, and height/width ratio were inversely correlated

with serum albumin.[34] Collectively, these studies suggest that the morphologic changes to the intestine may be at least as, if not more important than the lamina propria cellular infiltrate in the evaluation of intestinal tissue in dogs with chronic GI signs.

Lacteal dilation is a particularly important morphological criterion to examine, especially in cases of PLE. Previous studies have found varying incidence of lacteal dilation in histopathologic evaluation of intestinal tissue from dogs with chronic GI signs, ranging from 21% to 59%.[57, 58] In one study,[44] 100% of dogs with chronic GI signs and lymphoplasmacytic enteritis had concurrent moderate to severe lacteal dilation. The discordancy between studies might be explained by the fact that lymphangiectasia can be segmental, and in some cases, confined to deeper layers of the intestine that may not be sampled in endoscopic biopsies.[59] It is also likely that despite specific guidelines for interpretation, pathologists differ in their assessment of lacteal dilation. Unfortunately, gross endoscopic exam is not specific and only moderately sensitive for the identification of lymphangiectasia in dogs.[59] Other tissue-based methods of identifying and evaluating intestinal lymphatics may prove useful in making the assessment of lacteal dilation more consistent.

1.3 The Role Of Diet In The Pathogenesis And Therapeutic Management Of Chronic Enteropathy And Protein-Losing Enteropathy

1.3.1 Diet and the Pathogenesis of Chronic Enteropathy

Evidence that diet plays a role in the pathogenesis of canine CE is supported by a handful of prospective clinical trials[60-62] but is reinforced by overwhelming anecdotal clinical response to dietary alterations. In most cases, a dietary hypersensitivity and subsequent aberrant immune response is suspected.[11] Dietary hypersensitivities are defined by an adverse immune reaction

elicited by a particular food substance, most often a protein, and may be IgE-mediated, cell-mediated or mixed.[11] Nonimmunologic reactions, known as food intolerance, can also occur. Food intolerance may be caused by a pharmacologic reaction, metabolic problems, or be idiosyncratic.[11] Gluten-sensitive enteropathy of Irish Setters is an example of an idiosyncratic food intolerance. It is characterized by chronic intermittent diarrhea and poor weight gain in young Irish Setters fed a diet containing gluten, and is inherited as an autosomal recessive trait.[63] Some dogs respond to an elimination diet trial but do not experience recurrence of their clinical signs after re-introduction of their original diet, which suggests that they are benefitting from other advantageous properties of the diet.[60] Those properties may include higher bioavailability of nutrients, prebiotics supplemented in the diet, or an optimized n3:n6 fatty acid ratio,[11] among others.

The impact of diet on the development of human IBD is suggested by epidemiological studies. There has been a rapid increase in the incidence of human IBD in countries that have experienced Westernization, which implicates environmental factors in the pathogenesis.[64,65] One study revealed that immigrants who move from a country of low incidence to regions of higher incidence have the same risk of developing IBD as children of families who have resided in that region for many generations.[66] Furthermore, a case control study of Canadian children evaluated multiple dietary patterns and the risk of developing CD. Female children who ate a typical Western diet (meats, fatty foods and desserts) had an increased risk of CD and the consumption of fish, grains, nuts, olive oil, vegetables, and fruits were associated with a decreased risk of developing CD in both male and female children.[67] A number of theories have been suggested to explain the pathophysiological mechanisms behind the importance of diet in the development of IBD. First, certain foods and nutrients have been linked to mucosal

inflammation. For example, studies have shown that mice fed a high-fat diet have increased small intestinal or colonic inflammation.[68] The consumption of refined grains, red meat, and sugary desserts have been correlated with increased fecal calprotectin levels, a marker of mucosal inflammation.[64] Second, diet is known to have a pivotal impact on the microbes and microbial metabolites of the gut.[69] Because the gut microbiota is thought to have a crucial role in the development of IBD,[69,70] diet, through its influence on the microbiota, may influence the risk of developing IBD. Additionally, dietary components, including food additives can affect the mucus layer of the intestine and can influence mucosal barrier function. For example, carrageenans, used to thicken dairy products, sauces and incidentally, canned dog food, have been shown in in-vitro cell models to result in irregular tight-junction expression, leading to increased intestinal permeability.[71]

Additional evidence for the role of diet in the pathogenesis of human IBD is supported by the fact that most human patients believe their symptoms worsen when they eat certain foods, and food frequency surveys have identified intake of certain foods to be associated with a flare of disease.[72]

1.3.2 Diet in the Therapeutic Management of Chronic Enteropathy

Principles of dietary therapy of canine CE include reduction or restriction of exposure to antigens suspected to evoke sensitivity, improving nutrient absorption, and improving appetite.[5] Multiple studies have demonstrated a clinical response to diet change alone in cases of canine CE. In one of the earliest studies performed, 4/6 (67%) dogs with refractory CE had clinical improvement in response to a hydrolyzed protein diet.[62] In a prospective clinical trial, 39/65 (60%) dogs with idiopathic chronic diarrhea responded to a salmon and rice diet administered for

10 days.[60] In a retrospective examination of 203 cases of CE, 64% of cases were found to have food-responsive enteropathy (FRE). Among the dogs with FRE, elimination/novel diets (new protein and carbohydrate source), hydrolyzed diets (proteins broken down into peptides to reduce antigenic reaction), and home-cooked diets were utilized. This study found no difference in clinical responses between the diets used.[3] Taken together, these studies suggest that over 60% of dogs with CE will respond to a dietary change, and that to date there is no evidence-based benefit of using a hydrolyzed diet versus a novel protein diet. In some cases, a non-exclusion but easily digestible diet is attempted in cases of CE. One positively controlled field trial compared the use of a hydrolyzed protein diet to a non-exclusion, easily digestible diet in dogs with chronic small bowel enteropathies and serum albumin >2.0 g/dL. Both groups of dogs had good initial response to their diets, however, after a 3-year period only 1 of 6 dogs on the non-exclusion diet had clinical remission, versus 13 of 14 dogs on the hydrolysate. This study suggested that long-term control is more likely in dogs fed hydrolyzed diets when compared to those fed easily digestible, non-exclusion diets.[61] Finally, there is also some evidence that modifying the n3:n6 fatty-acid ratio in the diet can reduce the production of pro-inflammatory metabolites, thereby modulating the inflammatory response.[12]

Most veterinary studies primarily evaluate clinical response to therapy. However, in humans with IBD evidence of mucosal healing and histologic resolution of disease are becoming increasingly important in cases of IBD. A group of investigators set out to look at pathological changes in duodenal biopsies in 20 dogs with FRE. The investigators examined immunohistochemical, ultrastructural and histopathological changes in the duodenum before and after treatment. There was a significant improvement in ultrastructural lesions within 6 weeks of a diet change in this group of dogs, suggesting that brush border healing can occur with a diet

change alone in cases of CE. In this study no significant change was seen in the overall histological score following treatment, which may suggest that the ultrastructural lesions are more clinically significant than histological scores, or that more time may be needed to see resolution of inflammatory infiltrates.[55]

In short, the above handful of studies and an abundance of anecdotal evidence suggest that in many cases of canine CE a dietary trial should be considered as the first therapeutic approach unless contraindicated due to the clinical status of the patient.

Additionally, diet as a therapeutic strategy is receiving renewed attention in the management of the inflammatory bowel diseases of humans. The growing consensus that nutrition and diet play a role in the etiopathogenesis of IBD has renewed interest in the use of diet for treatment.[68] Exclusive enteral nutrition (EEN) with elemental, semi-elemental or formula diets has been the most widely studied dietary intervention in cases of IBD in human.[67] EEN been used to induce remission in new-onset CD, in children or adults with malnutrition perioperatively, and as a mainstay of therapy in pediatric CD in some parts of the world.[73,74] A variety of studies have demonstrated that EEN may induce remission in 60-86% of children with CD.[75] In pediatric patients with CD, a randomized controlled clinical trial showed that both glucocorticoids and EEN reduced symptoms, but only patients treated with EEN had evidence of mucosal healing.[73] EEN has also been associated with higher remission rates and better growth compared to glucocorticoids,[76,77] but has not shown to be superior in regard to relapse or long-term outcomes. Additionally, several studies have shown that tumor necrosis factor (TNF) antagonists are more effective when used in conjunction with EEN versus when they are used alone.[78]

Despite a plethora of evidence of benefits to human patients with IBD, the therapeutic use of EEN presents significant challenges to both the physician and patient. It should be noted that many of the studies that show benefits of EEN were performed in children.[68] Studies of adults have shown more variable results, which is suggested to be due to poor compliance to the therapy. Patients complain about the monotony of the diets, and when nasogastric tubes are needed for administration, some patients start to refuse therapy.[68] Further, many adults have trouble sticking to strict dietary recommendations, and discontinuation of dietary trials is common. Fortunately, even when only half of the daily calories are supplied by an elemental diet, benefits have still been seen in patients with CD.[79] In the last five years a variety of dietary therapies have been developed and recommended to patients with IBD, in an attempt to mimic the benefits of EEN while still allowing access to whole foods.

In order to develop dietary approaches for treatment of IBD that mimic the positive effects of EEN, it would be helpful to understand the mode of action of EEN. However, the exact mechanism central to the therapeutic response to EEN is unknown. Elemental formulas are fully hydrolyzed, therefore a low antigenic load has been suggested to be the mechanism underlying the response to EEN. One study showed that partially hydrolyzed formulas were equally effective to fully hydrolyzed ones, suggesting that antigenic load may not be the only factor.[80] It is important to keep in mind, however, that even partially hydrolyzed diets likely contain substantially less antigens than typical table food of a Western diet. Other possible mechanisms include influence of diet on the microflora, downregulation of mucosal pro-inflammatory cytokines, and the exclusion of foods that have been associated with an altered mucosal barrier (e.g. high fat foods, high sugar foods, gluten, food additives).[81]

The Crohn's disease exclusion diet excludes wheat, dairy and many food additives, and is low in animal fat, rich in complex carbohydrates and has a moderate amount of soluble fiber. In 21 children and adults with active clinical disease after loss of response to combinations of pharmaceuticals despite dose escalations, 61% achieved clinical remission and had a drop in inflammatory markers following adherence to this diet.[82] Other diets that have been developed include the specific carbohydrate diet (SCD), IBD anti-inflammatory diet (IBD-AID), a diet low in fermentable oigo-, di-, and monosaccarides and polyols (FODMAPs), and the Paleolithic diet. Small retrospective and prospective studies have generally shown positive effects of many of these diets, with the exception of the Paleolithic diet which has not been evaluated in a clinical research study.[68]

In the treatment of IBD, induction of remission is desired, but maintenance of remission is the ultimate goal. Dietary therapies show promise in their ability to help maintain homeostasis and prevent flares of symptoms in cases of IBD in humans. One study followed twenty postoperative adults with CD. Patients treated with nightly elemental diet and a low fat diet during the day were followed for 5 years. Sixteen of them maintained the diet. Only 2 of the 16 had disease recurrence compared to 9 of 20 in the normal-diet control group.[83]

In summary, dietary therapies are gaining significant traction as an important component of the management of human IBD, but mechanisms remain poorly understood as control of dietary studies in patients with IBD remains challenging. However, diet has emerged as a promising complementary therapy and future studies will likely help in clarifying the role of diet in the therapeutic management of IBD in humans, which may also be translatable to veterinary patients.

1.3.3 Diet in the Therapeutic Management of PLE

In all human patients with PLE, regardless of the cause, a high-protein diet is recommended. In order to achieve a positive protein balance in patients with PLE, protein requirement is typically 3-6 times that of a healthy individual. Increasing the protein intake is usually achieved through diet alone, but in some cases protein supplements are also deemed necessary.[29] In many cases of PLE in humans, a low fat diet is also recommended. A strict low fat diet is strongly recommended in cases of congenital intestinal lymphangiectasia, but use of a low fat diet is advocated in many different causes of PLE. For example, dilation of the lymphatics is common secondary to CD, and low fat diets diminish the need for lymphatic transport of fatty acids, thereby decreasing stress on the lymphatic system and reducing lymph leakage.[28] Furthermore, several animal models have demonstrated that high fat diets promote an inflammatory state, so in cases of IBD resulting in PLE, low-fat diets are often encouraged.[84] In humans with PLE, supplementation with water-soluble vitamins is preferred, and exclusion of long-chain fatty acids may also be recommended.[28]

Dietary alteration is considered one of the main components of therapy in dogs with PLE. Dogs with PLE are in a catabolic state, and may have marked negative energy and protein balance so adequate nutrition is essential.[25] Furthermore, treatment of the underlying disease causing PLE typically relies on alterations to the diet. For example, in a retrospective study of 24 dogs with intestinal lymphangiectasia that had either not responded to prednisolone or had relapsed once their prednisolone dose was decreased, 19/24(79%) responded satisfactorily to dietary fat restriction, allowing for their prednisolone dosage to be decreased without relapse.[85] A recent retrospective review found that some Yorkshire Terriers with presumptive or confirmed

PLE can be successfully managed with diet without the use of concurrent anti-inflammatory or immunosuppressives.[26]

While a low fat diet is recommended for dogs with intestinal lymphangiectasia, a novel protein, hypoallergenic, or hydrolyzed diet are often suggested for dogs with histologic evidence of inflammatory infiltrates. Therefore, difficulty may arise when managing the patient with histopathologic evidence of both inflammatory enteritis and lymphangiectasia. Prospective controlled clinical trials to evaluate the efficacy of various diets for the treatment of canine PLE have not been performed.

1.4 Overview of Intestinal Lymphatics

1.4.1 Intestinal Lymphatics in Health

Lymphatics are essential to the maintenance of homeostasis in the gastrointestinal system. They perform crucial transport and immune regulatory functions. Errors in lymphatic development and/or alteration of lymphatic flow frequently disturb GI functioning and physiology. Despite their critical functions, general understanding of lymphatic biology is lacking compared to vascular biology. This is due in part to the historical difficulties in visualizing and studying lymphatics, and a lack of appreciation for the importance of their functions. However, lymphatic biology is currently experiencing a renewal in interest in diseases outside of cancer and overt lymphatic disorders. Because the GI tract is highly dependent on adequate lymphatic functioning, the study of lymphatics in acute and chronic gastrointestinal diseases has been increasing.[86]

In the intestine, lymphatic vessels are responsible for the absorption of interstitial fluid, and are also the main route of absorption for fat, cholesterol, fat-soluble vitamins, gut hormones,

and food antigens. Lymphatic vessels are also central to the transport of intestinal immune and inflammatory cells including innate lymphoid cells and dendritic cells. Additionally, the lymphatic system has been shown to modulate the immune response as interactions between lymphatic endothelial cells (LECs) and leukocytes have been demonstrated to influence immune cell migration.[87-88]

The lymphatic vasculature is organized into initial lymphatic capillary networks and collecting lymphatic vessels. Interstitial fluid formed in tissues is first collected into initial blind-ended, permeable lymphatic capillaries that lack smooth muscle, are non-contractile, and lack valves. Initial lymphatic vessels are often attached to connective tissue within organs. Collection of fluid into the initial lymphatic capillaries is facilitated by lymphatic endothelial cells, which have overlapping flaps that serve as unidirectional valves during the filling. In the intestine, the lacteals draining individual villi connect with a network of lymphatics in the mucosal layer, and a seemingly non-connected network of lymphatics exists within the muscularis layer of the mucosa. Both systems of lymphatics drain into the lymphatic collecting vessels near the mesenteric border of the intestine. The lymphatic collecting vessels, which do contain smooth muscle or valves, then transport lymph through lymph nodes and eventually to the thoracic duct and back into blood circulation. These contractile units of lymphatics are known as 'lymphangions', which are individual segments between two adjacent valves, and are arranged in series in order to propel lymph forward.[86-88]

Normal lymphatic pumping is influenced by both extrinsic and intrinsic factors. Extrinsic factors include contraction of skeletal muscle, respiratory movements, pulsations of nearby blood vasculature, and variations in central venous pressure. Intrinsic factors also regulate pumping of lymphangions and some are increased after the consumption of a fat-containing meal.

Cholecystokinin, glucagon, endothelin, bradykinin, substance-P, serotonin, dopamine, and other substances are known to increase lymphatic pumping. Nitric oxide, anti-diuretic hormone, vasoactive intestinal peptide, prostacycline, acetylcholine, reactive oxygen metabolites, and cytokines are known to decrease lymphatic pumping. Notably, oxygen levels are relatively low in lymphatics, similar to venous blood. Although this low level is sufficient to support routine lymphatic pumping, the low oxygen tension may leave lymphatics susceptible to injury during ischemic stress.[86,89]

Lymphatics are the primary transporter of lipid and lipid-soluble substances from the intestine to the blood. Lipid is packaged into triglyceride-containing chylomicrons and transported first into the initial lymphatics and then through the rest of the lymphatic network as described previously. It is not well understood exactly how lipid enters the initial lymphatic vessels, both passive diffusion and active uptake of lipid vesicles into lymphatic endothelial cells have been proposed, and evidence exists for both theories. The specific molecular mechanisms that regulate the uptake of chylomicrons into lacteals have not been identified, however, it is known that if lymphatics are absent or non-functional, this task cannot be compensated for through portal blood absorption.[90]

1.4.2 Intestinal Lymphatics in Disease

In humans, various defects of GI lymphatics, including remodeling, obstruction, pump dysfunction, and changes in structure and pattern can result in or exacerbate several GI conditions. When the intestinal lymphatic system fails to function properly, lymphatic clearance will be inadequate, edema and dilated lymph vessels (lymphangiectasia) will occur, and immune cells can accumulate in the mucosa and submucosa.[86]

Intestinal lymphangiectasia (IL) in dogs is defined as a dilation of lymphatic vessels in the gastrointestinal tract. It can be a primary process, but is more typically a secondary process. Mechanisms known to result in IL include increased hydrostatic lymphatic pressure secondary to inflammatory or neoplastic intestinal mucosal infiltrates or increased venous pressure at the level of the thoracic duct which may be secondary to pericarditis, pericardial effusion, or right-sided heart failure.[25,59,91] Intestinal lymphangiectasia as a primary (idiopathic or congenital) process has been associated with certain dog breeds, including the Norwegian Lundehund, Yorkshire terrier, Rottweiler, Shar-Pei and Maltese.[25] Histologic evaluation in cases of IL often reveal variable degrees and types of inflammatory infiltrates, as well as other lesions including crypt abscesses, dilated crypts, and cystic crypts.[59] Because leakage of lymph from dilated or non-functional lymphatic vessels can result in an inflammatory reaction in the intestine, and inflammation can result in dysfunctional and dilated lymphatics the distinction between primary and secondary lymphangiectasia can be difficult.[91,92]

Intestinal lymphangiectasia can be diffuse or segmental, and can affect lymphatics at the villus level as well as in the deeper parts of the intestinal wall, such as the submucosa, muscularis, and serosal segments. Mesenteric lymphatics can also be affected. Definitive diagnosis of IL is via histopathology, however the ability to diagnose it can be limited if the disease is segmental or confined to the deeper parts of the intestine, especially if biopsies are obtained endoscopically.[57,59] The gross appearance of the mucosa is only moderately sensitive and not specific for the diagnosis of IL, and histopathologic exam is needed to confirm IL.[59]

The importance of lacteal changes in dogs with idiopathic IBD has been investigated in a small number of studies. One study investigated the significance of 'white spots' in 50 dogs with lymphoplasmacytic enteritis (LPE).[93] White spots are thought to represent intestinal villi

distended with lymph and are visualized during gross endoscopic examination. Dogs with white spots were more likely to be hypoproteinemic than dogs without white spots, and there was a significant correlation between the presence of white spots and histologic lymphatic dilation scores.[93] In another study, 100% of dogs with LPE showed histologic evidence of lacteal dilation and the height, width, and height/width ratio of the lacteals were inversely correlated with serum albumin levels.[44]

A seemingly discrete form of lymphangiectasia known as focal intestinal lipogranulomatous lymphangitis has been reported in a small number of dogs.[94,95] These cases are characterized by the presence of a small intestinal mass (typically located in the distal jejunum or ileum), often with the involvement of the adjacent mesentery, and histopathology of the mass reveals transmural granulomatous inflammation with extensive lipogranulomas mainly involving the muscularis, serosa and mesentery.[94,95]

Finally, intestinal lymphatic hypoplasia, a documented cause of PLE in human infants, has recently been reported in 3 dogs with PLE.[96] Small intestinal specimens from these 3 dogs showed a lack of immunohistochemical labeling of villous lacteals when compared with normal controls. The prevalence and significance of these changes needs further study.

Several syndromes associated with dysfunctional lymphatics have been identified in humans, with variable affects on the intestinal lymphatics.[86] These include Noonan syndrome, a congenital condition of lymphatic dysplasia,[97] and Nonne-Milroy syndrome, a hereditary form of lymphedema.[98] Hennekam's syndrome is a congenital autosomal recessive lymphedema, and IL is commonly present, as well as pleural lymphangiectasia and facial abnormalities.[99] Hypoplastic lymphatics resulting in poor lymphatic drainage is the suspected etiology of Yellow Nail syndrome. IL and PLE have been reported in Yellow Nail syndrome.[100]

Alteration to the intestinal lymphatics has been described in a variety of primary GI diseases of humans. Primary intestinal lymphangiectasia (PIL) in humans, eponymously referred to as Waldmann's disease,[101] is characterized by bilateral lower limb edema, nausea, abdominal pain, and diarrhea. PIL results from malformed lymphatic vessels and is generally diagnosed prior to 3 years of age. It is recognized histologically as distended and obstructed lymphatics and lymphopenia and hypoalbuminemia are common features.[29] Intestinal lymphatic hypoplasia is a congenital condition typically recognized in infancy. Histologically, this disease presents as significantly fewer lymphatics than normal and without obvious lymphatic obstruction or lymphangiectasia. Lymphopenia is typically not observed with this condition.[102]

Diseases that result in secondary lymphangiectasia in humans are numerous. Similar to dogs, they include pericarditis and congestive heart failure, as well as other cardiac conditions.[86] Abnormalities of the lymphatics can develop secondary to a procedure known as the Fontan operation, which is a cardiac surgical procedure to treat children with only a single effective ventricle. Elevated venous pressure and subsequent dilation of the lymphatics is the suspected mechanism resulting in IL following the Fontan procedure.[103] Obstruction of the lymphatics by sarcoidosis and lymphoma can also result in IL.[28] Lymphatic alterations are an important consequence of inflammation, therefore secondary lymphangiectasia can be seen in human IBD.[104]

As early as 1935, lymphatic abnormalities have been recognized as being associated with intestinal inflammation. Some gastroenterologists have even suggested that lymphatic dysfunction and lymphangitis may be the root cause of CD. Early pathologists studying CD repeatedly commented on alterations to the lymphatics including obstruction, generalized distension, and edema.[105] Furthermore, early experimenters found that when they obstructed

short segments of regional lymphatics in the intestine of pig and rats segmental intestinal disease histopathologically similar to what is seen in Crohn's disease developed.[106,107] More recently, the ablation of lymphatics in a mouse model resulted in mucosal barrier injury and allowed for the invasion of intestinal pathogens and subsequent intestinal inflammation, revealing a defensive role of the lymphatic vessels and suggesting intestinal inflammation could start with injury to the lymphatics.[108] The importance of functional lymphatics was also highlighted by a study in which lymphatic insufficiency was induced in mice, after which they were allowed to survive for 1.5 years. Following the 1.5 years in which only basal inflammation in the small intestine was noted, acute enteritis was promoted with indothelin. Mice with lymphatic insufficiency had persistent inflammation and morphologic changes to their intestine following recovery when compared to control mice.[109]

It is also understood that lymphatic abnormalities can be a consequence of intestinal inflammation, playing a role in the disease course of IBD. Remodeling of the lymphatic vasculature including growth of new initial lymphatics (lymphangiogenesis) has been associated with inflammation. Inflammation-induced lymphangiogenesis is mediated by vascular endothelial growth factor (VEGF), and occurs in both the draining lymph node and the inflamed tissue (Figure 1.2).[104] For example, in mice with experimental ileitis, lymphangiogenesis and lymphadenopathy persisted despite the apparent resolution of intestinal inflammation, leading investigators to speculate whether alterations of the intestinal lymphatics could play a role in the recurrence of CD.[110] Failure of lymphatic pumping can be also be induced by intestinal inflammation, which can lead to edema and lymphangitis.[111] Lymphangiogenesis is seen in both CD and UC, and has often been found in association with regions of fibrosis in the intestine.[112,113] In the case of UC, expansion of lymphatics into deeper portions of the intestine

where they are not normally found has been observed.[113] While lymphangiogenesis has been well described in both experimental and clinical IBD, it is unknown whether the proliferation of lymphatics is adaptive or maladaptive, and whether excess lymphatics are functional. This would be an important distinction, as if lymphatic remodeling and regenerating is beneficial, this could be an important target for therapy in cases of IBD.[114]

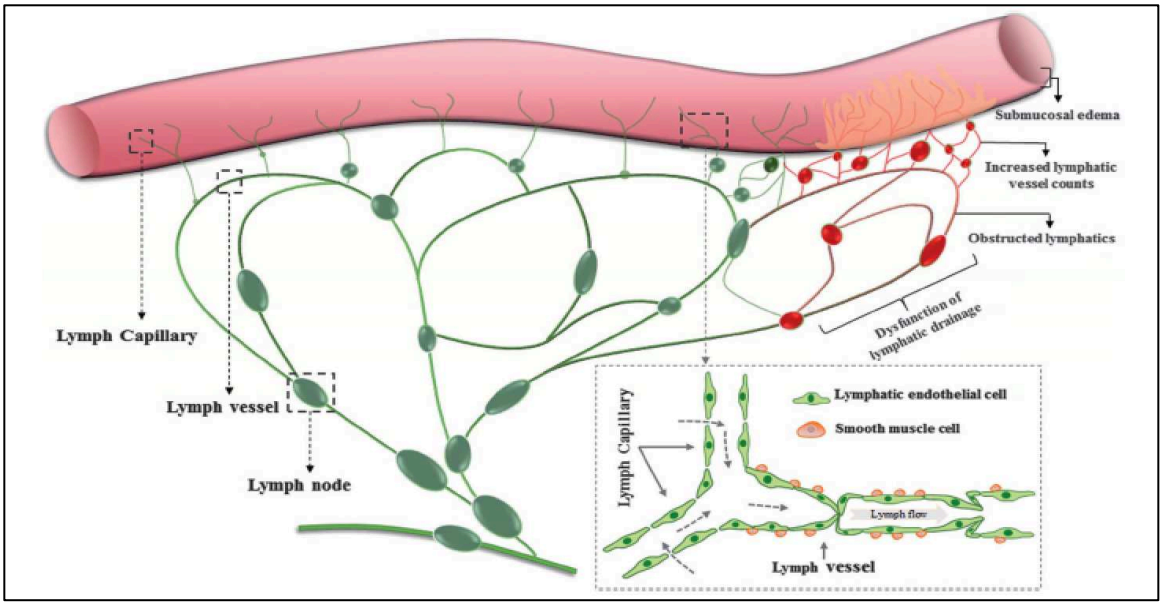

Figure 1.2 Lymphatic remodeling, obstruction, and associated lymphangiogenesis in humans with CD. Decreased drainage may result in lymphangiectasia, submucosal edema, and the accumulation of antigens and immune cells in the intestinal wall. From: Li Y, Zhu W, Zuo L, et al. The role of the mesentery in Crohn's disease: The contributions of nerves, vessels, lymphatics, and fat to the pathogenesis and disease course. Inflamm Bow Dis 2016;22:1483-1495. Used with permission.

1.4.3 Lymphatic Endothelial Cells

Lymphatic endothelial cells (LECs) are derived from venous progenitor cells.[115] The expression of 'lymphatic markers' differentiates LECs from blood endothelial cells. Lymphatic markers are numerous but include Prox-1, a nuclear transcription factor, vascular endothelial growth receptor-3 (VEGFR-3), lymphatic vascular endothelial hyaluronic acid receptor (LYVE-1), podoplanin, cadherin-13, and ZO-2.[86] Prox-1 is expressed by endothelial cells that stem from

primitive veins during embryogenesis and control lymphatic differentiation via a number of mechanisms.[116] Blood endothelial cell transcriptions are suppressed by Prox-1. Additionally, Prox-1 drives VEGFR-3 expression which permits LECs to respond and migrate towards VEGFR-3 ligands. Binding of some of these ligands appears to be essential for prenatal lymphatic patterning and post-natal control of lymphangiogenesis.[86] LYVE-1 is almost exclusively expressed in well-differentiated lymphatic vessels with no expression in blood vessels. It is a cell surface receptor for the extracellular matrix glycosaminoglycan hyaluronan (HA). After being rapidly degraded, HA is transported through the lymphatic networks to distant lymph nodes.[117]

1.4.4 Identification and Evaluation of the Form and Function of the Intestinal Lymphatic Vasculature

Historically, the lymphatic system has been considered difficult to visualize, which is one of the reasons why less is known about lymphatic biology when compared to vascular biology. Recently, a variety of techniques have been developed to better study the lymphatic system, including the development of lymphatic markers, as well as methods to study lymphatic function.

Immunohistochemical (IHC) markers, such as antibodies to podoplanin, Prox-1, and LYVE-1 have been developed in order to label lymphatics to make them readily identifiable in tissue. Since their development these markers have been used in human medicine to differentiate vascular tumors from lymphatic ones, identify lymphatic invasion into various tumors, study methods of lymphatic metastasis, and identify dysfunction of lymphatic development and proliferation in a variety of disease settings.[115]

With the help of IHC, lymphangiogenesis has been identified in chronic inflammation of the GI tract in humans, including in cases of CD and UC. Intestinal lymphatics have been shown to proliferate and distribute to every layer of the bowel.[112,113,118,119] One study evaluated 22 patients with CD and 16 with UC versus 11 controls. Quantification of lymphatic vessels was performed with IHC, and lymphatic vessel density was increased in both inflamed and non-inflamed tissues when compared with controls.[120] In addition, using IHC, investigators have attempted to correlate lymphatic density with disease behavior and post-operative recurrence in cases of CD with varied results. One group found that decreased lymphatic density and expression of Prox-1 in the mucosa of patients with CD was correlated with increased risk of post-operative endoscopic recurrence.[121] Another group specifically investigated the mesenteric lymphatic vasculature in patients with CD, and found that increased mesenteric lymphatic density was correlated with disease behavior, bowel wall thickness, and clinical recurrence of disease.[122] Despite the difference in their findings, both groups concluded that further investigations are warranted and a better understanding of the lymphatic systems role in the possible pathogenesis of CD could lead to novel therapies.

Lymphatic IHC markers have also been used to help differentiate cutaneous angiosarcomas in dogs,[123,124] and for definitive identification of lymphangiosarcoma in cats.[125] Prox-1 and LYVE-1 have been validated in canine tissues for this purpose, and in one study, lacteals from canine small intestine were immunolabeled to be used as positive controls.[123] Prox-1 was also recently used to identify three dogs with PLE and suspected intestinal lymphatic hypoplasia.[96]

Lymphatic function studies are very challenging to carry out in the clinical setting. In guinea pigs with experimental ileitis the contractile activity of lymphatics was found to be

impaired compared to sham-treated controls. Additionally, lymphatic dysfunction was well correlated with the degree of inflammation in the intestine.[126] Another group used the same animal model and duplicated these results, and then treated the guinea pigs with a non-selective COX inhibitor or a combination of COX-1 and COX-2 selective inhibitors. They found that lymphatic pumping returned to the level of the controls, suggesting a role for prostanoids in lymphatic contractive dysfunction in association with intestinal inflammation.[127] Whether lymphatic pumping dysfunction correlates with impaired lymph flow, removal of edema fluid, trafficking of immune cells, and/or movement of inflammatory cells is unknown but further study could lead to the development of new therapeutic approaches in IBD.

1.5 Hypovitaminosis D and Inflammatory Bowel Disease

1.5.1 Vitamin D Metabolism in Health

Vitamin D is a fat-soluble secosteroid hormone that has a well-understood role is in the regulation of bone, calcium, and phosphorus metabolism. In humans, vitamin D is present in two main forms, vitamin D2 (ergocalciferol, from plant sources, fish that feed on plants, and fortified foods) and D3 (cholecalciferol, from animal sources).[128] Both forms are absorbed from the diet in the small intestine. Dietary vitamin D is incorporated into chylomicrons and transported via lymphatics into the venous circulation. D3 is also endogenously synthesized in the epidermal layer of the skin in response to ultraviolet (UV) light. The suns UV rays convert 7-dehydrocholesterol to pre-vitamin D which subsequently undergoes swift thermal isomerization to vitamin D3 (Figure 1.3). Both forms are used in the body, however, it is thought that metabolites from D3 are far more potent than those of D2.[129] UV-mediated production of vitamin D is generally understood to be inconsequential in dogs, thus dogs are thought to obtain most if

not all of their vitamin D from diet (Figure 1.4). This is based on studies that have demonstrated little or no 7-dehydrocholesterol in the skin of dogs, and the experimental induction of rickets in puppies fed vitamin-D absent diets despite their exposure to UV light.[130] Increased activity of 7-dehydrocholesterol-Δ7-reductase has been demonstrated in the cat, and is thought to be responsible for the limited production of vitamin D in the skin in this species.[131] This phenomenon has not been studied in the dog.

Following endogenous synthesis (human) or intestinal absorption (human and dog), vitamin D is transported to the liver by carrier proteins, namely vitamin D binding protein (VDBP), and metabolized by cytochrome p450 enzymes 25-hydroxylases to form 25-hydroxy-vitamin D (25[OH]D). 25(OH)D is the major circulating metabolite, and is the most stable, with a circulating half-life estimated to be between 10 and 15 days. However, release from tissue stores results in an *in vivo* half-life of 2 to 3 months. It is also the form in which vitamin D is stored in adipose tissue and the liver.[132] 25(OH)D is accepted as the main marker of vitamin D status; however, it is not the active form. In the proximal tubule of the kidney, 1-α-hydroxylase converts 25(OH)D to the active form, 1,25-dihydroxy-vitamin D($1,25[OH]_2D$) (Figure 1.4). This last step is tightly regulated by fibroblast growth factor and parathyroid hormone (PTH) in response to serum calcium and phosphate levels.[129] Once in the active form, vitamin D can exert its effects though its receptor, vitamin D receptor (VDR). Its biologic effect is exerted through the traditional activation of transcription, and also through more rapid-acting membrane bound receptors. 25(OH)D and $1,25(OH)_2D$ are converted to inactive metabolites by the catabolic enzyme 24-hydroxylase and through a multi-step pathway to a water-soluble substrate which can undergo urinary and biliary excretion.[132]

Vitamin D metabolites are primarily transported through circulation bound to VDBP (85-90%), and to a lesser degree albumin (10-15%). Less than 1% of vitamin D is in the free form in circulation.[132] Although its main function is in binding, VDBP has also been found to have roles in the extracellular actin scavenging system, transport of endotoxins, T cell responses, and chemotaxis, among others. Serum VDBP levels have been positively correlated with lipid parameters, such as triglycerides and total cholesterol. An increased urinary VDBP concentration has been documented in human microalbuminuric patients and in humans with non-diabetic chronic kidney disease with overt proteinuria.[133]

Notably, recently studies have revealed that production of $1,25(OH)_2D$ by 1-α-hydroxylase occurs in non-renal tissues, such as the skin and pancreas, and in cells, including intestinal macrophages and monocytes.[132] Furthermore, numerous cell types in various tissues express the VDR. In dogs, VDR expression was highest in the duodenum, skin, kidney, ileum and spleen, and weakly expressed in the colon, heart, lymph node, liver lung and ovary.[134] This suggests that vitamin D has actions beyond its classic endocrine role, and may be important in a wide range of physiological processes.

The main action of vitamin D is to increase serum calcium and phosphate through stimulation of intestinal calcium and phosphate absorption, mobilization of calcium from bone, and with the help of PTH, increasing distal tubular calcium reabsorption. It is also crucial to the maintenance of skeletal health through its actions of promoting osteoblast differentiation and mineralization leading to a net increase in bone density.[132] In addition to its traditional roles, vitamin D has more recently been found to have distinct immunological functions. Specifically, vitamin D has been found to enhance phagocytic and chemotactic responses of macrophages, to increase the production of antimicrobial proteins, downregulate the production of many pro-

inflammatory cytokines, including interleukin(IL)-1, TNF-α, IL-6 and IL-8, and increase the production of anti-inflammatory cytokines (IL-4, IL-10 and TGF-β).[129] Vitamin D and VDR status also regulates T-cell development, function, and balance. For example, when stimulated with 1,25$[OH]_2$D, CD4+ T-cells from patients with CD displayed increased production of IL-10.[135] Increased levels of vitamin D have also been demonstrated to cause apoptosis of activated Th1 cells.[136] Vitamin D has also been demonstrated to have a protective effect on the gut epithelial barrier, and to influence and modulate the gut microbiota.[137]

The discovery of the distinct immunological roles has generated a huge interest in vitamin D in a variety of disease states, including autoimmune conditions, cancer, infectious disease, diabetes, as well as Crohn's disease and ulcerative colitis.[138] Veterinary studies have evaluated vitamin D in the context of congestive heart failure,[139] renal disease,[140] cancer,[141] spirocercosis,[142] and canine CE and PLE.[143,144]

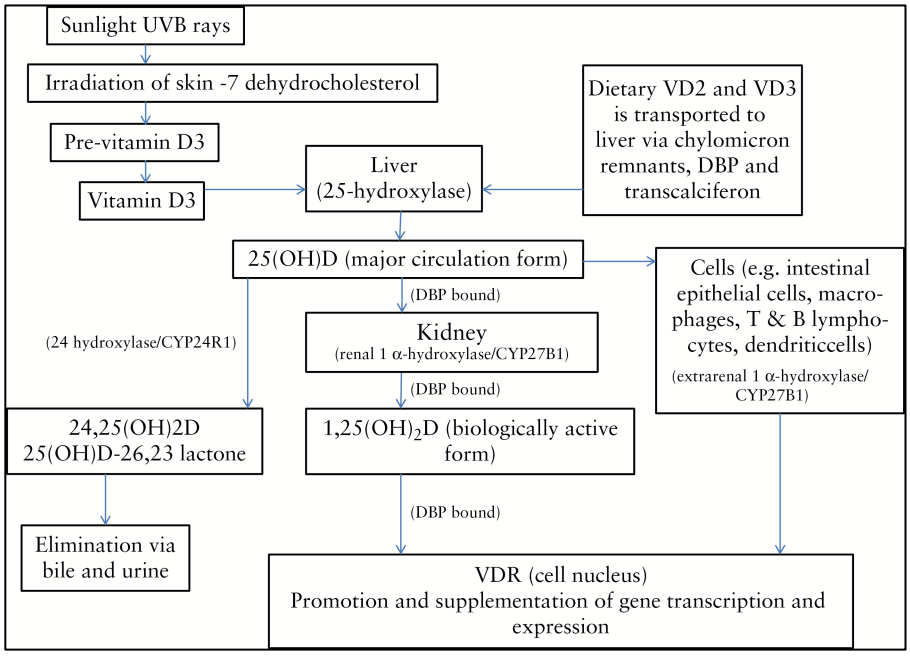

Figure 1.3 The synthesis and metabolism of vitamin D in humans. BDP: vitamin D-binding protein;VD2: vitamin D2;VD3: vitamin D3;VDR :vitamin D receptor; UVB: ultraviolet B rays. From: Nielsen OH, Rejnmark L, Moss AC. Role of Vitamin D in the natural history of inflammatory bowel disease. J Crohns Col 2018;12:742-752. Used with permission.

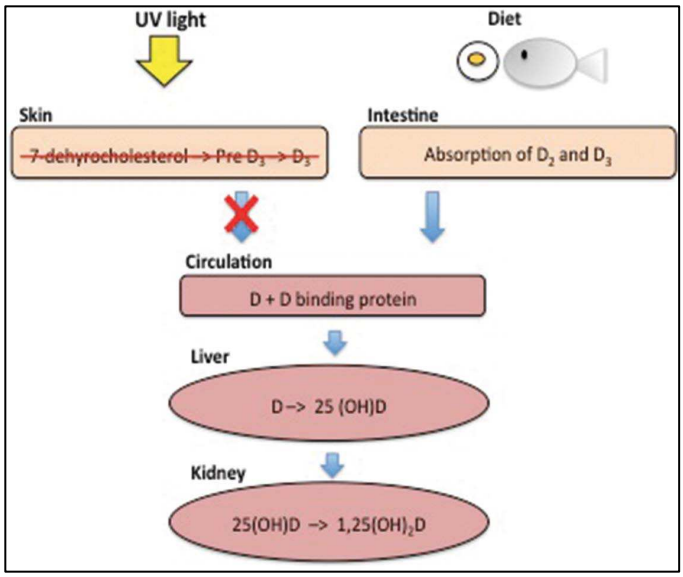

Figure 1.4 Basic schematic of vitamin D metabolism in dogs indicating the production of vitamin D in the skin of dogs is insignificant. From: Weidner N, Verbrugghe A. Current knowledge of vitamin D in dogs. Crit Rev Food Sci Nutr 2017;57:3850-3859. Used with permission.

1.5.2 Vitamin D and Inflammatory Bowel Disease

Considering the influence of vitamin D on the gut epithelial barrier, effects on the microbiota, and impact on immune function, a causal association between vitamin D and IBD has been suggested and investigated.[137] Vitamin D receptor knock out mice (VDR $^{-/-}$) have been demonstrated to be more susceptible to experimental forms of IBD compared to controls,[146] and feeding mice a diet deficient in vitamin D has been shown to predispose to IBD.[147] Furthermore, VDR $^{-/-}$ mice with experimentally induced colitis develop worse clinical disease when compared to wild-type controls.[148] Additionally, administration of exogenous vitamin D or a VDR agonist in a mouse model of IBD was shown to suppress colitis and reduce TNF- α levels.[149,150] Direct evidence linking vitamin D to the pathogenesis of IBD has not been found, but there is data supporting the relationship. In geographic regions where sunlight exposure and natural vitamin D synthesis are lower, the incidence of IBD is higher.[151] A Nurses' Health Study prospectively

collected data and found that a higher intake of vitamin D was associated with a lower risk of developing CD.[152] A direct link between the pathogenesis of CE and vitamin D status has similarly not been found in the dog, but the relationship between hypovitaminosis D and the development of GI disease has been suggested.[143]

A systemic review and meta-analysis of 14 observational studies with a total of 938 humans with IBD demonstrated that the prevalence of hypovitaminosis D was 38.1% in CD and 31.6% in UC. Vitamin D deficiency was defined as serum 25(OH)D ≤25 ng/ml. Both CD and UC had significantly higher odds of vitamin D deficiency when compared with controls. The prevalence among IBD patients was greater when compared with other groups at risk of vitamin D deficiency.[153] Other studies have found prevalence rates ranging from 16% to 95% and generally, an increased prevalence of hypovitaminosis D in cases of CD compared to UC.[129,154-156]

Vitamin D deficiency also appears to have an effect on clinical outcome in humans with IBD. Hypovitaminosis D was found to be an independent risk factor for hospitalizations and surgery in a retrospective review of 3217 patients with IBD in Massachusetts.[157] Increased IBD activity scores and lower quality of life scores have also been reported in vitamin D-deficient patients with IBD when compared to patients with IBD and normal vitamin D levels.[128] In another study performed over a 5 year period, 965 IBD patients were followed and categorized according to their mean serum 25(OH)D level. Patients with a low mean vitamin D required significantly more glucocorticoids, biologics, narcotics, CT scans, hospital admissions, emergency visits, and surgery compared to patients with normal vitamin D levels. In this same study, patients with low vitamin D had worse disease activity scores, pain, and quality of life. Additionally, the patients who received vitamin D therapy had a significant reduction in their

utilization of health care.[158] Correcting vitamin D levels may also be important in optimizing treatment with other therapies such as TNF-α inhibitors. A retrospective review of 384 patients with IBD treated with TNF inhibitors found that if IBD patients had a normal serum vitamin D level at the time of initiation of therapy they were 2.64 times more likely to reach remission within 3 months when compared to patients with low vitamin D.[159] Other studies evaluating the effect of vitamin D therapy in patients with IBD have shown variable results. In a placebo-controlled, randomized clinical trial, 94 patients with CD received vitamin D3 or placebo daily for 12 months. Clinical relapse rate was decreased from 29% to 13% in the subjects taking D3, although the difference was not statistically significant ($p=0.06$).[160] An association between intake of higher doses of vitamin D and reduced inflammatory markers, such as IL-6 and CRP has also been observed.[161] A few more clinical trials have been performed, but there are concerns with under-dosing of vitamin D preparations, variation in assays performed, and defined cut-offs for deficiency and target treatment levels. When higher target levels have been used, symptom-based activity scores improve significantly, but some of these studies have been underpowered and lack a control group.[129] Based on the prevalence, and links to disease activity, relapse, and poorer quality of life, the general consensus is that therapy with vitamin D is warranted in patients with IBD, but more work needs to be performed to determine the optimal therapeutic level and the exact effects of vitamin D in patients with IBD.

The mechanism of hypovitaminosis D in cases of IBD is not well understood and may be multifactorial. In humans, proposed mechanisms have included decreased exposure to sunlight, decreased intake, malabsorption, GI loss, and an active inflammatory state resulting in reduced hepatic production of VDBP and total vitamin D levels.[162] Exposure to sunlight and decreased intake have not been definitively proven as factors for hypovitaminosis D in IBD.[162] Intestinal

absorption of vitamin D was has been evaluated in a number of studies. Twelve hours after ingesting 50,000 IU of vitamin D2, circulating levels were significantly lower in CD compared with healthy controls; an approximately 30% reduction in the ability to absorb vitamin D was seen.[163] In other studies, intestinal absorption of vitamin D was normal in most patients with varying severities of IBD.[164] Loss of VDBP has been proposed as a theory for hypovitaminosis D, particularly in patients with hypoalbuminemia, since VDBP belongs to the same family of binding proteins.[165] One recent study found that VDBP levels were decreased in a cohort of children with IBD, compared to healthy controls, but no correlation was found with serum vitamin D levels.[166] Direct loss of vitamin D has also been proposed, although very small amounts of vitamin D actually circulate unbound. Finally, polymorphisms in the VDR have been investigated in humans with IBD with contradictory results.[162] Understanding the mechanism of hypovitaminosis D in some case of IBD may help reveal specific clinical phenotypes that can lead to a better understanding of IBD.

Notably, other fat-soluble vitamins, including vitamin A, E, and K have been found to be decreased in patients with CD compared to healthy controls. Additionally, patients with a longer course of disease were found to have lower levels of vitamins compared to those who were more recently diagnosed.[167]

Vitamin D deficiency has been well documented in dogs with chronic enteropathies and protein-losing enteropathy and several studies have found an association between low serum vitamin D levels and poor outcome. [41,143-145] Correlation between serum 25(OH)D levels and serum albumin have been inconsistent.[41,143-145] Low vitamin D status has been correlated with markers of systemic and gastrointestinal inflammation, including duodenal histopathology scores and serum IL-2 and IL-8 concentrations. A negative correlation with neutrophil and monocyte

counts has been reported.[168] Clinical trials evaluating the effects of vitamin D therapy in cases of chronic GI disease in the dog have not been performed. The mechanism of hypovitaminosis D in dogs with CE is even less well understood than in humans with IBD. Since dogs do not produce meaningful levels of vitamin D in response to sunlight, reduced dietary intake could be considered as an especially important factor in hypovitaminosis D in cases of CE. However, one study found that dogs with CE had significantly lower levels of 25[OH]D compared to ill hospitalized dogs who had reduced appetite, and so the influence of dietary intake remains unclear.[144] A comprehensive investigation into the mechanism(s) of hypovitaminosis D in dogs with chronic GI disease has not been performed. Considering the potential role of vitamin D in the pathogenesis, progression, and outcome of disease, further studies are warranted.

REFERENCES

1. Allenspach K, Wieland A, Grone A, et al. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med 2007;21:700-708.
2. Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? J Sm Anim Pract 2016;57:589-599.
3. Allenspach K, Culverwell C, Chan D. Long-term outcome in dogs with chronic enteropathies: 203 cases. Vet Rec 2016;178:368
4. Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. Vet Clin: Small Anim Pract 2011;41:381-398.
5. Jergens AE, Simpson KW. Inflammatory bowel disease in veterinary medicine. Frontiers in bioscience (Elite edition) 2012;4:1404-1419.
6. Schreiner N, Gaschen F, Grone A, et al. Clinical signs, histology and CD-3 positive cells before and after treatment of dogs with chronic enteropathies. J Vet Intern Med 2008;22:1079–1083.
7. Jergens AE, Moore FM, Haynes JS, et al. Idiopathic inflammatory bowel disease in dogs and cats: 84 cases (1987-1990). J Am Vet Med Assoc 1992;201:1603-1608.
8. Craven M, Simpson JW, Ridyard AE, et al. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). J Small Anim Pract 2004;45:336-342.
9. German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. J Vet Intern Med 2003;17:8-20.
10. German AJ, Hall EJ, Day MJ. Immune cell populations within the duodenal mucosa of dogs with enteropathies. J Vet Intern Med 2001;15:14-25.
11. Gaschen FP, Merchant SR. Adverse food reactions in dogs and cats. Vet Clin North Am Small Anim Pract 2011;41:361-79.
12. Ontsouka CE, Burgener IA, Mani O, et al. Polyunsaturated fatty acid-enriched diets used for the treatment of canine chronic enteropathies decrease the abundance of selected genes of cholesterol homeostasis. Dom Anim Endo 2010;38:32-37
13. Manchester AC, Hill S, Sabatino B, et al. Association between granulomatous colitis in French Bulldogs and invasive Escherichia coli and response to fluoroquinolone antimicrobials. J Vet Intern Med 2013;27:56-61.
14. Simpson KW, Dogan B, Rishniw M, et al. Adherent and invasive Escherichia coli is associated with granulomatous colitis in boxer dogs. Infection and immunity 2006;74:4778-4792.
15. Cerquetella M, Spaterna A, Laus F, et al. Inflammatory bowel disease in the dog: differences and similarities with humans. World J of Gastroenterology 2010;16:1050-1056.
16. Shouval DS, Rufo PA. The role of environmental factors in the pathogenesis of inflammatory bowel diseases: A review. JAMA Pediatrics 2017;171:999-1005.
17. D'Aiessio S, Tacconi C, Danese S. Targeting lymphatics in inflammatory bowel disease. Oncotarget 2015;6:34047.

18. Kathrani A, House A, Catchpole B, et al. Polymorphisms in the TLR4 and TLR5 gene are significantly associated with inflammatory bowel disease in German shepherd dogs. PloS one 2010;5:e15740.
19. Locher C, Tipold A, Welle M, et al. Quantitative assessment of mast cells and expression of IgE protein and mRNA for IgE and interleukin 4 in the gastrointestinal tract of healthy dogs and dogs with inflammatory bowel disease. Am J Vet Res 2001;62:211-216.
20. Burgener IA, König A, Allenspach K, et al. Upregulation of toll‐like receptors in chronic enteropathies in dogs. J Vet Intern Med 2008;22:553-560.
21. Luckschander N, Hall JA, Gaschen F, et al. Activation of nuclear factor-κB in dogs with chronic enteropathies. Vet Immunol Immunopath 2010;133:228-236.
22. Vázquez-Baeza Y, Hyde ER, Suchodolski JS, et al. Dog and human inflammatory bowel disease rely on overlapping yet distinct dysbiosis networks. Nature Micro 2016;1:16177.
23. Suchodolski JS. Fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. In: Encyclopedia of Metagenomics 2015: pp.183-186. Springer, Boston, MA.
24. Frank DN, Robertson CE, Hamm CM, et al. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. Inflamm Bowel Diseases 2010;17:179-184.
25. Dossin, Olivier, and Rachel Lavoué. Protein-losing enteropathies in dogs. Vet Clin: Small Anim Pract 2011;41:399-418.
26. Rudinsky AJ, Howard JP, Bishop MA, at al. Dietary management of presumptive protein‐losing enteropathy in Yorkshire terriers. J Small Anim Pract 2017;58:103-108.
27. Volkmann M, Steiner JM, Fosgate GT, et al. Chronic diarrhea in dogs–retrospective study in 136 cases. J Vet Intern Med 2017;31:1043-1055.
28. Umar SB, DiBaise JK. Protein-losing enteropathy: case illustrations and clinical review. Am J of Gastro 2010;105:43-49.
29. Braamskamp MJ, Dolman KM, Tabbers MM. Clinical practice: Protein-losing enteropathy in children. Euro J Pediatrics 2010;169:1179-1185.
30. Takeda H, Ishihama K, Fukui T, et al. Significance of rapid turnover proteins in protein-losing gastroenteropathy. Hepato-gastroenterology 2003;50:1963-1965.
31. Ferrante M, Penninckx F, De GH, et al. Protein-losing enteropathy in Crohn's disease. Acta gastro-enterologica Belgica 2006;69:384-389.
32. Annese V, Duricova D, Gower-Rousseau C, et al. Impact of new treatments on hospitalisation, surgery, infection, and mortality in IBD: a focus paper by the epidemiology committee of ECCO. J Crohns Col 2015;10:216-225.
33. Westermarck E, Skrzypczak T, Harmoinen J, et al. Tylosin‐responsive chronic diarrhea in dogs. J Vet Intern Med 2005;19:177-186.
34. Rossi G, Pengo G, Caldin M, et al. Comparison of microbiological, histological, and immunomodulatory parameters in response to treatment with either combination therapy with prednisone and metronidazole or probiotic VSL# 3 strains in dogs with idiopathic inflammatory bowel disease. PloS one 2014;9:e94699.
35. Pietra M, Fracassi F, Diana A, at al. Plasma concentrations and therapeutic effects of budesonide in dogs with inflammatory bowel disease. Amer J Vet Res 2013;74:78-83.

36. Jergens AE, Crandell J, Morrison JA, et al. Comparison of oral prednisone and prednisone combined with metronidazole for induction therapy of canine inflammatory bowel disease: a randomized-controlled trial. J Vet Intern Med 2010;24:269-277.
37. Simmerson SM, Armstrong PJ, Wünschmann A, et al. Clinical features, intestinal histopathology, and outcome in protein-losing enteropathy in yorkshire terrier dogs. J Vet Intern Med 2014;28:331-337.
38. Dye TL, Diehl KJ, Wheeler SL, et al. Randomized, controlled trial of budesonide and prednisone for the treatment of idiopathic inflammatory bowel disease in dogs. J Vet Intern Med 2013;27:1385-1391.
39. Allenspach K, Rüfenacht S, Sauter S, et al. Pharmacokinetics and clinical efficacy of cyclosporine treatment of dogs with steroid-refractory inflammatory bowel disease. J Vet Intern Med 2006;20:239-244
40. Dandrieux JR, Noble PJ, Scase TJ, et al. Comparison of a chlorambucil-prednisolone combination with an azathioprine-prednisolone combination for treatment of chronic enteropathy with concurrent protein-losing enteropathy in dogs: 27 cases (2007–2010). A Amer Vet Med Assoc 2013;242:1705-1714.
41. Allenspach K, Rizzo J, Jergens AE, et al. Hypovitaminosis D is associated with negative outcome in dogs with protein losing enteropathy: a retrospective study of 43 cases. BMC Vet Res 2017;13:96.
42. Jergens AE,Schrreiner CA, Frank DE, et al. A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med 2003;17:291-297.
43. Nakashima K, Hiyoshi S, Ohno K et al. Prognostic factors in dogs with protein-losing enteropathy. The Vet J 2015;205:28-32.
44. Rossi G, Cerquetella M, Antonelli E, et al. The importance of histologic parameters of lacteal involvement in cases of canine lymphoplasmacytic enteritis. Gastroenterol Hepatol Bed Bench 2015;8:33.
45. Washabau RJ, Day MJ, Willard MD, et al. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med 2010;24:10–26.
46. Willard MD, Jergens AE, Duncan RB, et al. Interobserver variation among histopathologic evaluations of intestinal tissues from dogs and cats. J Am Vet Med Assoc 2002;220:1177–1182.
47. Willard M, Mansell J. Correlating clinical activity and histopathologic assessment of gastrointestinal lesion severity: current challenges. Vet Clin: Small Anim Pract 2011;41:457-463.
48. Willard, M.D., Lovering, S.L., Cohen, N.D., et al. Quality of tissue specimens obtained endoscopically from the duodenum of dogs and cats. J Am Vet Med Assoc;219: 474–479.
49. Wilcock, B. Endoscopic biopsy interpretation in canine or feline enterocolitis. Seminars in Veterinary Medicine and Surgery (Small Animal) 1992; 7:162–171.
50. Waly, N.E., Stokes, C.R., Gruffydd-Jones, T.J., eat al. Immune cell populations in the duodenal mucosa of cats with inflammatory bowel disease. J Vet Intern Med 2004;18:816–825.
51. Jergens AE, Willard MD, Allenspach K. Maximizing the diagnostic utility of endoscopic biopsy in dogs and cats with gastrointestinal disease. Vet J 2016;214:50-60.
52. Garcia-Sancho M, Rodriguez-Franco F, Sainz A, et al. Evaluation of clinical,

macroscopic, and histopathologic response to treatment in nonhypoproteinemic dogs with lymphocytic-plasmacytic enteritis. J Vet Intern Med 2007;21:11–17.
53. McCann T, Ridyard A, Else R, et al. Evaluation of disease activity markers in dogs with idiopathic inflammatory bowel disease. J Small Anim Pract 2007;48:620–625.
54. Münster M, Hörauf A, Bilzer T. Assessment of disease severity and outcome of dietary, antibiotic, and immunosuppressive interventions by use of the canine IBD activity index in 21 dogs with chronic inflammatory bowel disease Berl Munch Tierarztl Wochenschr 2006;119:493–505 [in German].
55. Walker D, Knuchel‐Takano A, McCutchan A, et al. A comprehensive pathological survey of duodenal biopsies from dogs with diet‐responsive chronic enteropathy. J Vet Intern Med 2013;27:862-874.
56. Stroda K, Wakamatsu N, Gaschen L, et al. Histopathological, clinical, endoscopic, and ultrasound features of dogs with chronic enteropathies and small intestinal crypt lesions. J Vet Intern Med 2012;26:767–768.
57. Kleinschmidt S, Meneses F, Noltr I, et al. Retrospective study on the diagnostic value of full-thickness biopsies from the stomach and intestine of dogs with chronic gastrointestinal disease symptoms. Vet Pathol 2006;43:1000–1003.
58. Van der Gaag I, Happ_e RP. The histological appearance of peroral small intestinal biopsies in clinically healthy dogs and dogs with chronic diarrhea. Zentralbl Veterinarmed A 1990;37:401–416.
59. Larson RN, Ginn JA, Bell CM, et al. Duodenal endoscopic findings and histopathologic confirmation of intestinal lymphangiectasia in dogs. J Vet Intern Med 2012;26:1087–1092.
60. Luckschander N, Allenspach K, Hall J, et al. Perinuclear antineutrophilic cytoplasmic antibody and response to treatment in diarrheic dogs with food responsive disease or inflammatory bowel disease. J Vet Intern Med 2006 Mar;20:221-227.
61. Mandigers PJ, Biourge V, Van Den Ingh TS, et al. A randomized, open‐label, positively‐controlled field trial of a hydrolyzed protein diet in dogs with chronic small bowel enteropathy. J Vet Intern Med 2010;24:1350-1357.
62. Marks S, Laflamme DP, McAloose D. Dietary trial using a commercial hypoallergenic diet containing hydrolyzed protein for dogs with inflammatory bowel disease. Vet Ther: Res App Vet Med 2002.
63. Hall EJ, Batt RM. Dietary modulation of gluten sensitivity in a naturally occurring enteropathy of Irish setter dogs. Gut 1992;33:198-205.
64. Khalili H, Chan SS, Lochhead P, et al. The role of diet in the aetiopathogenesis of inflammatory bowel disease. Nature Reviews Gastroenterology & Hepatology 2018;22:1.
65. Molodecky NA, Soon S, Rabi DM, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology. 2012;142:46-54.
66. Charlebois A, Rosenfeld G, Bressler B. The impact of dietary interventions on the symptoms of inflammatory bowel disease: a systematic review. Crit Rev Food Sci Nut 2016;56:1370-1378.
67. D'souza S, Levy E, Mack D, et al. Dietary patterns and risk for Crohn's disease in children. Inflamm Bow Dis 2007;14:367-373.
68. Lewis JD, Abreu MT. Diet as a trigger or therapy for inflammatory bowel diseases. Gastro 2017;152:398-414.

69. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun1998;66:5224-5231.
70. Hudcovic T, Štěpánková R, Cebra J, et al. The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice. Folia Micro 2001;46:565-72.
71. Fahoum L, Moscovici A, David S, et al. Digestive fate of dietary carrageenan: evidence of interference with digestive proteolysis and disruption of gut epithelial function. Mol Nutr Food Res 2017;61:16.
72. Jowett SL, Seal CJ, Pearce MS, et al. Influence of dietary factors on the clinical course of ulcerative colitis: a prospective cohort study. Gut 2004;53:1479-1484.
73. Borrelli O, Cordischi L, Cirulli M, et al. Polymeric diet alone versus corticosteroids in the treatment of active pediatric Crohn's disease: a randomized controlled open-label trial. Clin Gastro Hepato 2006;4:744-753.
74. Grover Z, Muir R, Lewindon P. Exclusive enteral nutrition induces early clinical, mucosal and transmural remission in paediatric Crohn's disease. J Gastro 2014;49:638-645.
75. Levine A, Boneh RS, Wine E. Evolving role of diet in the pathogenesis and treatment of inflammatory bowel diseases. Gut 2018:gutjnl-2017.
76. Connors J, Basseri S, Grant A, et al. Exclusive enteral nutrition therapy in paediatric crohn's disease results in long-term avoidance of corticosteroids: results of a propensity-score matched cohort analysis. J Crohns Col 2017;11:1063–1070.
77. Cohen-Dolev N, Sladek M, Hussey S, et al. Differences in outcomes over time with exclusive enteral nutrition compared with steroids in children with mild to moderate crohn's disease: results from the GROWTH CD Study. J Crohns Col 2018;12:306–312.
78. Nguyen DL, Palmer LB, Nguyen ET, et al. Specialized enteral nutrition therapy in Crohn's disease patients on maintenance infliximab therapy: a meta-analysis. Thera Adv Gastro 2015;8:168-175.
79. Van Limbergen J, Haskett J, Griffiths AM, et al. Toward enteral nutrition in the treatment of pediatric Crohn disease in Canada: A workshop to identify barriers and enablers. Can J Gastro Hepato2015;29:351-356.
80. Zachos, Mary, Melody Tondeur, and Anne Marie Griffiths. Enteral nutritional therapy for induction of remission in Crohn's disease 2007: CD000542-CD000542.
81. Wędrychowicz A, Zając A, Tomasik P. Advances in nutritional therapy in inflammatory bowel diseases. World J Gastro 2016;22:1045-1066.
82. Sigall Boneh R, Sarbagili Shabat C, Yanai H, et al. Dietary therapy with the Crohn's disease exclusion diet is a successful strategy for induction of remission in children and adults failing biological therapy. J Crohns Col 2017;11:1205-1212.
83. Yamamoto T, Shiraki M, Nakahigashi M, et al. Enteral nutrition to suppress postoperative Crohn's disease recurrence: a five-year prospective cohort study. Intern J of Colo Dis 2013;28:335-340.
84. Shivashankar R, Lewis JD. The role of diet in inflammatory bowel disease. Curr Gastroenterol Rep 2017;19:22.
85. Okanishi H, Yoshioka R, Kagawa Y, et al. The clinical efficacy of dietary fat restriction in treatment of dogs with intestinal lymphangiectasia. J Vet Intern Med 2014;28:809-817.

86. Alexander JS, Ganta VC, Jordan PA, et al. Gastrointestinal lymphatics in health and disease. Pathophysiology 2010;17:315-335.
87. Miller MJ, McDole JR, Newberry RD. Microanatomy of the intestinal lymphatic system. Ann NY Acad Sci 2010;1207:21-28.
88. Ge Y, Li Y, Gong J, et al. Mesenteric organ lymphatics and inflammatory bowel disease. Ann Anat 2018;218:199-204.
89. Chakraborty S, Davis MJ, Muthuchamy M. Emerging trends in the pathophysiology of lymphatic contractile function. In: Seminars in cell & developmental biology 2015;38:55-66.
90. Dixon JB. Lymphatic lipid transport: sewer or subway? Trends Endocrinol Metab 2010;21:480-487.
91. Kull PA, Hess RS, Craig LE, et al. Clinical, clinicopathologic, radiographic, and ultrasonographic characteristics of intestinal lymphangiectasia in dogs: 17 cases (1996–1998). J Am Vet Med Assoc 2001;219:197–202.
92. Van Kruiningen HJ, Lees GE, Hayden DW, et al. Lipogranulomatous lymphangitis in canine intestinal lymphangiectasia. Vet Pathol 1984;21:377–383.
93. Villaescusa A. White spots on the mucosal surface of the duodenum in dogs with lymphocytic plasmacytic enteritis. J Vet Sci 2011;12:165-170.
94. Watson VE, Hobday MM, Durham AC. Focal intestinal lipogranulomatous lymphangitis in 6 dogs (2008–2011). J Vet Intern Med 2014;28:48-51.
95. Lecoindre A, Lecoindre P, Cadoré JL, et al. Focal intestinal lipogranulomatous lymphangitis in 10 dogs. J Small Anim Pract 2016;57:465-471.
96. Malatos JM, Kurpios NA, Duhamel GE. Small intestinal lymphatic hypoplasia in three dogs with clinical signs of protein-losing enteropathy. J Comp Path 2018;160:39-49.
97. Ferrell RE, Finegold DN, Research perspectives in inherited lymphatic disease: an update, Ann NY Acad Sci 2008;1113:134–139.
98. Irrthum, A, Karkkainen, MJ, Devriendt K, et al. Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. Am J Hum Genet 2000;67:295–301.
99. Hennekam RC, Geerdink RA, Hamel BC. Autosomal recessive intestinal lymphangiectasia and lymphedema, with facial anomalies and mental retardation. Am J Med Gen 1989;34(4):593-600.
100. Malek NP, Ocran K, Tietge UJ, et al. A case of the yellow nail syndrome associated with massive chylous ascites, pleural and pericardial effusions. Zeitschrift fur Gastro 1996;34:763-766.
101. Waldmann TA, Steinfeld JL, Dutcher TF et al. The role of the gastrointestinal system in "idiopathic hypoproteinemia". Gastro 1961;41:197–207.
102. Hardikar W, Smith AL, Chow CW. Neonatal protein-losing enteropathy caused by intestinal lymphatic hypoplasia in siblings. J Ped Gastro Nutr 1997;25:217-221.
103. Feldt RH, Driscoll DJ, Offord KP et al. Protein-losing enteropathy after the Fontan operation. J Thorac Cardiovasc Surg 1996;112:672–680
104. Liao S, von der Weid PY. Inflammation-induced lymphangiogenesis and lymphatic dysfunction. Angiogenesis 2014;17:325-334.
105. Van Kruiningen HJ, Colombel JF. The forgotten role of lymphangitis in Crohn's disease. Gut. 2008;57:1-4.
106. Kalima T. Experimental lymphatic obstruction in the ileum. Ann Chir Gyanec Fenn

1970;59:187–201.
107. Kalima T, Saloniemi H, Rahko T. Experimental regional enteritis in pigs. Scand J Gastroenterol 1976;11:353–362.
108. Jang JY, Koh YJ, Lee SH, et al. Conditional ablation of LYVE-1+ cells unveils defensive roles of lymphatic vessels in intestine and lymph node. Blood 2013.
109. Davis RB, Kechele DO, Blakeney ES, et al. Lymphatic deletion of calcitonin receptor–like receptor exacerbates intestinal inflammation. JCI insight 2017;2.
110. Rehal S, Stephens M, Roizes S, et al. Acute small intestinal inflammation results in persistent lymphatic alterations. Am J Phys-Gastro Liv Path 2017;314:G408-417.
111. Wu TF, MacNaughton WK, Von Der Weid PY. Lymphatic vessel contractile activity and intestinal inflammation. Memorias do Inst Oswaldo Cruz 2005;100:107-110.
112. Geleff S, Schoppmann SF, Oberhuber G. Increase in podoplanin-expressing intestinal lymphatic vessels in inflammatory bowel disease. Virchows Archiv 2003;442:231-237.
113. Kaiserling E, Krober S, Geleff S. Lymphatic vessels in the colonic mucosa in ulcerative colitis. Lymphology 2003;36:52-61.
114. Alexander JS, Chaitanya GV, Grisham MB, et al. Emerging roles of lymphatics in inflammatory bowel disease. Ann NY Acad Sci 2010;1207:E75-85.
115. Podgrabinska S, Braun P, Velasco P, et al. Molecular characterization of lymphatic endothelial cells. Proc Nat Acad Sci 2002;99:16069-74.
116. Oliver G, Sosa-Pineda B, Geisendorf S, et al. Prox 1, a prospero-related homeobox gene expressed during mouse development. Mech Dev 1993;44:3-16.
117. Banerji S, Ni J, Wang SX, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Bio 1999;144:789-801.
118. Fogt F, Pascha TL, Zhang PJ, et al. Proliferation of D2-40-expressing intestinal lymphatic vessels in the lamina propria in inflammatory bowel disease. Intern J Mol Med 2004;13:211-214.
119. Pedica F, Ligorio C, Tonelli P, et al. Lymphangiogenesis in Crohn's disease: an immunohistochemical study using monoclonal antibody D2-40. Virchows Archiv 2008;452:57-63.
120. Rahier JF, De Beauce S, Dubuquoy L, et al. Increased lymphatic vessel density and lymphangiogenesis in inflammatory bowel disease. Aliment Pharmacol Ther 2011;34:533-543.
121. Shen W, Li Y, Cao L, et al. Decreased expression of Prox1 is associated with postoperative recurrence in crohn's disease. J Crohn's Col 2018.
122. Li Y, Ge Y, Gong J, et al. Mesenteric lymphatic vessel density is associated with disease behavior and postoperative recurrence in crohn's disease. J Gastroi Surg 2018 Jul 24:1-8.
123. Halsey CH, Worley DR, Curran K, et al. The use of novel lymphatic endothelial cell-specific immunohistochemical markers to differentiate cutaneous angiosarcomas in dogs. Vet Comp Onc 2016;14:236-244.
124. Curran KM, Halsey CH, Worley DR. Lymphangiosarcoma in 12 dogs: a case series (1998–2013). Vet Comp Onc 2016;14:181-190.
125. Galeotti F, Barzagli F, Vercelli A, et al. Feline lymphangiosarcoma–definitive identification using a lymphatic vascular marker. Vet Derm 2004;15:13-18.
126. Wu TF, Carati CJ, MacNaughton WK, et al. Contractile activity of lymphatic vessels is altered in the TNBS model of guinea pig ileitis. Amer J Phys-Gastro Liver Phys 2006 ;291:G566-74.

127. von der Weid PY, Rehal S. Lymphatic pump function in the inflamed gut. Ann NY Acad Sci 2010;1207.
128. Limketkai BN, Mullin GE, Limsui D, et al. Role of vitamin D in inflammatory bowel disease. Nut Clin Pract 2017;32:337-345.
129. Nielsen OH, Rejnmark L, Moss AC. Role of Vitamin D in the natural history of inflammatory bowel disease. J Crohns Col 2018;12:742-752.
130. Weidner N, Verbrugghe A. Current knowledge of vitamin D in dogs. Crit Rev Food Sci Nutr 2017;57:3850-3859.
131. Morris, JG. Ineffective vitamin D synthesis in cats is reversed by an inhibitor of 7-dehydrocholestrol-D7-reductase. J Nutr 1999;129:903–908.
132. Garg M, Lubel JS, Sparrow MP, et al. Vitamin D and inflammatory bowel disease–established concepts and future directions. Aliment Pharmacol Ther 2012;36:324-344.
133. Delanghe JR, Speeckaert R, Speeckaert MM. Behind the scenes of vitamin D binding protein: more than vitamin D binding. Best Pract Res Clin Endo Met 2015;29:773-786.
134. Cartwright JA, Gow AG, Milne E, et al. Vitamin D Receptor Expression in Dogs. J Vet Intern Med 2018;32:764-774.
135. Bartels LE, Jørgensen SP, Agnholt J, et al. 1, 25-dihydroxyvitamin D3 and dexamethasone increase interleukin-10 production in CD4+ T cells from patients with Crohn's disease. Intern Immunopharm 2007;7:1755-1764.
136. Martinesi M, Treves C, d'Albasio G, et al. Vitamin D derivatives induce apoptosis and downregulate ICAM-1 levels in peripheral blood mononuclear cells of inflammatory bowel disease patients. Inflamm Bow Dis 2008;14:597-604.
137. Gubatan J, Moss AC. Vitamin D in inflammatory bowel disease: more than just a supplement. Curr Op Gastro 2018;34:217-225.
138. Autier P, Boniol M, Pizot C, et al. Vitamin D status and ill health: a systematic review. The Lancet: Diab Endo 2014;2:76-89.
139. Kraus MS, Rassnick KM, Wakshlag JJ, et al. Relation of vitamin D status to congestive heart failure and cardiovascular events in dogs. J Vet Intern Med 2014;28:109-115.
140. Gerber B, Hässig M, Reusch CE. Serum concentrations of 1, 25-dihydroxycholecalciferol and 25-hydroxycholecalciferol in clinically normal dogs and dogs with acute and chronic renal failure. Am J Vet Res 2003;64:1161-1166.
141. Selting KA, Sharp CR, Ringold R, et al. Serum 25‐hydroxyvitamin D concentrations in dogs–correlation with health and cancer risk. Vet Comp Onc 2016;14:295-305.
142. Rosa CT, Schoeman JP, Berry JL, et al. Hypovitaminosis D in dogs with spirocercosis. J Vet Intern Med 2013;27:1159-1164.
143. Titmarsh H, Gow AG, Kilpatrick S, et al. Association of vitamin D status and clinical outcome in dogs with a chronic enteropathy. J Vet Intern Med 2015;29:1473-1478.
144. Gow AG, Else R, Evans H, et al. Hypovitaminosis D in dogs with inflammatory bowel disease and hypoalbuminaemia. J Sm Anim Pract 2011;52:411-418.
145. Mellanby RJ, Mellor PJ, Roulois A, et al. Hypocalcaemia associated with low serum vitamin D metabolite concentrations in two dogs with protein‐losing enteropathies. J Sm Anim Pract 2005;46:345-51.
146. Froicu M, Cantorna MT. Vitamin D and the vitamin D receptor are critical for control of the innate immune response to colonic injury. BMC Immunol 2007;8:5.
147. Lagishetty V, Misharin AV, Liu NQ, et al. Vitamin D deficiency in mice impairs colonic antibacterial activity and predisposes to colitis. Endocrinology 2010;151:2423–2432.

148. Froicu M, Weaver V, Wynn TA, et al. A crucial role for the vitamin D receptor in experimental inflammatory bowel diseases. Mol Endocrinol 2003;17:2386–2392
149. Cantorna MT, Munsick C, Bemiss C, et al. 1,25-Dihydroxycholecalciferol prevents and ameliorates symptoms of experimental murine inflammatory bowel disease. J Nutr 2000;130:2648-2652.
150. Laverny G, Penna G, Vetrano S, et al. Efficacy of a potent and safe vitamin D receptor agonist for the treatment of inflammatory bowel disease. Immunol Lett 2010;131:49-58.
151. Nerich V, Monnet E, Etienne A, et al. Geographical variations of inflammatory bowel disease in France: a study based on national health insurance data. Inflamm Bowel Dis 2006;12:218-226.
152. Ananthakrishnan AN, Khalili H, Higuchi LM, et al. Higher predicted vitamin D status is associated with reduced risk of Crohn's disease. Gastroenterology 2012;142(3):482-489.
153. Del Pinto R, Pietropaoli D, Chandar AK, et al. Association between inflammatory bowel disease and vitamin D deficiency: a systematic review and metaanalysis. Inflamm Bowel Dis 2015; 21:2708–2717.
154. Sadeghian M, Saneei P, Siassi F, et al. Vitamin D status in relation to Crohn's disease: meta-analysis of observational studies. Nutrition 2016;32:505–14.
155. Ulitsky A, Ananthakrishnan AN, Naik A, et al. Vitamin D deficiency in patients with inflammatory bowel disease: association with disease activity and quality of life. JPEN J Parenter Enteral Nutr 2011;35:308–316.
156. Suibhne TN, Cox G, Healy M, et al. Vitamin D deficiency in Crohn's disease: prevalence, risk factors and supplement use in an outpatient setting. J Crohns Colitis 2012;6:182–188.
157. Ananthakrishnan AN, Cagan A, Gainer VS, et al. Normalization of plasma 25-hydroxy vitamin D is associated with reduced risk of surgery in Crohn's disease. Inflamm Bowel Dis 2013;19:1921–1927.
158. Kabbani TA, Koutroubakis IE, Schoen RE, et al. Association of vitamin D level with clinical status in inflammatory bowel disease: a 5-year longitudinal study. Am J Gastro 2016;111:712-719.
159. Winter RW, Collins E, Cao B, et al. Higher 25-hydroxyvitamin D levels are associated with greater odds of remission with anti-tumour necrosis factor-α medications among patients with inflammatory bowel diseases. Aliment Pharmacol Ther 2017;45:653–9.
160. Jorgensen SP, Agnholt J, Glerup H, et al. Clinical trial: vitamin D3 treatment in Crohn's disease – a randomized double-blind placebo controlled study. Aliment Pharmacol Ther 2010; 32: 377–383.
161. Pappa HM, Mitchell PD, Jiang H, et al. Maintenance of optimal vitamin D status in children and adolescents with inflammatory bowel disease: a randomized clinical trial comparing two regimens. J Clin Endocrinol Metab 2014;99:3408–3417.
162. Ardesia M, Ferlazzo G, Fries W. Vitamin D and inflammatory bowel disease. BioMed research international 2015;470805.
163. Farraye FA, Nimitphong H, Stucchi A, et al. Use of a novel vitamin D bioavailability test demonstrates that vitamin D absorption is decreased in patients with quiescent Crohn's disease. Inflamm Bow Dis 2011;17:2116-2221.
164. Vogelsang H, Schofl R, Tillinger W, et al. 25-hydroxyvitamin D absorption in patients with Crohn's disease and with pancreatic insufficiency. Wien Klin Wochenschr. 1997; 109:678–682.

165. Pappa HM, Gordon CM, Saslowsky TM, et al. Vitamin D status in children and young adults with inflammatory bowel disease. Pediatrics 2006;118:1950-1961.
166. Strisciuglio C, Cenni S, Giugliano FP, et al. The role of inflammation on Vitamin D levels in a cohort of pediatric patients with inflammatory bowel disease. J Ped Nutr Gastro 2018.
167. Fabisiak N, Fabisiak A, Watala C, et al. Fat-soluble vitamin deficiencies and inflammatory bowel disease. J Clin Gastro 2017;51:878-889.
168. Titmarsh HF, Gow AG, Kilpatrick S, et al. Low vitamin D status is associated with systemic and gastrointestinal inflammation in dogs with a chronic enteropathy. PloS one 2015;10:e0137377.

CHAPTER 2: RESEARCH OVERVIEW

2.1 Research Overview

The goals of the research described in this dissertation were to explore a set of clinical, clinicopathologic, histopathologic, and immunohistochemical features of dogs with CE with and without concurrent PLE, and improve our understanding of the etiopathogenesis of CE and PLE. Determining the etiopathogenesis of this disease could lead to novel therapeutic protocols and better outcomes in this group of dogs. Chapter 3 retrospectively examines the histological findings of dogs with CE with and without concurrent PLE. Chapter 4 examines a group of dogs with glucocorticoid-resistant CE and PLE that had clinical and clinicopathologic improvement following a novel therapeutic dietary approach. The response to diet calls for an examination of the role of diet in the pathology and treatment of CE and concurrent PLE. Chapter 5 prospectively evaluates alterations to the lymphatic vasculature with the use of immunohistochemical lymphatic markers in dogs with CE with and without concurrent PLE in an attempt to better understand the role of the lymphatic network in cases of CE and PLE. Chapter 6 investigates the prevalence of hypovitaminosis D in cases of CE with and without PLE, and explores possible mechanisms underlying the etiology of hypovitaminosis D in canine CE.

CHAPTER 3: HISTOPATHOLOGIC CHARACTERISTICS OF INTESTINAL BIOPSY SAMPLES FROM DOGS WITH CHRONIC INFLAMMATORY ENTEROPATHY WITH AND WITHOUT HYPOALBUMINEMIA[a]

3.1 Overview

Background: Previous studies have identified hypoalbuminemia as a risk factor for negative outcome in dogs with chronic enteropathy (CE), but it has not been determined whether histopathology differs between CE dogs with and without hypoalbuminemia.

Objective: To compare histopathologic findings in dogs with biopsy-diagnosed inflammatory CE with and without hypoalbuminemia.

Animals: 83 dogs that had intestinal biopsy performed between January 2010–July 2015. Dogs had signs compatible with CE of at least 3-weeks' duration and no evidence of clinically relevant extra-gastrointestinal (GI) disease or potential non-GI causes of hypoalbuminemia. Dogs had primary diagnosis of inflammatory enteritis based on histopathology.

Methods: Dogs were grouped into CE with normoalbuminemia (CEN; serum albumin concentration ≥3.0 g/dL, N = 46) or chronic enteropathy with hypoalbuminemia (CEH; serum albumin concentration <3.0 g/dL, N = 37). A pathologist blinded to the groups reviewed biopsy samples and applied the World Small Animal Veterinary Association scoring system to all samples.

Results: Intestinal biopsy samples from dogs in the CEH group were significantly more likely to display villous stunting, epithelial injury, crypt distension, and lacteal dilatation, and were more likely to have intraepithelial lymphocytes and lamina propria neutrophils than biopsy samples

[a] Wennogle SA, Priestnall SL, Webb CB. Histopathologic characteristics of intestinal biopsy samples from dogs with chronic inflammatory enteropathy with and without hypoalbuminemia. . J Vet Intern Med 2017;31:371-6

from dogs in the CEN group. Additionally, higher scores for each of the above listed histopathologic criteria were associated with a lower serum albumin concentration.

Conclusions and Clinical Importance: Histopathologic features of chronic inflammatory enteropathy differ between dogs that are hypo- versus normoalbuminemic. Additional work is needed to elucidate the clinical relevance of these differences.

3.2 Introduction

Chronic enteropathy (CE) is a term used to describe various inflammatory conditions of the intestinal tract.[1] It is characterized by the presence of gastrointestinal (GI) signs such as weight loss, vomiting, diarrhea, and decreased appetite of at least several weeks' duration, and is associated with histologic evidence of inflammation in the small intestine.[1–3] In dogs, the type of CE often is determined by response to treatment and can include antibiotic-responsive disease, food-responsive disease, and idiopathic inflammatory bowel disease, which may be steroid responsive.[1] The prognosis is reported to be highly variable[1–5] and dependent on response to treatment.[6]

When a GI disorder results in hypoalbuminemia as a consequence of excessive loss of plasma proteins through the intestinal mucosa, it is commonly referred to as protein-losing enteropathy (PLE).[7] Causes of PLE are numerous, including diseases that result in infiltration, inflammation, hemorrhage, or edema of the GI tract,[7] including intestinal lymphangiectasia, alimentary lymphoma, hookworm infestation, infection by *Histoplasma capsulatum*, and intestinal intussusception.[8] Chronic enteropathies can result in PLE,[8] and for the purposes of this study, we refer to this type of PLE as chronic enteropathy with hypoalbuminemia (CEH).

Although several previous studies have identified hypoalbuminemia as a risk factor for negative outcome in cases of CE, it is still unclear whether histopathology differs between CE dogs with and without hypoalbuminemia.[1,4,5] Historically, substantial interobserver variation in histopathologic evaluation of intestinal tissue has compounded this problem, [9] making it difficult to compare changes among dogs with different categories of intestinal disease. Recently, the GI Standardization Group developed guidelines for the interpretation of inflammatory change in the GI mucosa of the dog and cat. These standards, known as the World Small Animal Veterinary Association (WSAVA) scoring system, include classification and scoring of morphologic and inflammatory changes in the canine intestinal mucosa.[10]

The objective of our study was to compare histopathologic findings (as determined by the WSAVA scoring system) in dogs with biopsy-diagnosed CE with and without hypoalbuminemia. Our hypothesis was that dogs with CE and hypoalbuminemia would have different histopathologic features than CE dogs without hypoalbuminemia.

3.3 Materials and methods

Electronic medical records at Colorado State University were reviewed for dogs that had intestinal biopsy performed between January 2010 and July 2015. Included dogs had clinical signs compatible with CE of at least 3-weeks' duration, including weight loss, diarrhea, vomiting, and decreased appetite, and intestinal biopsy samples that indicated variable types and degrees of inflammatory infiltrate. Dogs were included if their primary diagnosis was an inflammatory enteritis on their original histopathologic evaluation. Additionally, all dogs had an appropriate history of fecal testing or deworming, and most (67/83; 80%) had abdominal ultrasonography performed before intestinal biopsy to screen for intestinal or abdominal masses.

Dogs with clinically relevant concurrent extra GI disease, with potential non-GI causes of hypoalbuminemia, with causes of GI disease other than inflammatory enteritis (eg, intestinal lymphoma), or for which a complete medical record could not be obtained, were excluded. Specifically, hypoalbuminemic dogs that were not screened for proteinuria, had substantial proteinuria (UPC >1.0), or had clinically abnormal serum bile acid concentrations (preprandial >20 lmol/L; postprandial >40 lmol/L) were excluded. Dogs were placed in either the CE with normoalbuminemia (serum albumin concentration ≥3.0 g/dL) group (chronic enteropathy with normoalbuminemia, CEN) or the CE with hypoalbuminemia (serum albumin concentration <3.0 g/dL) group (CEH). Serum albumin concentration <3.0 g/dL was defined as hypoalbuminemia based on the Colorado State University Diagnostic Laboratory's normal reference interval for serum albumin concentration in dogs (3.0–4.3 g/dL).

Recorded data included age, breed, sex, clinical signs, additional clinicopathologic abnormalities, tissue types available, and biopsy method. After cases were selected for study, histopathologic evaluation of previously obtained intestinal tissue was performed by a single pathologist blinded to clinical group. The pathologist established that the biopsy samples were adequate for evaluation, and then determined the presence of morphologic criteria (villous stunting, epithelial injury, crypt distension, lacteal dilatation, mucosal fibrosis) and inflammatory criteria (intraepithelial lymphocytes [IEL], lamina propria eosinophils, lamina propria lymphocytes or plasma cells, lamina propria neutrophils) and scored the degree of change based on WSAVA guidelines. For the degree of each change, the following scores were applied based on established criteria: 0 = normal, 1 = mild, 2 = moderate, 3 = marked. In cases where >1 tissue type was available, the tissue type with the highest total WSAVA score was used in the analysis.

3.3.1 Statistical Analysis

The proportion of each variable present was compared between the 2 groups (CEN versus CEH) using Fisher's exact test. A Spearman (rank-based) correlation also was performed to evaluate for a correlation between score for each biopsy variable and serum albumin concentration. A multiple logistic regression analysis then was performed using those variables found to be significantly associated with hypoalbuminemia based on Fisher's exact test (crypt distention, IEL, lacteal dilatation, neutrophils, villous epithelial injury, and villous stunting). Specifically, hypoalbuminemia (yes or no) was the response variable and the histopathologic variables (presence/absence) were used as predictors. Backward elimination then was used to decrease the number of predictors in the logistic regression model. For Fisher's exact test and Spearman's correlation analyses, a Bonferroni adjustment was used to account for multiple testing.

Statistical significance for all analyses was defined as the probability of the null hypotheses (ie, no relationship or no difference) being true at <5.0% ($p < 0.05$).

3.4 Results

Medical records of 270 dogs that underwent intestinal biopsy at Colorado State University (CSU) from January 2010 to July 2015 were reviewed. Of the 270, 83 met the inclusion criteria. Ninety-six of the excluded dogs had either evidence of clinically relevant concurrent extra-GI disease, or clinically relevant concurrent extra-GI disease could not be excluded. Sixty-four of the excluded dogs had a primary diagnosis other than inflammatory enteritis as the cause of their chronic GI signs (e.g., chronic foreign body and neoplasia). In 27 excluded cases, no intestinal tissue or previously prepared slide was available for evaluation.

Of the included dogs, 37/83 (45%) were hypoalbuminemic (CEH group; serum albumin concentration<3.0 g/dL), and 46/83 (55%) were normoalbuminemic (CEN group; serum albumin concentration ≥3.0 g/dL). The median age of dogs in the CEH group was 3 years (range, 1–12 years). The median age of dogs in CEN group was 2 years (range, 5 months–14 years). In the CEH group, 3 dogs were intact males, 15 dogs were castrated males, 1 dog was an intact female, and 18 dogs were spayed females. In the CEH group, 2 dogs were intact males, 30 dogs were castrated males, 2 dogs were intact females, and 12 dogs were spayed females. There was no significant difference in age or sex status between groups.

Breeds included mixed breed (15), Labrador retriever (9), German shepherd dog (4), boxer (3), Cocker spaniel (3), Pomeranian (3), West Highland white terrier (3), Australian shepherd (2), border collie (2), English bulldog (2), French bulldog (2), golden retriever (2), Great Pyrenees (2), Newfoundland (2), Yorkshire terrier (2), and 1 each of the following: American eskimo, Airedale terrier, Akita, Bernese mountain dog, Boston terrier, Chesapeake Bay retriever, Doberman, miniature dachshund, German shorthaired pointer, giant schnauzer, Irish setter, Maltese, Norwich terrier, Lundehund, Rottweiler, soft coated Wheaten terrier, Samoyed, Shar Pei, Shetland sheepdog, shiba inu, shih tzu, Siberian husky, Saint Bernard, standard poodle, Tibetan terrier, and Weimaraner.

In the CEH group, biopsy samples were obtained by endoscopy in 33/37 (89%) cases, with 24/33 (73%) having only gastroduodenoscopy performed and 9/33 (27%) having both gastroduodenoscopy and ileocolonoscopy performed. Three dogs in the CEH group had intestinal biopsy samples obtained celiotomy, and 1 dog by laparoscopy. In the CEN group, biopsy samples were obtained by endoscopy in 42/46 (91%) cases, with 22/42 (52%) having only gastroduodenoscopy performed and 20/42 (48%) having both gastroduodenoscopy and

ileocolonoscopy performed. Four dogs in CEN group had intestinal biopsy samples obtained by celiotomy. Method of biopsy procurement was not different between groups. Median serum albumin concentration in the CEH group was 1.9 g/dL (range, 0.8–2.8 g/dL). Median serum albumin concentration in the CEN group was 3.7 g/dL (range, 3.0–4.4 g/dL).

A Fisher's exact test indicated that dogs in the CEH group had a greater proportion of villous stunting, epithelial injury, crypt distension, and lacteal dilatation than did dogs in the CEN group. Additionally, dogs in the CEH group had a greater proportion of IEL and lamina propria neutrophils than did dogs in the CEN group. Proportions of lamina propria eosinophils and lamina propria lymphocytes or plasma cells were not different between groups. The criterion of mucosal fibrosis was not represented often enough to be suitable for statistical analysis (Figure 3.1).

A rank-based Spearman's correlation indicated that a higher score for villous stunting, epithelial injury, crypt distension, lacteal dilatation, IEL, or lamina propria neutrophils was moderately associated with a lower serum albumin concentration (Table 3.1).

After backward elimination (using alpha = 0.05), multiple logistic regression analysis indicated that the following variables remained significant: crypt distension (odds ratio [OR], 12.487; $p < 0.001$), intraepithelial lymphocytes (OR, 10.060; $p = 0.013$), and lacteal dilatation (OR, 5.037; $p = 0.009$). The OR represents the odds of hypoalbuminemia comparing the presence versus the absence of each histopathologic variable, with other variables held constant.

Table 3.1 Scores for histopathologic variables in dogs with chronic enteropathy and correlation with hypoalbuminemia (Spearman rank-based)

Variable	Group	Med	Min	Max	*p* Value	Adjusted *p* Value
Crypt distension	CEH	1	0	3	<0.001	<0.001
	CEN	0	0	2		
Intraepithelial lymphocytes	CEH	0	0	3	0.001	0.01
	CEN	0	0	1		
Lacteal dilation	CEH	1	0	3	<0.001	<0.001
	CEN	0	0	3		
Eosinophils	CEH	0	0	2	0.77	1
	CEN	0	0	3		
Lymphocytes and plasma cells	CEH	1	0	2	0	1
	CEN	0	0	2		
Neutrophils	CEH	0	0	3	0.002	0.04
	CEN	0	0	2		
Mucosal fibrosis	CEH	0	0	1	NA	NA
	CEN	0	0	0		
Villous epithelial injury	CEH	0	0	3	0.001	0.005
	CEN	0	0	1		
Villous stunting	CEH	1	0	3	<0.001	<0.001
	CEN	0.5	0	2		

CEH, chronic enteropathy with hypoalbuminemia; CEN, chronic enteropathy with normoalbuminemia; LP, lamina propria; NA, not applicable: statistical tests unreliable due to low expression of this trait. [a]After Bonferroni correction.

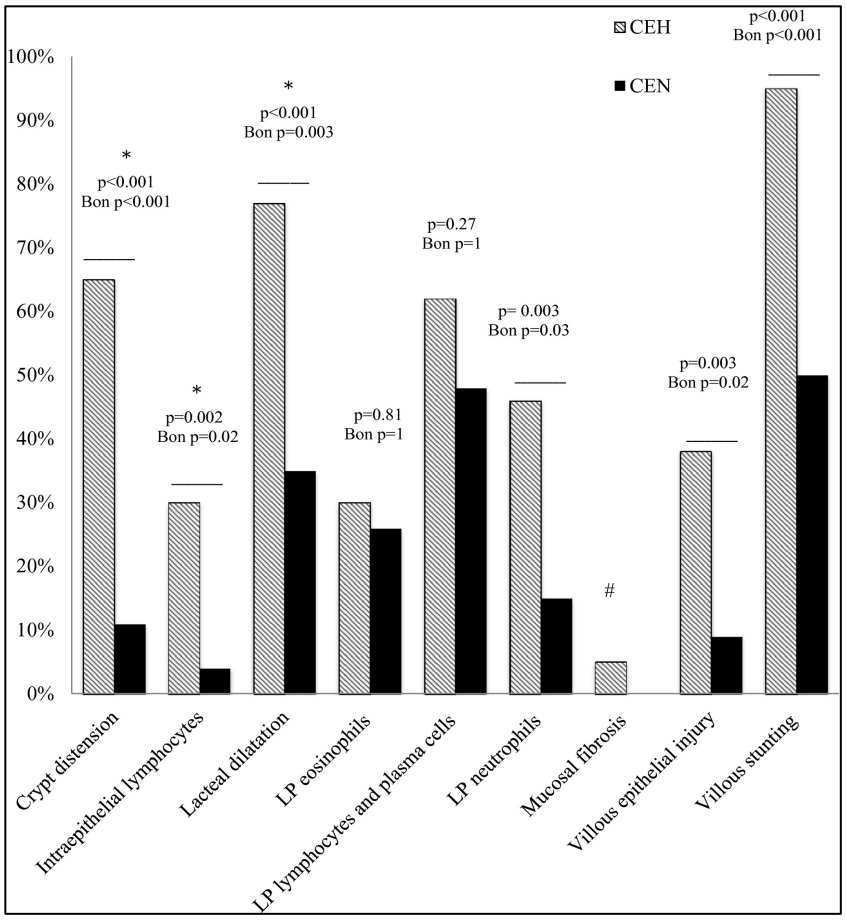

Figure 3.1 The presence of histopathologic variables in dogs with CEH versus dogs with CEN. *P* values (Fischer's exact test) and adjusted *P* values (after Bonferroni correction) shown for significant and nonsignificant variables. *Retained significance in multivariate analysis; #Statistical tests unreliable because of low expression of this trait; LP: lamina propria; CEH: chronic enteropathy and hypoalbuminemia; CEN: chronic enteropathy and normoalbuminemia.

3.5 Discussion

Lymphoplasmacytic or eosinophilic intestinal infiltrates or both are considered the hallmark histopathological findings of idiopathic CE. In our population of dogs with CE, the proportions of lamina propria lymphoplasmacytic or eosinophilic infiltrates were not different between the hypoalbuminemic and normoalbuminemic groups. Additionally, the proportions and severity of these infiltrates were not correlated with serum albumin concentration. This finding was not surprising, and it is in agreement with previous studies, in which the histologic scores of infiltrates of eosinophils, lymphocytes, or plasma cells were not correlated with the patient's

serum albumin concentration or outcome.[1,5] Interestingly, the cellular infiltrates that were different between CEH and CEN dogs included IEL and lamina propria neutrophils.

Intraepithelial lymphocytes are a component of the first line of defense in the GI immune system and have both pro- and anti-inflammatory roles.[11,12] We found that IEL were more likely to be present ($P < .001$) in dogs with hypoalbuminemia versus normoalbuminemic dogs with CE, but as a consequence of the retrospective nature of our study, we were not able to classify these cells further. Additional study is needed to determine the role of these cells in dogs with CE.

Lamina propria neutrophils were more likely to be present ($P = .003$) in dogs with hypoalbuminemia (17/ 37; 46%) compared to dogs with normoalbuminemia (7/46; 15%). Whether the neutrophils are representative of an underlying etiology, are present because of adhesion and possible translocation of microbial flora or pathogens, or are simply part of the inflammatory response is not well understood and deserves further study.

Crypt distension also was found more often in dogs with CEH versus dogs with CEN ($P < .001$). Previous studies have reported on crypt lesions, sometimes referred to as crypt abscesses, in dogs with PLE.[13] In a case series of 6 dogs with PLE,[14] only 2/6 had inflammatory infiltrates or lymphangiectasia histologically. Interestingly, both dogs that had inflammatory infiltrates had neutrophils noted in the lamina propria, and 5/6 dogs had neutrophils present in distended crypts. In another series of 58 dogs with CE, dogs with crypt abscesses were found to have more severe intestinal protein loss and shorter survival times than dogs with crypt distension or no crypt lesions.[15] The clinical relevance of crypt lesions in dogs with CE also deserves further study.

Dilated lacteals were present in a high proportion of dogs overall (44/83; 53%), but were significantly more likely ($P < .001$) to be present in dogs with hypoalbuminemia (28/37; 76%)

compared to dogs with normal serum albumin concentration (16/46; 35%). A previous retrospective analysis of full-thickness intestinal biopsy samples in dogs with chronic GI disease showed that, in 38/64 dogs (59%), the major histopathologic abnormality was intestinal lymphangiectasia (dilated lacteals). Lymphoplasmacytic enteritis (LPE) was identified in only 5/64 dogs (8%).[16] These findings are in contrast to a study that evaluated histologic findings of endoscopic biopsy samples in 368 dogs with chronic diarrhea, where LPE was identified in approximately 25% of cases, and lymphangiectasia was not common.[17] The serum albumin concentration of dogs in these studies was not compared. A more recent study evaluated the histologic features of 136 dogs with chronic GI signs, 94 with LPE, and 42 with GI disease not caused by inflammatory bowel disease. All 94 dogs with LPE had lacteal dilatation graded as moderate to severe by WSAVA standards. Additionally, lacteal height, width, and height/width ratio were inversely correlated with serum albumin concentration in this group of dogs.[18] The reason for the discrepancies among the studies likely is multifactorial, but could include the fact that intestinal lymphangiectasia may be segmental or multifocal and biopsies may miss these lesions if the affected area is not sampled. Most of the biopsy samples in our study were obtained endoscopically with the potential to miss lesions deep within the submucosal and muscularis layers of the intestinal wall.[19] Nonetheless, the high prevalence of intestinal lymphangiectasia in dogs with a primary diagnosis of inflammatory enteritis may be important. Although secondary lymphangiectasia is thought to resolve with treatment targeting idiopathic inflammatory bowel disease, it may be that dogs not responding to this treatment would benefit from fat restriction because of persistent intestinal lymphangiectasia. Also, lymphatic dysfunction and lymphangiogenesis have long been suspected as a component of the pathology of inflammatory

bowel diseases in humans, and therapeutic interventions to stimulate lymphatic function have shown some promise in recent experimental murine models of inflammatory bowel disease.[20–23]

In a previous study of dogs with diet-responsive enteropathy, WSAVA histopathologic scoring identified more villous stunting in the enteropathy dogs versus controls.[24] Ultrastructural lesions of the mitochondria and brush border also were evaluated and in those dogs clinically responsive to food, and the microvillus height and additional ultrastructural lesions improved. The authors postulated that the recovery of enterocyte health after clinical response to diet suggests that architectural changes may be at least as important as more standard measures of pathology in the intestine, namely inflammatory infiltrates. It was suggested that this might explain why previous studies have failed to identify improvement in the inflammatory infiltrates of dogs with CE, despite clinical improvement.[24] Our study is in agreement that the morphologic lesions appear to be important in dogs with CE. Additionally, because these lesions were more common in hypoalbuminemic dogs, we suspect they may indicate a more severe form of CE. Whether the presence or relative severity of these lesions indicates a possible alternative etiology or the need for alternative treatment approaches is unknown. A prospective study in which clinical data and response to treatment can be monitored based on the histologic criteria is warranted.

Our study had several limitations. The median age of dogs in our CEH and CEN groups was younger (CEH, 3 years; CEN, 2 years) when compared to other studies of dogs with CE.[1, 25–28] Therefore, our population of dogs with CE may not reflect those seen at other institutions. Additionally, the retrospective design of our study did not allow us to determine the dogs that had received proper food or antibiotic trials before intestinal biopsy so that we could better classify them. Therefore, we applied the term CE to these cases, rather than idiopathic

inflammatory bowel disease. However, at our institution, it is common for food and antibiotic trials to be completed before the recommendation of biopsy, provided the patient is stable. Response to treatment and prognosis as it relates to the above histopathologic features could not be determined because of incomplete follow-up of many of the cases. Most biopsy samples were obtained endoscopically, which is the standard of practice at our institution but does have limitations, as described above.[19] Also, ileal biopsy samples (via ileocolonoscopy, celiotomy, or laparoscopy) were obtained in just 35% of dogs with CEH and 57% of dogs with CEN. The method of biopsy sample procurement was not different between groups, but a larger proportion of the CEN dogs had ileal biopsy samples obtained, which may have affected the results. Pathology can differ among sections of small intestine,[26,29] and ideally, ileal biopsy samples would have been available for evaluation in all dogs. Finally, despite the use of the WSAVA guidelines for histopathologic scoring of endoscopic intestinal biopsy samples, substantial interobserver variability occurs, controversy still exists, and scoring is still considered relatively subjective.[30]

In conclusion, many histopathologic features of chronic inflammatory enteropathy differ between dogs that are hypo- versus normoalbuminemic. The classic features of CE, lymphoplasmacytic cellular infiltrate and eosinophilic infiltrates, were not different between groups, whereas other inflammatory infiltrates and many of the morphologic features were different. These morphologic lesions may simply indicate a more severe disease process occurring in the intestine. Additional work is needed to elucidate the clinical relevance of these differences and to determine whether the presence or relative severity of these lesions indicates a possible alternative etiology or the need for alternative or supplemental treatment strategy in some cases of CE in dogs.

REFERENCES

1. Allenspach K, Wieland B, Gracone A, et al. Chronic enteropathies in dogs: Evaluation of risk factors for negative outcome. J Vet Intern Med 2007;21:700–708.
2. Jergens AE, Moore FM, Haynes JS, et al. Idiopathic inflammatory bowel disease in dogs and cats: 84 Cases (1987–1990). J Am Vet Med Assoc 1992;201:1603–1608.
3. German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. J Vet Intern Med 2003;17:8–20.
4. Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. Vet Clin North Am Small Anim Pract 2011;41:381–398.
5. Craven M, Simpson JW, Ridyard AE, et al. Canine inflammatory bowel disease: Retrospective analysis of diagnosis and outcome in 80 cases (1995–2002). J Small Anim Pract 2004;45:336–342.
6. Nakashima K, Hiyoshi S, Ohno K, et al. Prognostic factors in dogs with protein-losing enteropathy. Vet J 2015;205:28–32.
7. Equilino M, Theodoloz V, Gorgas D, et al. Evaluation of serum biochemical marker concentrations and survival time in dogs with protein-losing enteropathy. J Am Vet Med Assoc 2015;246:91–99.
8. Dossin O, Lavoue R. Protein-losing enteropathies in dogs. Vet Clin North Am Small Anim Pract 2011;41:399–418.
9. Willard MD, Jergens AE, Duncan RB, et al. Interobserver variation among histopathologic evaluations of intestinal tissues from dogs and cats. J Am Vet Med Assoc 2002;220:1177–1182.
10. Washabau RJ, Day MJ, Willard MD, et al. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med 2010;24:10–26.
11. Haas E, Reutgen BC, Gerner W, et al. Phenotypic characterization of canine intestinal intraepithelial lymphocytes in dogs with inflammatory bowel disease. J Vet Intern Med 2014;28:1708–1715.
12. Kawaguchi-Miyashita M, Shimada S, Kurosu H, et al. An accessory role of TCRcd+ cells in the exacerbation of inflammatory bowel disease in TCRa mutant mice. Eur J Immunol 2001;31:980–988.
13. Simmerson SM, Armstrong PJ, Weunschmann A, et al. Clinical features, intestinal histopathology, and outcome in protein-losing enteropathy in yorkshire terrier dogs. J Vet Intern Med 2014;28:331–337.
14. Willard MD, Helman G, Fradkin JM, et al. Intestinal crypt lesions associated with protein-losing enteropathy in the dog. J Vet Intern Med 2000;14:298–307.
15. Stroda K, Wakamatsu N, Gaschen L, et al. Histopathological, clinical, endoscopic, and ultrasound features of dogs with chronic enteropathies and small intestinal crypt lesions. J Vet Intern Med 2012;26:767–768.
16. Kleinschmidt S, Meneses F, Noltr I, et al. Retrospective study on the diagnostic value of full-thickness biopsies from the stomach and intestine of dogs with chronic gastrointestinal disease symptoms. Vet Pathol 2006;43:1000–1003.

17. Van der Gaag I, Happ_e RP. The histological appearance of peroral small intestinal biopsies in clinically healthy dogs and dogs with chronic diarrhea. Zentralbl Veterinarmed A 1990;37:401–416.
18. Rossi G, Cerquetella M, Antonelli E, et al. The importance of histologic parameters of lacteal involvement in cases of canine lymphoplasmacytic enteritis. Gastroenterol Hepatol Bed Bench 2015;8:33.
19. Larson RN, Ginn JA, Bell CM, et al. Duodenal endoscopic findings and histopathologic confirmation of intestinal lymphangiectasia in dogs. J Vet Intern Med 2012;26:1087–1092.
20. Alexander JS, Chaitanya GV, Grisham MB, et al. Emerging roles of lymphatics in inflammatory bowel disease. Ann NYAcad Sci 2010;1207:E75–E85.
21. Tonelli F, Giudici F, Liscia G. Is lymphatic status related to regression of inflammation in Crohn's disease? World J Gastrointest Surg 2012;4:228–233.
22. D'Alessio S, Correale C, Arena V, et al. Stimulation of lymphatic function via VEGFR-3 as a novel therapy for chronic experimental intestinal inflammation. Gastroenterology 2012;142: S84–S85.
23. D'Alessio S, Tacconi C, Fiocchi C, et al. Advances in therapeutic interventions targeting the vascular and lymphatic endothelium in inflammatory bowel disease. Curr Opin Gastroenterol 2013;29:608–613.
24. Walker D, Knuchel-Takano A, McCutchan A, et al. A comprehensive pathological survey of duodenal biopsies from dogs with diet responsive chronic enteropathy. J Vet Intern Med 2013;27:862–874.
25. Toresson L, Steiner JM, Suchodolski JS, et al. Oral cobalamin supplementation in dogs with chronic enteropathies and hypocobalaminemia. J Vet Intern Med 2016;30:101–107.
26. Procoli F, Motskula PF, Keyte SV, et al. Comparison of histopathologic findings in duodenal and ileal endoscopic biopsies in dogs with chronic small intestinal enteropathies. J Vet Intern Med 2013;27:268–274.
27. Titmarsh HF, Gow AG, Kilpatrick S, et al. Low vitamin D status is associated with systemic and gastrointestinal inflammation in dogs with a chronic enteropathy. PLoS ONE 2015;10: e0137377.
28. Heilmann RM, Volkmann M, Otoni CC, et al. Fecal S100A12 concentration predicts a lack of response to treatment in dogs affected with chronic enteropathy. Vet J 2016;215:96–100.
29. Casamian-Sorrosal D, Willard MD, Murray JK, et al. Comparison of histopathologic findings in biopsies from the duodenum and ileum of dogs with enteropathy. J Vet Intern Med 2010;24:80–83.
30. Jergens AE, Evans RB, Ackermann M, et al. Design of a simplified histopathologic model for gastrointestinal inflammation in dogs. Vet Pathol 2014;51:946–950.

CHAPTER 4: CLINICAL EFFECT OF A DIETARY CHANGE IN TEN DOGS WITH GLUCOCORTICOID-RESISTANT CHRONIC ENTEROPATHY AND PROTEIN-LOSING ENTEROPATHY

4.1 Overview

Objective: To describe the clinical effect and outcome of a dietary alteration as a sole change in dogs with glucocorticoid-resistant chronic enteropathy (CE) and protein-losing enteropathy (PLE).

Design: Prospective case series.

Animals: Ten dogs with glucocorticoid-resistant CE and concurrent PLE treated from January 2015-May 2018 at the James L. Voss Veterinary Teaching Hospital at Colorado State University.

Results: Following a dietary alteration, 8/10 dogs achieved a complete remission (CR) as defined by improvement in Canine Chronic Enteropathy Clinical Activity Index (CCECAI) to ≤3, 1 dog achieved partial remission (PR) defined by improvement in CCECAI without complete resolution of signs, and 1 dog had no response (NR) and was euthanized. 7/8 dogs achieving a CR have remained in remission up to 3.5 years after initial dietary alteration, 1 dog experienced a relapse at 12 months and was subsequently euthanized. The dog achieving PR experienced relapse 6 months following enrollment and was euthanized.

Conclusions and clinical relevance: Dogs with CE and PLE with previous lack of response to a combination of dietary therapies, glucocorticoids and immunosuppressive medications can achieve remission following a specific dietary change. An individualized, novel dietary approach may be an alternative to further immunosuppression or anti-inflammatory strategies in some dogs with difficult to treat CE with PLE.

4.2 Introduction

Protein-losing enteropathy (PLE) is a syndrome of excessive intestinal protein loss secondary to disease affecting the enteric mucosa and surrounding structures. While the exact mechanisms resulting in the protein loss have not been well described in veterinary medicine, it is likely the result of altered intestinal permeability, direct mucosal erosion or ulceration and secondary loss of protein, in association with altered lymphatic function and direct loss of protein-rich lymph.[1] Potential causes of PLE are numerous including parasitic enteritis, fungal enteritis, neoplasia, intestinal obstruction, intestinal erosions and ulcerations, intestinal lymphangiectasia (IL), and idiopathic CE.[2]

Dogs with PLE caused by CE have a variable prognosis and may fail to respond adequately to standard CE therapy regimens.[3] The traditional therapy for PLE is often a combination of dietary management and immunosuppressive or anti-inflammatory medications.[2] Why some dogs with PLE fail to adequately respond to this standard regimen is not well understood.[4]

Part of the difficulty of managing dogs with CE and PLE may be that the choice of diet in these cases can be challenging. A recent retrospective review found that 53% of dogs with chronic inflammatory enteropathies had some degree of lacteal dilation present on their intestinal biopsies. Of the dogs in this retrospective study who were hypoalbuminemic, 76% had some degree of lacteal dilation.[5] Also, a number of different breeds known to have a higher incidence of PLE (e.g. Yorkshire terrier, soft-coated Wheaton terrier) have been described to have varied lesions in their intestinal biopsies, including but not limited to lacteal dilation, lymphangitis, mixed degrees and types of intestinal inflammation, crypt lesions, and crypt abscesses.[6-8] While a novel or hydrolyzed protein diet are often suggested for dogs with inflammatory CE, strict fat

restriction is often recommended for dogs with intestinal lymphangiectasia. Unfortunately, the commercially available hydrolyzed diets generally have greater fat content than what is typically recommended for dogs with lymphangiectasia. Therefore, difficulty may arise with the dietary choice when managing a patient with histopathologic evidence of both inflammatory CE and lymphangiectasia. The ideal diet for many of these dogs may be unknown at the start of therapy, and trial and error is often necessary to determine the best diet in any individual case.

In cases of refractory CE and PLE, recommendations may be made to try alternative immunosuppressive or anti-inflammatory approaches instead of dietary interventions. While diet is considered an important component of the initial management of this condition, it may not be recognized as a therapeutic target in refractory cases because studies have shown that dogs with food-responsive enteropathy tend to have normal serum albumin and lower CCECAI scores.[2,4,9]

The purpose of this prospective study was to assess the efficacy of a dietary intervention as the single change in therapy in dogs with glucocorticoid-refractory CE with PLE. The success of the dietary change was measured by change in serum albumin and improvement in clinical signs. CCECAI[2] scores were monitored. We hypothesized that dogs with glucocorticoid-resistant CE and PLE would be clinically responsive to a change in diet.

4.3 Materials and Methods

4.3.1 Case selection criteria

Dogs presenting to the Colorado State University Veterinary Teaching Hospital with a complaint of persistent clinical signs (CCECAI ≥5) despite standard therapy for previously diagnosed CE and PLE were considered eligible for inclusion in the study. The original

diagnosis of PLE was based on serum albumin <2.5 g/dL and exclusion of other causes of hypoalbuminemia including renal loss of protein based on either negative urine dipstick or urine protein:creatinine ratio <0.5, and liver disease based on normal pre- and postprandial serum bile acids or normal synthetic liver function and enzyme activity on routine biochemistry panel in all dogs. Diagnosis of inflammatory CE was based on endoscopic exam and gastrointestinal histopathology with all dogs having variable types and degrees of inflammatory infiltrate. Only dogs with a primary diagnosis of inflammatory enteropathy based on their histopathologic evaluation were eligible for the study. Dogs were not eligible for the study if their primary diagnosis was intestinal neoplasia or IL. Dogs were excluded from the study if all non-gastrointestinal causes of hypoalbuminemia had not been excluded based on original diagnostic work-up or if significant concurrent disease was present (e.g. extra-intestinal disease, neoplasia).

Dogs were eligible for inclusion if they had been treated with prednisone or prednisolone at a dosage of at least 1 mg/kg (0.45mg/lb), PO, q 24 h, or divided q 12 h for at least 30 days with persistence of CCECAI score ≥5. A total of at least 30 consecutive days of therapy with prednisone or prednisolone within the previous 3 months was required for inclusion. If dogs were currently receiving prednisone or prednisolone at the time of enrollment the dose was not adjusted for the first 30 days of the study, unless significant adverse effects were occurring. Following 30 days, tapering of glucocorticoid was allowed. No dose increases were permitted at any time during the study.

In order to be eligible for the study, no other major changes to the dogs' current therapies outside of the dietary adjustment were allowed at the time of enrollment. This included changes to glucocorticoid therapy, immunosuppressive therapy, or the addition of new therapies such as cobalamin or calcitriol. The type of dietary adjustment was determined by the primary attending

clinician and a board-certified veterinary nutritionist (JS) and was partly based on the avoidance of specific and general types of diets that were previously attempted but determined to be ineffective, or were diets that the dog had not accepted. Diets chosen were at the discretion of the primary attending clinician and veterinary nutritionist, however, Royal Canin® diets including Royal Canin Veterinary Diet® Canine Hydrolyzed Protein Adult HP, Royal Canin Veterinary Diet® Canine Selected Protein Adult PW Moderate Calorie, and Royal Canin Veterinary Diet® Gastrointestinal Low Fat LF were available at no cost to the owner for the duration of the initial study period (30 days). Each dog underwent a one-week diet transition period where the new diet was gradually introduced in increasing proportion towards a complete transition. If the dogs were receiving immunosuppressive therapy (e.g. azathioprine, chlorambucil, cyclosporine) at the time of enrollment they were eligible for inclusion only if that therapy had been administered for >30 days at appropriate doses and with the provision that dose could not be altered for the first 30 days of the study. Tapering of these drugs was allowed following the initial 30-day period. Dose increases were not allowed. Dogs had to accept the diet and owners agreed to feed the diet exclusively. Owners provided informed consent and agreed to recheck evaluations for their dogs at 14 -30 days, and 3 months following study enrollment. The Institutional Animal Care and Use Committee at Colorado State University approved this project.

4.3.2 Monitoring and determination of clinical response

Dogs were re-evaluated at 14-30 days and 3 months to determine clinical response by use of CCECAI score (Figure 4.1) and biochemical parameters. A partial response (PR) was defined as improvement of clinical signs but persistence of CCECAI score >3. A complete response (CR) was defined as resolution of clinical signs as evidenced by CCECAI score decreasing to

insignificant levels (0-3)[2] within the study period. Non-response was defined by failure to improve CCECAI score. The decision to intervene with significant treatment changes in the first 30 days of the study would result in removal from the study.

4.3.3 Statistical analysis

Descriptive statistics were calculated for age, body weight, sex and clinical variables. Continuous variables were tested for normality using the Shapiro-Wilk test. Albumin was compared between the study time-points using a one-way repeated measure ANOVA. CCECAI scores were compared using Wilcoxson signed-rank test. P-values <0.05 were considered significant for all analyses.

	Clinical Inflammatory Bowel Disease Index (CIBDAI)	Canine Chronic Enteropathy Clinical Activity Index (CCECAI)
Attitude/Activity	0 *normal* 1 *slightly decreased* 2 *moderately decreased* 3 *severely decreased*	0 *normal* 1 *slightly decreased* 2 *moderately decreased* 3 *severely decreased*
Appetite	0 *normal* 1 *slightly decreased* 2 *moderately decreased* 3 *severely decreased*	0 *normal* 1 *slightly decreased* 2 *moderately decreased* 3 *severely decreased*
Vomiting	0 *normal* 1 *mild (1x/week)* 2 *moderate (2-3x/week)* 3 *severe (>3x/week)*	0 *normal* 1 *mild (1x/week)* 2 *moderate (2-3x/week)* 3 *severe (>3x/week)*
Stool consistency	0 *normal* 1 *slightly soft feces* 2 *very soft feces* 3 *watery diarrhea*	0 *normal* 1 *slightly soft feces* 2 *very soft feces* 3 *watery diarrhea*
Stool frequency	0 *normal* 1 *slightly increased (2-3x/d) or fecal blood, mucus, or both* 2 *moderately increased (4- 5x/d)* *3 severely increased (>5x/d)*	0 *normal* 1 *slightly increased (2-3x/d) or fecal blood, mucus, or both* 2 *moderately increased (4-5x/d)* *3 severely increased (>5x/d)*
Weight loss	0 *none* 1 *mild (<5%)* 2 *moderate (5-10%)* 3 *severe (>10%)*	0 *none* 1 *mild (<5%)* 2 *moderate (5-10%)* 3 *severe (>10%)*
Albumin levels		0 *albumin ≥2.0 g/dL* 1 *albumin 1.5-1.9 g/dL* 2 *albumin 1.2-1.4 g/dL* 3 *albumin <1.2 g/dL*
Ascites or peripheral edema		0 *none* 1 *mild amount ascites or peripheral edema* 2 *moderate amount ascites or peripheral edema* 3 *severe amount ascites or peripheral edema*
Pruritus		0 *no pruritus* 1 *occasional episodes of licking* 2 *regular episodes of licking, but stops when dog is asleep* 3 *dog regularly wakes up to itch*

Figure 4.1 Comparison of clinical activity indices (CIBDAI versus CCECAI).

4.4 Results

Twenty dogs with glucocorticoid-refractory CE with PLE were eligible for enrollment in the study over the time period of January 2015- February 2018. In 3 cases, the owners would not commit to feeding the diet exclusively, and in 5 additional cases at the time of would-be enrollment the primary attending clinician recommended a significant change in therapy in addition to a dietary alteration (e.g. increase in glucocorticoid dose, addition of immunosuppressive drug, addition of calcitriol), and the owner accepted this recommendation making the dog ineligible for the study. Therefore, 12 dogs were enrolled. Two dogs did not eat the diet exclusively following the allowed one-week transition period; therefore 10 dogs completed the study.

Seven dogs were neutered males, 2 dogs were spayed females, and 1 dog was an intact female. Median age was 5 years (range, 1 to 8 years). Median body weight was 24 kg (range 2.6-46 kg). Median body condition score (BCS) (0-9 scale)[10] was 4 (range 2-5). The diagnosis of PLE as a result of CE had been made in all dogs via intestinal biopsy and exclusion of other possible causes of hypoalbuminemia. All dogs had significant proteinuria excluded with a negative dipstick (n=8), or with a urine protein:creatinine ratio <0.5 (n=2). Three dogs had fasted and post-prandial bile acids performed, 2/3 were within normal limits for both (fasted <5 umol/L; post prandial <25 umol/L) and 1 dog had mild elevations of both fasted and post prandial bile acids (fasted 56 umol/L; post-prandial 41 umol/L). The dog with mildly elevated bile acids was panhypoproteinemic with no other significant suggestion of liver dysfunction. Three dogs had only fasted bile acids performed all the within normal reference interval (<5 umol/L). The other 4 dogs had significant liver disease excluded as the cause of hypoalbuminemia based on the presence of normal synthetic liver function and enzyme activity on routine biochemistry panel. 8/10 dogs had a basal cortisol >2 ug/dL or normal

adrenocorticotropin hormone (ACTH) stimulation test to exclude hypoadrenocorticism as the cause of hypoalbuminemia. The other two dogs were administered glucocorticoid therapy prior to testing for hypoadrenocorticism and remained on glucocorticoid therapy at the time of enrollment. All dogs had exocrine pancreatic insufficiency excluded by fasted serum canine trypsin-like immunoreactivity (TLI) > 5.0 ng/ml. Additionally, all dogs had fecal testing with or without deworming, and all dogs had abdominal ultrasound by a board-certified radiologist to screen for intestinal or abdominal masses prior to endoscopic biopsy.

Five dogs had endoscopic biopsy of both the duodenum and ileum; 5 dogs had duodenal biopsy only. Duodenal cellular infiltrate was described as lymphoplasmacytic in 6 dogs, lymphoplasmacytic with eosinophils in 2 dogs, lymphoplasmacytic with neutrophils in 1 dog, and lymphoplasmacytic with eosinophils and neutrophils in 1 dog. The degree of duodenal cellular infiltrate was classified as mild in 5 dogs, moderate in 4 dogs, and severe in 1 dog (lymphoplasmacytic with neutrophils) based on WSAVA criteria.[11] Ileal cellular infiltrate was lymphoplasmacytic in 2 dogs, lymphoplasmacytic with eosinophils in 2 dogs, and lymphoplasmacytic with eosinophils and neutrophils in 1 dog. The degree of ileal cellular infiltrate was described as mild in all 5 dogs. The ileal cellular infiltrate type differed from the duodenal cellular infiltrate type in 1/5 dogs. The degree of ileal cellular infiltrate differed from the degree of duodenal cellular infiltrate in 2/5 dogs. All dogs had WSAVA scoring for morphologic changes of the duodenum performed. Lacteal dilation was absent in 3 dogs, noted as mild in 6 dogs, and moderate in 1 dog. Crypt distension was mild in 3 dogs, and marked in 1 dog. Further descriptive data on the ten study dogs from the time of original diagnosis is shown in Table 4.1.

At the time of enrollment in the study, all ten dogs were exhibiting diarrhea. Other complaints included lethargy (n=6), weight loss (n=7), and vomiting (n=1). At enrollment, 8/10 dogs were being administered prednisone or prednisolone with a median dose of 0.86 mg/kg (0.39 mg/lb), PO, q 24 h, or divided q 12 h (range 0.5 mg/kg [0.23 mg/lb], PO, q 24 h, or divided q 12 h to 1.5 mg/kg [0.68 mg/lb], PO, q 24 h, or divided q 12 h). The median duration of therapy was 1.75 months (range 1-3 months). All dogs had been administered prednisone or prednisolone as an initial therapy at the time of diagnosis. The median starting dose was 1.5 mg/kg (0.68 mg/lb), PO, q 24 h, or divided q 12 h (range 1.0 mg/kg [0.45 mg/lb], PO, q 24 h, or divided q 12 h to 2.0 mg/kg [0.9 mg/lb], PO, q 24 h, or divided q 12 h) and all dogs were administered this dosage for a minimum of one month.

One dog not being administered prednisolone or prednisone at the time of enrollment had been weaned off prednisone one week prior, following 4 months of therapy with an initial dosage of 1.5 mg/kg (0.68 mg/lb), PO, q 24 h for one month and subsequent tapering, with no noted clinical or clinicopathologic improvement. Additionally, this dog developed a splenic thrombosis diagnosed via abdominal ultrasound, which influenced the decision to wean off prednisone therapy in this case. The second dog not being administered prednisolone or prednisone at the time of study entry had been weaned off prednisolone 2 months prior, following 2 months of therapy with an initial dosage of 1.1 mg/kg (0.5 mg/lb), PO, q 24 h for 5 weeks and subsequent tapering following limited clinical or clinicopathologic improvement and the perception of significant adverse effects (polyuria, polydipsia, polyphagia, and behavioral changes) reported by the owner.

At the time of enrollment, 5 dogs were being administered immunosuppressive medications for > 30 days including chlorambucil (n=1; 5 mg/m^2, PO, q 24 h), azathioprine (n=1,

2.2 mg/kg [1 mg/lb], PO, q 48 h), leflunomide (n=1, 1 mg/kg (0.45 mg/lb), PO, q 24 h, and cyclosporine (n=2, 5.5-8.1 mg/kg [2.52-3.68 mg/lb], PO, q 24 h). Two additional dogs had been treated with cyclosporine (6-10 mg/kg [2.72-4.5 mg/lb), PO, q 24 h) but were no longer receiving it. One other dog had been administered leflunomide (2 mg/kg [0.9 mg/lb], PO, q 24 h), but it was discontinued prior to the study.

Other medications that were being utilized at the time of enrollment included aspirin (n=3, 0.5-1.1 mg/kg [0.23-0.5 mg/lb], PO, q 24 h), calcium carbonate (n=1, 20 mg/kg [9 mg/lb], PO, q 12 h), clopidogrel (n=3, 2 mg/kg [0.9 mg/lb], PO, q 24 h), cobalamin (n=9, 250- 1000 mcg, SQ, q weekly), calcitriol (n=2, 5-10 ng/kg, PO, q 12 h), famotidine (n=1, 0.6 mg/kg [0.27 mg/lb], PO, q 12 h), furosemide (n=1, 1 mg/kg [0.45 mg/lb], PO, q 12 h), magnesium sulfate (n=2, 1-2 meq/kg, PO, q 24 h), maropitant (n=1, 1.6 mg/kg [0.7 mg/lb], PO, q 24 h), metronidazole (n=1, 12.8 mg/kg [5.8 mg/lb], PO, q 12 h), Proviable® (n=2, 1 capsule, PO, q 24 h), tylosin (n=4, 15-25 mg/kg [6.8-11.4 mg/lb], PO, q 12 h), and sucralfate (n=1; 1 gram, PO as slurry, q 8 h).

Diets utilized in the study included Royal Canin Veterinary Diet® Gastrointestinal Low Fat LF (n=4), Royal Canin Veterinary Diet® Canine Selected Protein Adult PW Moderate Calorie (n=1), and a veterinary nutritionist-formulated home cooked diet (n=5). A comparison of these diets is shown in Table 4.2. Prescription diets that had been attempted at various times prior to enrollment in the study included Royal Canin Veterinary Diet® Gastrointestinal Low Fat LF (n=4), Royal Canin Veterinary Diet® Canine Selected Protein Adult PW Moderate Calorie (n=2), Royal Canin Veterinary Diet® Canine Hydrolyzed Protein Adult HP (n=7), Purina® Pro Plan® Veterinary Diet HA Hydrolyzed® (n=3), Hills Prescription Diet® i/d® Low Fat (n=1), Royal Canin Veterinary Diet® Ultimino® (n=1), Royal Canin Veterinary Diet® Canine Selected

Protein Adult PV (n=1), and Hills Prescription Diet® z/d® Canine (n=2). All dogs for which Royal Canin Veterinary Diet® Gastrointestinal Low Fat LF was selected as their study diet had previously been treated with various hydrolyzed diets with limited to no clinical response seen. The decision for a formulation of a customized home cooked diet formulated by a boarded veterinary nutritionist was based on expected patient acceptability of the diet or previous non-response to both a hydrolyzed diet and low-fat diet.

At enrollment, median CCECAI score was 11.5 (range 6-14). Median albumin for all ten dogs was 1.9 g/dL (range 1.5-2.7 g/dL). At 2-4 weeks median CCECAI score was 4 (range 1-11), and median serum albumin was 2.6 g/dL (range 1.6-3.1 g/dL). Data was available for all dogs at 3 months except for the dog who had no response and was euthanized at 30 days. At 3 months, median CCECAI score was 1, and median serum albumin was 2.8 g/dL. Data is shown for the 9 dogs with a complete data set in Figures 4.2 & 4.3 (box and whiskers). CCECAI scores and serum albumin concentration were available for all dogs at all time points in the study with the exception of the dog who was euthanized at 30 days. A significant improvement in CCECAI score was seen between time of enrollment and 14-30 days ($p=0.004$) and between time of enrollment and 3 months ($p=0.004$), but not between 14-30 days and 3-month time point ($p=0.156$). A significant improvement in serum albumin was seen between time of enrollment and 14-30 days ($p<0.001$) and between time of enrollment and 3 months ($p=0.005$), but not between 14-30 days and 3-month time point ($p=0.3228$). Data regarding the response and outcome of individual dogs is shown in Table 4.3.

Table 4.1 Selected clinicopathologic data at time of original diagnosis

Laboratory Value	Median (Range)	Reference Interval
Albumin	1.6 (1.3-2.2)	3.0-4.3 g/dL
Globulin	1.55 (1.3-3.5)	1.5-3.2 g/dL
Cholesterol	97.5 (80-174)	130-300 mg/dL
Total calcium	8 (3.9-9.9)	9-11.5 mg/dL
Ionized calcium	1.19 (0.7-1.4)*	1.25-1.45 mmol/L
Magnesium	1.6 (1.1-1.9)	1.8-2.4 mg/dL
Lymphocyte	1.3 (0.5-3.2)	1 - 4.8 x10^3/ul
Platelet	344 (176-636)	200 - 500 x10^3/ul
Cobalamin	258 (<150-794)	251-908 ng/L
Folate	10.5 (3.5-20)	7.7-24.4 mcg/L

*data available for 7 dogs.

Table 4.2 Composition of the diets used in the study

Diet	Protein Source	% Fat*	% Pro*	% CHO*
Royal Canin Veterinary Diet® Canine Selected Protein Adult PW Moderate Calorie Dry	Whitefish	25	26	49
Royal Canin Veterinary Diet® Canine Selected Protein Adult PW Canned	Whitefish	41	25	34
Royal Canin Veterinary Diet® Gastrointestinal Low Fat LF Dry	Chicken	17	24	59
Royal Canin Veterinary Diet® Gastrointestinal Low Fat LF Canned	Chicken, pork	16	29	55
Tilapia/quinoa^	Tilapia	16	44	40
Turkey/cous cous^	Turkey	12	39	49
Tuna/potato^	Tuna	10	53	37

*% metabolizable energy (ME) Pro: protein, CHO: carbohydrate

^Full recipe not included so not complete and balanced as described, should not be fed exclusively for extended period of time without appropriate supplements.

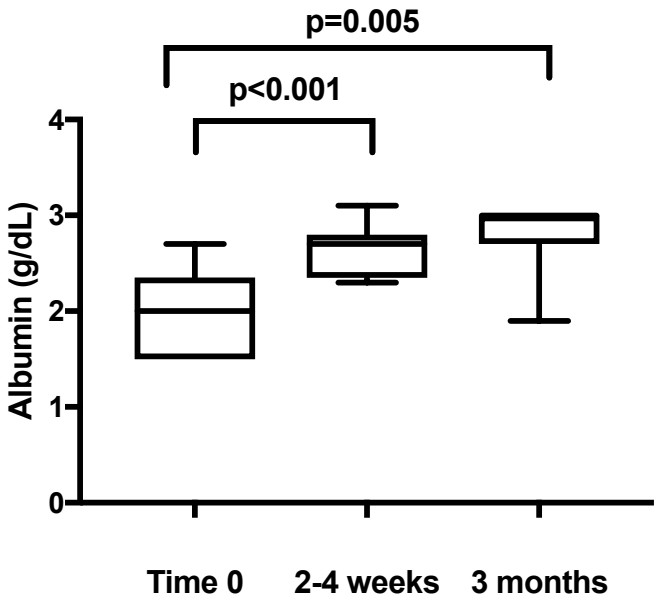

Figure 4.2 Change in serum albumin over time period of study. Whiskers depict minimum and maximum values. Horizontal bar represents median. Lower and upper quartiles also shown.

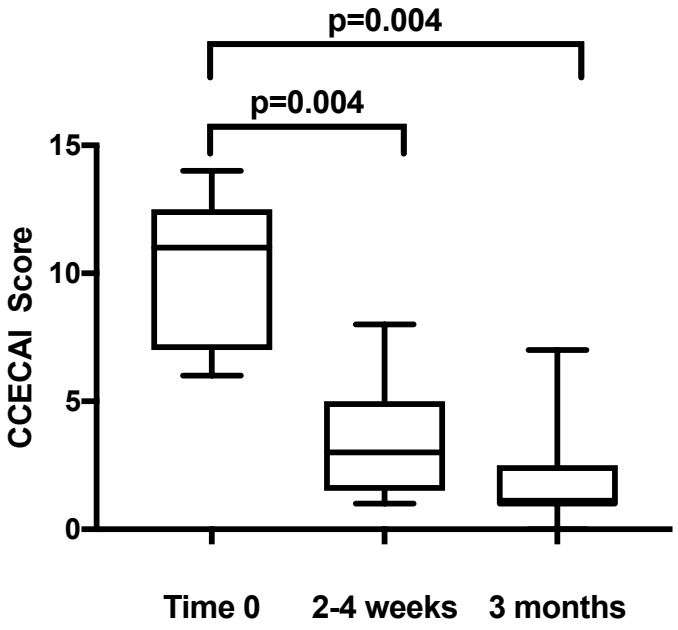

Figure 4.3 Change in CCECAI score over time period of study. Whiskers depict minimum and maximum values. Horizontal bar represents median. Lower and upper quartiles also shown.

Table 4.3 General descriptive data on the 10 dogs in the study

Response	Age (years)	Sex	Breed	Biopsy duodenum (infiltrate^/degree)	Diet	Final outcome
CR1	5	MC	Australian shepherd	LP/Moderate	Royal Canin GI LF*	Remission at 30 months (lost to follow-up) Albumin (30 months): 3.1 g/dL Tx: RC GI LF, B12, psyllium
CR2	5	FS	Bernese mountain dog	LP/ Mild	Royal Canin GI LF*	Remission at 28 months (lost to follow-up) Albumin (24 months): 3.9 g/dL Tx: RC GI LG, B12
CR3	6	MC	Yorkshire terrier mix	LPN/Severe	Royal Canin PW%	Remission at 6 months (at publication) Albumin (6 months): 2.8 g/dL Tx: RC WP, pred 0.25 mg/kg/day, B12, tylosin
CR4	3	MC	Rottweiler mix	LPE/Moderate	Tuna/potato$	Relapse at 12 months, euthanized at 15 months
CR5	6	MC	Bernese mountain dog	LPEN/Mild	Tilapia/quinoa$	Remission at 12 months (at publication) Albumin (10 months): 3.1 g/dL Tx: turkey/oat$, budesonide 0.025 mg/kg/day, B12
CR6	1	FI	Samoyed	LPE/Mild	Tuna/potato$	Remission at 40 months (at publication) Albumin (36 months): 4.1 g/dL Tx: tuna/potato, pred 0.125mg/kg/day, azathioprine 2.2 mg/kg/q 48 h, B12
CR7	3	MC	Labrador retriever	LP/Mild	Turkey/cous cous$	Remission at 4 months (at publication) Albumin (4 months): 2.8 g/dL Tx: turkey/cous cous, B12, calcitriol
CR8	7	FS	Yorkshire terrier	LP/Mild	Tilapia/quinoa$	Remission at 12 months (at publication) Albumin (10 months): 3.2 g/dL Tx: tilapia/quinoa, pred 0.25 mg/kg q 48 h, aspirin
PR1	8	MC	Bernese mountain dog	LP/Moderate	Royal Canin GI LF*	PR at 3 months, relapse and euthanasia at 6 months Albumin (3 & 6 months): 2.4 g/dL& 1.9 g/dL
NR1	2	MC	Yorkshire terrier	LP/ Mild	Royal Canin GI LF*	No response at 30 days (euthanasia) Necropsy: endocardial thrombosis

CR: complete response, PR: partial response, NR: no response, MC: male castrated, FS: female spayed, FI: female intact, ^LP: lymphoplasmacytic, LPE: lymphoplasmacytic with eosinophils, LPN: lymphoplasmacytic with neutrophils, LPEN: lymphoplasmacytic with eosinophils and neutrophils, * Royal Canin Veterinary Diet® Gastrointestinal Low Fat LF, % Royal Canin Veterinary Diet® Canine Selected Protein Adult PW Moderate Calorie, $ full recipe not included so not complete and balanced as described, should not be fed exclusively for extended period of time without appropriate supplement.

4.5 Discussion

This study evaluated the clinical effects of a dietary change in 10 cases of glucocorticoid-resistant CE with PLE. Eight dogs achieved a complete remission with medium to long-term survival reported in 7 of those dogs. Of those 7 dogs, 3 responded to a veterinary prescription diet, and 4 responded to a veterinary nutritionist-formulated home cooked diet.

The exact reason for the response to dietary change in these cases is unknown. In 8/9 dogs that achieved a complete or partial response during the study period, the dietary therapy recommended and implemented was more fat-restricted than the diets the dogs had previously been fed. In the cases of the veterinary nutritionist-formulated home cooked diets, the dietary fat restriction was also beyond what is found in commercially available diets (Table 4.2). Dietary fat restriction has previously been shown to be effective in allowing for the reduction in prednisone dosage in 24 dogs with IL and PLE.[12] In addition to IL, intestinal inflammatory infiltrates were noted in 18/24 dogs in that study.[12] Because IL can occur primarily or secondary to inflammatory CE[1], it can sometimes be difficult to determine which lesion is most responsible for the clinical disease. While lymphatic dilation based on WSAVA scoring was generally minimal in our dogs, the presence or extent of lymphatic disease may have been underappreciated. The diagnosis of lymphangiectasia can be hindered by the fact that the disease can be segmental, and in some cases confined to the deeper parts of the intestinal wall.[13] Therefore lesions can be missed, particularly when endoscopic biopsies are obtained.[14] Furthermore, of the 9 dogs that responded partially or completely, 4 did not have ileal or jejunal biopsies obtained. It is possible that more significant lacteal dilation would have been observed on ileal[15,16], jejunal, or full-thickness surgical biopsies. In addition, lymphatic function and flow has not been assessed in dogs with CE with or without concurrent PLE. In humans with Crohn's disease it is thought that

improvement in the flow and function of the lymphatic system may contribute to a reduction in the inflammatory process in the intestine.[17] This has been demonstrated in mouse models of chronic intestinal inflammation.[18] If concurrent lymphatic disease was under-recognized in this group of dogs with CE and concurrent PLE, dietary fat restriction may have played a significant role in the positive responses seen. In this instance, it is expected that other commercially available low fat diets would have produced the same response. It is worth noting that a dietary fat restriction beyond what is found in commercially available diets may be necessary in some cases of PLE, and may have resulted in the clinical response seen in the dogs fed a veterinary nutritionist-formulated home cooked diet.

The clinical improvement in the dogs in this study may also be attributed to providing a novel protein source compared to the diet the dogs were eating previously, and a subsequent reduction in some component of an adverse food reaction.[19] In some cases, the improvement may be due to the diet providing both a novel protein source and a significant reduction in dietary fat compared to the diet the dogs were treated with previously. This may be especially true for those cases that utilized a veterinary nutritionist to formulate a homemade diet, as their individual diet was formulated with the intention to identify a novel protein in each case, and to adjust the fat content as dictated by a complete dietary history. Determining if the lymphangiectasia is a primary or secondary process may help determine the best initial diet choice, however, because loss of lymph into the intestinal lumen can induce secondary inflammation[1] the distinction between primary and secondary lymphangiectasia can be challenging. While some dogs with inflammatory CE and secondary lymphangiectasia will respond to dietary treatment of inflammatory CE alone, it is possible that in some cases dogs need dietary management of both conditions, which is difficult to achieve with the commercial diets currently available.

Improved digestibility of the new diet compared to the previously fed diet could also explain clinical improvement in this group of dogs, however that is difficult to assess in individual client-owned dogs. Veterinary nutritionist-formulated home cooked diets typically contain few ingredients, so it could also be considered that the absence of certain ingredients could be responsible for the improvement,[20] rather than the dietary protein source or fat content.

The number of cases in this study limits any attempt to identify factors that may have predicted the dietary response in these cases of CE with PLE. As discussed previously lacteal dilation was generally absent to mild in these dogs, the importance of which is difficult to determine due to the challenges of diagnosing lymphangiectasia and lymphatic dysfunction. A variety of types of inflammatory infiltrates were seen including lymphoplasmacytic, lymphoplasmacytic with eosinophils, lymphoplasmacytic with neutrophils, and lymphoplasmacytic with eosinophils and neutrophils. The degree of inflammatory infiltrate in the duodenum and ileum (where available) varied. However, notably among the 7 dogs that maintained complete remission through the study period and beyond, mild was the most common degree of inflammatory infiltrate in the duodenum (4/7 dogs) and ileum (4/4). Anecdotally, at our institution we often find that dogs with the highest CCECAI scores at the time of diagnosis, and those with inconsistent response to glucocorticoids and immunosuppressives tend to have the mildest degree of inflammatory infiltrate on intestinal biopsy. This would fit with previous studies that have found that the degree of inflammatory infiltrate often does not correlate with clinical disease.[2,3] Whether a mild infiltrate tends to predict lack of glucocorticoid response or a certain dietary response is unknown. This possible phenomenon deserves further study in prospective clinical trials with histopathology performed by a single blinded pathologist.

Following the change in diet, 3 dogs have maintained control of their disease beyond the study period without glucocorticoids or other immunosuppressives for 4-30 months (last known or time of publication). The other 4 surviving dogs have maintained remission with a combination of therapies including diet, low doses of glucocorticoids, immunosuppressive drugs (1 dog) and supportive therapies (Table 4.3). Yorkshire terriers with PLE can respond to dietary management without the use of anti-inflammatory or immunosuppressive therapies[6] and dietary fat restriction was effective in in the management of dogs with intestinal lymphangiectasia and PLE.[12] The ability to manage PLE dogs without the use of glucocorticoids or with low doses of glucocorticoids is desirable, as glucocorticoids can have significant side effects[21] and can negatively affect quality of life. Furthermore, a hypercoagulable state has been identified in dogs with PLE,[22] which could be worsened with the use of glucocorticoids. Additionally, the use of long-term immunosuppressive drugs can increase the risk of opportunistic infections, lymphoproliferative disorders, and other adverse effects.[23] Dietary modifications have limited side effects, and known efficacy for the treatment of gastrointestinal disease.[19] Prospective clinical trials are needed in a variety of dogs with CE and PLE to determine whether certain specific dietary approaches (e.g. hydrolyzed, low fat, or veterinary nutritionist-formulated home cooked diet) with or without the use of concurrent anti-inflammatory drugs and/or immunosuppressives are best, perhaps in relation to clinical, clinicopathologic, or histologic characteristics.

Food trials are often considered a preliminary treatment in dogs with CE based on evidence that 50% of dogs will be responsive to intervention with food alone[19] and some dogs with PLE may be responsive to diet alone.[6] However, there is a tendency in clinical practice to consider dogs to be non-responsive to food after a single trial with one type of diet, and often the

validity of these trials cannot be determined. In one study of 23 dogs with CE responding to a hydrolyzed or easily digestible diet, 9 had been previously determined to be non-responsive to food.[24] Furthermore, the tendency to distinguish dogs as either food-responsive, glucocorticoid/immunosuppressive-responsive, or non-responsive may undermine the complexity of the disease process in cases of CE with or without concurrent PLE. Although only a small number of dogs, the results of this study would suggest that it may be just as important to optimize dietary therapy as it is to adjust anti-inflammatory or immunosuppressive therapy in some cases of CE with PLE.

A significant improvement in CCECAI score and serum albumin was seen between time of enrollment and 14-30 days and between time of enrollment and 3 months, but not between 14-30 days and 3-month time point. Although this is just a small number of dogs, this suggests that clinical and biochemical improvement that may be attributed to a dietary change in dogs with CE and concurrent PLE would likely be seen within 14-30 days of the dietary change, which is in line with previous studies.[2,9,25] Tapering of medications was allowed following the initial 30-day period of the study, therefore it is possible that the tapering of those medications could have played a role in the changing CCECAI score or serum albumin level following the 30-day time point.

This study has several limitations. First, the owners of the dogs participating in this study committed to continued intensive treatment despite the refractory nature of their dogs' disease and unknown prognosis. Therefore, case selection may have been bias towards highly dedicated owners that may be predisposed to placebo effect. Two dogs would not eat the diet exclusively and so had to be excluded from the study which may have also skewed the data. The enrollment of dogs with significant intestinal disease in a prospective study requiring adherence to a strict

diet and limiting the changes to therapy that are allowed made case recruitment challenging. Dogs were enrolled in the study after the diagnosis of CE and PLE had been established which limited some aspects of the study. Gross endoscopic scores could not be obtained for all dogs. Ileal biopsies were not obtained in all dogs. Pathology can differ between sections of intestine therefore this may have limited the understanding of the disease process in those cases.[14,15] Additionally, even with the use of the WSAVA guidelines for histopathologic scoring[11] of endoscopic intestinal biopsy samples, substantial interobserver variability occurs,[26] and scoring is still considered subjective.[27] Ideally, a single pathologist blinded to the case data would have evaluated all of these samples, however samples were not available for further review in all cases.

It is impossible to be certain whether the remission achieved in these cases was due to the dietary change alone because many dogs were receiving other treatments as outlined above. Furthermore, a control group was not included so spontaneous improvement or response to other treatments cannot be entirely ruled out. Prior to enrollment all dogs had received prednisone or prednisolone at 1-2 mg/kg/day for a time period of at least 30 days, but at enrollment in the study various doses of glucocorticoids were being utilized in addition to other medications. Four dogs that responded were receiving immunosuppressive therapies that may also have influenced their response. Neither drug levels nor suppression of T-cell function were measured for dogs on immunosuppressive therapies, so efficacy of those drugs was not determined. However, all dogs had been receiving their immunosuppressive drugs for >30 days prior to enrollment and 3/4 dogs were able to be weaned off these drugs following their diet change, suggesting that these drugs may not have been the most important component of their therapy. Many dogs were also treated with supportive therapies prior to enrollment and continued on those medications after

enrollment. The contribution of those therapies cannot be determined. Nine dogs were being supplemented with cobalamin, however levels were not re-assessed in all cases so it is unknown if hypocobalaminemia may have contributed to inadequate or lack of response in some dogs. In 8/10 dogs, a basal cortisol or ACTH stimulation test was performed and excluded hypoadrenocorticism as the cause of clinical signs and hypoalbuminemia. Hypoadrenocorticism could not be completely excluded in the other 2 dogs due to current glucocorticoid therapy. However, previous lack of response to glucocorticoid administration would suggest that hypoadrenocorticism was unlikely in these cases. The dog with NR, a Yorkshire terrier, had mild-moderately elevated fasting and post-prandial serum bile acids, however this dog was panhypoproteinemic suggesting that intestinal disease was at least a component of his hypoalbuminemia. Based on his synthetic liver function tests and liver enzyme activities liver failure was considered unlikely in his case. Considerations for the cause of the dog's elevated serum bile acids include portal vein hypoplasia, common in Yorkshire Terriers,[28] or ileal disease.[29] Four dogs had hepatic failure excluded on the basis of synthetic liver function tests and liver enzyme activities alone as fasting and post-prandial serum bile acids were not performed. However, all of these dogs responded to the dietary change, which would not be expected in cases of liver failure.

PLE has a variable but guarded prognosis,[30] and euthanasia is common after repeated failure of multiple therapies. If clients are willing and able, consultation with a board-certified veterinary nutritionist and new dietary approaches should be considered in dogs refractory to standard treatments. Further studies are needed to assess the contribution of lymphatic disease in cases of CE with or without concurrent PLE and identification of biomarkers that may predict the best dietary approach in cases of PLE would be useful. Prospective clinical trials are also

needed to evaluate the optimal dietary therapy and to determine if dietary therapy alone or in combination with other therapies is best for individual dogs with CE and PLE.

REFERENCES

1. Dossin O, Lavoué R. Protein-losing enteropathies in dogs. Vet Clin North Am Small Anim Pract 2011; 41:399-418.
2. Allenspach K, Wieland B, Gröne A, et al. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med 2007;21:700-8.
3. Craven M, Simpson JW, Ridyard AE, et al. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995–2002). J Small Anim Pract 2004;45:336-42.
4. Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? J Small Anim Pract 2016;57:589-99.
5. Wennogle SA, Priestnall SL, Webb CB. Histopathologic characteristics of intestinal biopsy samples from dogs with chronic inflammatory enteropathy with and without hypoalbuminemia. . J Vet Intern Med 2017;31:371-6.
6. Rudinsky AJ, Howard JP, Bishop MA, et al. Dietary management of presumptive protein‐losing enteropathy in Yorkshire terriers. J Small Anim Pract 2017; 58:103-8.
7. Littman MP, Dambach DM, Vaden SL, et al. Familial protein‐losing enteropathy and protein‐losing nephropathy in Soft Coated Wheaten Terriers: 222 cases (1983–1997). J Vet Intern Med 2017;14:68-80.
8. Simmerson SM, Armstrong PJ, Wünschmann A, et al. Clinical features, intestinal histopathology, and outcome in protein‐losing enteropathy in yorkshire terrier dogs. J Vet Intern Med 2014;28:331-7.
9. Allenspach K, Culverwell C, Chan D. Long-term outcome in dogs with chronic enteropathies: 203 cases. Vet Rec 2016:vetrec-2015.
10. Laflamme, D. R. P. C. Development and validation of a body condition score system for dogs. Canine Pract. 1997;22:10-15.
11. Washabau RJ, Day MJ, Willard MD, et al. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med 2010;24:10–26.
12. Okanishi H, Yoshioka R, Kagawa Y, et al. The clinical efficacy of dietary fat restriction in treatment of dogs with intestinal lymphangiectasia. J Vet Intern Med 2014; 28:809-17.
13. Kull PA, Hess RS, Craig LE, et al. Clinical, clinicopathologic, radiographic, and ultrasonographic characteristics of intestinal lymphangiectasia in dogs: 17 cases (1996–1998). J Am Vet Med Assoc 2001; 219:197-202.
14. Larson RN, Ginn JA, Bell CM, et al. Duodenal endoscopic findings and histopathologic confirmation of intestinal lymphangiectasia in dogs. J Vet Intern Med 2012;26:1087-92.
15. Procoli F, Motskula PF, Keyte SV, et al. Comparison of histopathologic findings in duodenal and ileal endoscopic biopsies in dogs with chronic small intestinal enteropathies. J Vet Intern Med 2013;27:268-74.
16. Casamian‐Sorrosal D, Willard MD, Murray JK, et al. Comparison of histopathologic findings in biopsies from the duodenum and ileum of dogs with enteropathy. J Vet Intern Med 2010;24:80-3.

17. Tonelli F, Giudici F, Liscia G. Is lymphatic status related to regression of inflammation in Crohn's disease? World J GI Surg 2012:228.
18. D'alessio S, Correale C, Arena V, et al. P016 Stimulation of lymphatic function via VEGFR-3 as a novel therapy for chronic experimental intestinal inflammation. J Crohn's and Col 2012;6:S17.
19. Gaschen FP, Merchant SR. Adverse food reactions in dogs and cats. Vet Clin North Am Small Anim Pract 2011;41:361-79.
20. Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. Vet Clin North Am Small Anim Pract 2011; 41:381-98.
21. Cohn LA. Glucocorticoid therapy. In: Ettinger SG, Feldman EC, eds. Textbook of veterinary internal medicine. 7th ed. St Louis: Saunders Elsevier, 2010;602–608.
22. Goodwin LV, Goggs R, Chan DL, et al. Hypercoagulability in dogs with protein‐losing enteropathy. J Vet Intern Med 2011;25:273-7.
23. Viviano KR. Update on immunosuppressive therapies for dogs and cats. . Vet Clin North Am Small Anim Pract 2013;43:1149-70.
24. Mandigers PJ, Biourge V, Van Den Ingh TS, et al. A randomized, open‐label, positively‐controlled field trial of a hydrolyzed protein diet in dogs with chronic small bowel enteropathy. J Vet Intern Med 2010;24:1350-7.
25. Marks S, Laflamme DP, McAloose D. Dietary trial using a commercial hypoallergenic diet containing hydrolyzed protein for dogs with inflammatory bowel disease. Vet Therapeutics 2002.
26. Willard MD, Jergens AE, Duncan RB, et al. Interobserver variation among histopathologic evaluations of intestinal tissues from dogs and cats. J Am Vet Med Assoc 2002;220:1177–1182.
27. Jergens AE, Evans RB, Ackermann M, et al. Design of a simplified histopathologic model for gastrointestinal inflammation in dogs. Vet Path 2014;51:946-50.
28. Hirose N, Uchida K, Kanemoto H, Ohno K, et al. A retrospective histopathological survey on canine and feline liver diseases at the University of Tokyo between 2006 and 2012. J Vet Med Sci 2014;76:1015-20.
29. Lawrence YA, Steiner JM. Laboratory evaluation of the liver. Vet Clin North Am Small Anim Pract 2017;47:539-53.
30. Nakashima K, Hiyoshi S, Ohno K, et al. Prognostic factors in dogs with protein-losing enteropathy. The Veterinary Journal. 2015;205:28-32.

CHAPTER 5: LYMPHATIC ENDOTHELIAL CELL IMMUNOHISTOCHEMICAL MARKERS FOR EVALUATION OF THE INTESTINAL LYMPHATIC VASCULATURE IN DOGS WITH CHRONIC ENTEROPATHY WITH AND WITHOUT PROTEIN-LOSING ENTEROPATHY AND HEALTHY CONTROLS

5.1 Overview

Background: Lymphatic endothelial cell (LEC) immunohistochemical markers have identified intestinal lymphatic vasculature abnormalities in humans with inflammatory bowel disease (IBD), but have not been used to evaluate the intestinal lymphatic vasculature in a group of dogs with chronic enteropathy (CE).

Objectives: Utilize LEC markers to identify, quantify, and measure intestinal lymphatic vasculature in endoscopic biopsies of CE dogs and evaluate whether changes to the lymphatic vasculature correlate to serum albumin concentrations.

Animals: 24 dogs with CE (n=13 serum albumin concentration <2.5 g/dL [CE-PLE], n=11 serum albumin concentration >2.5 g/dL [CE-N]) and 4 healthy controls.

Methods: Prospective cohort study. LEC immunolabeling with Prox-1 and LYVE-1 performed on endoscopic biopsies from 24 dogs with CE and 4 healthy controls (HC). Duodenal and ileal (CE dogs) or duodenal only (HC dogs) villous lacteal width (VLW), mucosal lacteal width (MLW), and number of mucosal LEC (MLEC) determined for each case and analyzed for correlation to serum albumin. Lymphatic parameters were also compared between CE-PLE, CE-N and healthy controls dogs.

Results: Serum albumin concentrations correlated with the MLEC in the duodenum (p= 0.0332) and ileum (p=0.0038) and with villous (p=0.0223) and mucosal (p=0.0006) lacteal width in the ileum. Villous (p=0.0218) and mucosal (p=0.0031) lacteals in the ileum of dogs with CE-PLE were wider than dogs with CE-N.

Conclusions and Clinical Importance: LEC immunolabeling identified mucosal lymphangiectasia in dogs with CE, particularly in the ileum of dogs with CE-PLE. Evaluation of villous lacteals alone is likely underestimating abnormalities of the lymphatic vasculature in dogs with CE.

5.2 Introduction

The term chronic enteropathy (CE) refers to various conditions of the intestinal tract that are characterized by the presence of gastrointestinal signs of at least 3 weeks duration.[1-3] In up to 20% of cases of canine CE, intestinal protein loss occurs as a result of the disorder, which is referred to as protein-losing enteropathy (PLE).[1,4] While CE is among the most common of the many causes of PLE, the reasons why some dogs with CE develop concurrent PLE while others maintain normal protein levels are not well understood. Intestinal lymphangiectasia (IL), a disorder of dilated lymphatic vasculature within the intestinal tract, is another common cause of PLE.[5] While IL can be a primary process, it can also occur as a result of increased lymphatic pressure due to inflammatory or neoplastic infiltrates in the intestine, which can result in direct loss of protein-rich lymph and/or lymphatic dysfunction.[6,7]

Several recent studies have found a relationship between serum albumin concentrations and IL in cases of idiopathic CE in the dog.[8-10] Lymphangiectasia can be underappreciated on routine histopathologic exam of the intestine due to the fact that it can have a segmental distribution, and in some cases, be confined to deeper layers of the intestine that may not be sampled endoscopically.[7] Lymphatic abnormalities have long been described in humans with Crohn's disease (CD)[11-13], a type of inflammatory bowel disease (IBD) characterized by idiopathic inflammation and formation of granulomas that can be localized to the ileum or found

diffuse throughout the small intestine.[14] In some cases of CD, dilated lymphatics and lymphatic granulomas have been identified in the deeper mucosa, submucosa, and muscularis layers of the intestine, as well as in the mesentery.[15-17]

Lymphatic endothelial cells (LEC) are derived from venous progenitor cells. Their expression of various markers distinguishes them from blood endothelial cells.[18] In humans with CD, immunolabeling with LEC-specific markers has been demonstrated to be superior to standard microscopy for revealing abnormalities of the lymphatic vasculature, including lymphangiectasia and obstructed lymphatics.[16] Lymphatic markers are numerous and include, human prospero homeobox (Prox-1), a nuclear transcription factor,[19] and LYVE-1, a lymphatic vascular endothelial hyaluronic acid receptor.[20] Immunolabeling with Prox-1 and LYVE-1 have previously been used to differentiate types of angiosarcomas in the dog,[21] but to our knowledge have not been used to evaluate the intestinal lymphatic vasculature in a group of dogs with CE.

The objective of this study was to utilize LEC markers to identify, quantify, and measure the intestinal lymphatic vasculature in endoscopic biopsies of dogs with CE, and to determine whether abnormalities associated with the lymphatic vasculature were related to serum albumin concentrations in dogs with idiopathic CE. A second objective was to explore how the use of LEC markers correlated to traditional methods of identifying lymphangiectasia in cases of canine CE.

5.3 Materials and Methods

5.3.1 Study Population

Client-owned dogs presenting to Colorado State University Veterinary Teaching Hospital for evaluation of chronic gastrointestinal signs (decreased appetite, vomiting, diarrhea, weight

loss) of >3 weeks duration were recruited for participation in the study. In order to be eligible for inclusion, dogs were required to have fecal screening (fecal floatation, *Giardia* IFA, and *Cryptosporidium* IFA) with no parasites detected, no evidence of significant non-gastrointestinal illness as assessed via routine hematology and biochemical profile, and a histopathologic diagnosis of inflammatory enteritis for which no distinct cause could be identified. Dogs with histopathologic evidence of intestinal neoplasia were excluded. In order to be eligible for the study, exclusion of exocrine pancreatic insufficiency as a cause of their clinical signs with a fasted serum canine trypsin-like immunoreactivity concentration >5.0 ng/ml was required. Hypoalbuminemic dogs were also required to have no significant proteinuria (negative urine dipstick or urine protein:creatinine ratio <0.5) and no evidence of significant hepatic disease based on normal fasted and post-prandial bile acids or normal synthetic liver function and enzyme activity on routine biochemistry panel.

Recorded data included age, breed, sex, weight, duration of illness, clinicopathological data, and results of any diagnostic imaging performed. Additionally, at the time of enrollment, owners were asked to score appetite, activity level, vomiting, fecal consistency, fecal frequency, weight loss, and pruritus for each dog. Following the results of the biochemical profile (serum albumin) and abdominal ultrasound (peritoneal effusion), and using the owner's scores, a CCECAI[1] was calculated for each dog. The Clinical Review Board at Colorado State University approved all procedures and written consent was obtained from the owners of each of the study participants.

5.3.2 Healthy Controls

Four client-owned dogs were recruited to serve as healthy controls. To be established as clinically healthy, dogs had to have no significant history of gastrointestinal signs (canine inflammatory bowel disease activity index [CIBDAI][22] score of 0), normal physical exam, and no significant abnormalities on hematology and biochemical profile. To be eligible for inclusion, dogs had to be undergoing uncomplicated elective dental prophylaxis as recommended by their primary care veterinarian performed by Colorado State University Veterinary Teaching Hospital Community Practice service. Gastroduodenoscopy and biopsy of the duodenum was performed routinely by the investigators prior to elective dental prophylaxis. Duodenal samples (n=12) were placed in formalin for routine histopathologic analysis via haematoxylin and eosin (H&E) and immunohistochemical analysis of lymphatic endothelial cell markers (described below). The Colorado State University Institutional Animal Care and Use Committee (IACUC) approved all procedures for healthy controls (protocol ID# 18-7829A) and written consent was obtained from the owners of each of the healthy control dogs.

5.3.3 Endoscopic Examination and Histopathologic Evaluation

For clinical cases, gross lymphatic dilation endoscopic scores were assigned and recorded in each section of the intestine by a single investigator. Endoscopic scoring of lacteal dilation was modeled after a previously published scoring system.[23] In brief, the absence of any white foci in the intestine resulted in a score of 0, focal to multifocal white foci resulted in a score of 1, and if diffuse white foci were appreciated, a score of 2 was assigned. Twelve duodenal and 5 ileal biopsies were obtained from each case for histopathologic evaluation. Histopathologic evaluation of endoscopically-obtained intestinal tissue from clinical cases (duodenum and ileum)

and healthy control dogs was performed by a board-certified veterinary pathologist and pathologist-in-training blinded to clinical data, clinicopathologic information, and groups. Biopsy samples were assessed as adequate for evaluation, and both evaluators reviewed duodenal and ileal tissues and reached a consensus for the presence and degree of morphologic criteria (villous stunting, epithelial injury, crypt distension, lacteal dilation, mucosal fibrosis) and inflammatory criteria (intraepithelial lymphocytes, lamina propria eosinophils, lamina propria lymphocytes/plasma cells, lamina propria neutrophils) based on WSAVA guidelines.[24] For the degree of each change, the following scores were applied based on established criteria: 0 = normal, 1 = mild, 2 = moderate, 3 = marked. Scores for lymphatic dilation were based on the most severely affected villous in each case. If the lymphatic vessel occupied 0-25% of the villous width a score of 0 was given, 25-50% was a score of 1, 50-75% was a score of 2, and greater than 75% of the width of the villous resulted in a score of 3.

5.3.4 Immunohistochemistry

All IHC labeling was performed on the Leica Bond III[a] immunostainer. Formalin-fixed paraffin embedded tissues were sectioned at 5 micrometers and mounted on positively charged slides for IHC. Dewaxing and epitope retrieval were performed on the Bond III[a] instrument. Epitope retrieval was performed using the ER1 solution, a pH 6.0 citrate buffer. Antibodies against Prox-1 (rabbit polyclonal; Angiobio[b]) and LYVE-1 (rabbit polyclonal; Abcam[c]) were diluted 1:100 in PowerVision IHC Super Block (Leica) and incubated on tissue sections for 20 minutes. All wash steps were performed in triplicate using Leica Bond wash buffer. Tissue

[a] Leica Biosystems Inc.,IL, USA
[b] Angiobio, Delmar, CA, USA
[c] Abcam, Cambridge, MA, USA

sections were then incubated for 20 min with goat secondary antibody at 1:200 dilution and alkaline phosphatase polymer. The Leica Fast Red chromogenic substrate for alkaline phosphatase was used to detect specific immunoreactivity of each antibody. Upon completion of immunolabeling, samples were counterstained with hematoxylin. Isotype-matched irreverent primary antibodies were used as negative controls.

5.3.5 Immunohistochemical Evaluation

Prox-1 and LYVE-1 IHC slides for clinical cases and healthy controls, as well as negative control slides were digitally scanned via Phillips® Ultra Fast Scanner slide[d] and analyzed with the use of Phillips® Image Management System viewer (version 2.4.1.2)[e] by a single evaluator, who was blinded to the case data. The viewing trail feature was used to ensure that all areas of the slide were assessed. In order to be counted or measured as a lymphatic vessel, a visible lumen was required in addition to immunolabeling with the LEC markers. On low power, 10 well-oriented villi associated with cryptal tissue and dispersed throughout the slide were selected for measurement of villous lymphatic vessel width (VLW). Lacteal width (um) was taken as the distance from one side of the Prox-1 or LYVE-1 labeled lacteal to the other, measuring roughly perpendicular to the midline of the lacteal. Next, at low power the mucosa was evaluated for immunolabeling. Well-oriented mucosal areas were identified for examination. In 10, distinct, 20x fields in the mucosa, lymphatic vessels labeled with Prox-1 or LYVE-1 were identified and lacteal width (um) was taken, measuring roughly perpendicular to the midline of the lacteal (mucosal lymphatic vessel width [MLW]). If no lacteals were identified in the field, the next field was examined. No more than 2 lacteals were measured per 20x field. If

[d] Phillips IntelliSite Pathology Solution, Phillips Electronics, The Netherlands
[e] Phillips IntelliSite Pathology Solution, Phillips Electronics, The Netherlands

>2 lacteals were identified in the field, all visible lacteals were measured, and the widest and most narrow lacteal of the group were used in the analysis. Additionally, numbers of mucosal lymphatic endothelial cells (MLEC) labeled with Prox-1 were counted to assess lymphatic vessel density. Counted areas were outlined with the Image Management Systems[b] drawing tool to ensure that 20x fields were not counted more than once

For all the lymphatic variables (VLW, MLW, MLEC) the mean of the ten measurements was recorded for each tissue in each case. In 2/24 clinical cases, and 1/4 healthy control cases, only 5 definitively measurable mucosal lymphatics could be identified, therefore those cases had their mucosal lacteal width scores calculated as the mean of the 5 measurements, rather than 10. Finally, on high power, well-oriented villi were examined to find the villous most severely affected with lymphangiectasia. Lacteal width (as described above) and villous width, taken as maximum distance from one side of the villous to the other and measuring perpendicular to midline, were measured in the most severely affected villous for each case and in each section of intestine. These measurements were used to determine the width of the lacteal as a percentage of the width of the villous. This was then translated to a score of 0, 1, 2, or 3, corresponding to 0-25%, 25-50%, 50-75%, or greater than 75%, respectively, and recorded as the lacteal dilation score as assessed via IHC (IHC-LD).

5.3.6 Statistical Analysis

Descriptive statistics were calculated for age, sex, weight, duration of illness, CCECAI scores, and histopathological scores. The distribution of data for statistical analysis was assessed by the Shapiro-Wilk test. Data was not normally distributed. Spearman (rank-based) correlation was used to evaluate relationships between the lymphatic variables (villous lacteal vessel width

(VLW) mucosal lymphatic width [MLW] and number of mucosal LEC [MLEC]) and albumin in each section of intestine. Spearman (rank-based) correlation was also performed to assess the relationships between the above lymphatic variables and histologic and endoscopic scores for lacteal dilation. In addition, the lacteal dilation score as assessed via IHC (IHC-LD) was compared to the lacteal dilation score as assessed via H&E with a Spearman (rank-based) correlation. Data from healthy control dogs were not included in the Spearman (rank-based) correlations.

For group comparisons, dogs with serum albumin concentration <2.5 g/dL were placed in the CE-PLE group (n=13), and those with serum albumin concentrations ≥2.5 g/dL were placed in the CE-N group (n=11). Duodenal lymphatic variable scores were also available for 4 healthy dogs. One-way ANOVA with post hoc pairwise comparisons was performed to determine if there were differences between groups for age and body weight. Fischer's exact test was used to determine if there were differences between groups for sex. Duodenal lymphatic variable scores were compared between CE-PLE, CE-N, and healthy dogs via Kruskal-Wallis test with post hoc pairwise comparisons. Ileal lymphatic variable scores were compared between CE-PLE and CE-N dogs via Mann Whitney-*U*. All statistical analysis was performed using GraphPad Prism scientific statistic software (Graph Pad Prism, GraphPad Software, Inc, San Diego, CA). Statistical significance for all statistical comparisons was set at $p < 0.05$.

5.4 Results

Thirty dogs were screened for inclusion in the study. Following routine histopathologic evaluation, one dog was diagnosed with intestinal lymphoma and was therefore excluded from the study. Ileal biopsies were unable to be obtained in two dogs, which prompted exclusion from

the study. In three additional cases, the quality of the endoscopic biopsies were considered inadequate for IHC evaluation of LEC markers due to either the presence of only villous tips, or the absence of any well-oriented tissue limiting the ability to accurately examine the lymphatic vasculature. Therefore, 24 clinical cases were included in the final data analysis. The majority of dogs (n=22) had hypoadrenocorticism excluded by a serum basal cortisol >2 ug/ml or normal response to ACTH. All dogs had routine abdominal ultrasonography performed by or under the supervision of a board-certified veterinary radiologist to evaluate for extra-intestinal disease or extra-luminal intestinal masses prior to endoscopic exam.

Of the 24 cases, 13 dogs had a serum albumin concentration <2.5 g/dL, and 11 dogs had a serum albumin concentration > 2.5 g/dL. Breeds in the CE-PLE group included Bernese mountain dog (2), Labrador retriever (2), mixed breed dog (2), and one each of the following: Australian shepherd, English bulldog, Great Pyrenees, Pembroke Welsh corgi, pug, Rottweiler, and Yorkshire terrier. Breeds in the CE-N group included mixed breed dog (3), and one each of Australian terrier, Bernese mountain dog, Brittany spaniel, Cavalier King Charles spaniel, German shepherd dog, German shorthaired pointer, Labrador retriever, and Siberian husky. The healthy control group included 3 mixed breed dogs and one Australian shepherd dog; 3 dogs were spayed females, and one dog was a neutered male. The CE-PLE group consisted of 7 neutered males and 5 spayed females, and the CE-N group consisted of 8 neutered males and 3 spayed females. Sex was not different between any of the groups. Descriptive statistics of interest for clinical cases and healthy controls are presented in Table 5.1.

Prox-1 and LYVE-1 immunolabeling of lymphatics from a non-study dog with a histopathologic diagnosis of marked lymphangiectasia in the duodenum and ileum was used as a positive control. Prox-1 has been reported to be variably expressed in enteroendocrine epithelial

cells in the crypts. In our cases, Prox-1 labeling was visible in some individual crypt epithelial cells, but was easily differentiated from lymphatic vessel labeling. LEC labeling was not observed on any of the negative control slides. Examples of immunolabeled villous lymphatics in CE-PLE, CE-N, and healthy control dogs are shown in Figure 5.1. Examples of immunolabeled mucosal lymphatics in CE-PLE and CE-N dogs, as well as healthy controls are shown in Figure 5.2. Examples of mucosal lymphangiectasia in CE-PLE dogs are shown in Figure 5.3.

The number of mucosal lymphatic endothelial cells (MLEC) in the duodenum ($p=0.0332$) and ileum ($p=0.0038$) were correlated with serum albumin concentrations. Villous lacteal width (VLW) and mucosal lacteal width (MLW) in the ileum were also correlated with serum albumin ($p=0.0223$, and $p=0.0006$, respectively). Summary statistics and correlation data for all lymphatic variables are presented in Table 5.2.

Villous lacteals were wider in the duodenum of CE dogs with PLE ($p=0.0042$) and normoalbuminemic CE dogs ($p=0.0341$) when compared to healthy controls. Villous lacteal width in the duodenum was not different between hypoalbuminemic and normoalbuminemic dogs with CE (Figure 5.4). Mucosal lacteals in the duodenum were wider in hypoalbuminemic CE dogs when compared to healthy controls ($p=0.0017$; Figure 5.5). Hypoalbuminemic dogs had a higher number of LEC in the duodenal mucosa compared to healthy control dogs ($p=0.0008$; Figure 5.6). Villous ($p=0.0218$) and mucosal ($p=0.0031$) lacteals were wider in the ileum of hypoalbuminemic dogs with CE when compared to normoalbuminemic dogs with CE (Figures 5.7 and 5.8, respectively). Additionally, hypoalbuminemic dogs with CE had a higher number of mucosal LEC's in the ileum when compared to normoalbuminemic dogs with CE ($p=0.0014$; Figure 5.9).

Duodenal lacteal dilation scores as assessed via blinded H&E review were correlated to duodenal lacteal dilation scores as assessed via blinded IHC (Spearman r=0.6742; p=0.0003) and villous lacteal width in the duodenum (Spearman r=0.4634; p= 0.0226). Duodenal endoscopic lacteal dilation scores were correlated to duodenal lacteal dilation scores as assessed via blinded IHC (Spearman r=0.4907; p=0.0223). Ileal lacteal dilation scores as assessed via blinded H&E review were correlated to ileal lacteal dilation scores as assessed via blinded IHC (Spearman r=0.5952; p=0.0022) and villous lacteal width in the ileum (Spearman r=0.5292; p=0.0078). Ileal endoscopic lacteal dilation scores were weakly correlated to ileal lacteal dilation scores as assessed via blinded IHC (Spearman r=0.4176; p=0.0423). Endoscopic scores were not correlated to mucosal lymphatic scores or to villous lymphatic width. Lacteal dilation scores as assessed via routine H&E were not correlated with the number of mucosal LEC's or the mucosal lymphatic width scores.

Table 5.1 Selected descriptive statistics for dogs with chronic enteropathy with and without protein-losing enteropathy and healthy controls

Variable	CE-PLE (n=13) median (range)	CEN (n=11) median (range)	Healthy (n=4) median (range)
Age	7 (1-10)	4 (1-12)	5 (4-8)
Body weight (kg)	24 (5-42)	21 (6-43)	18.5 (14-30)
CCECAI	11 (5-19)	8 (4-11)	NA
Duration of illness	3 (1-12)	6 (2-24)	NA
Albumin (g/dL)	1.7 (0.9-2.4)	3.3 (2.6-3.9)	4 (3.7-4.2)
Duodenal endoscopic LD score	0 (0-2)	0 (0-1)	NA
Ileal endoscopic LD score	0 (0-2)	0 (0-2)	NA
Duodenal histologic LD score	1 (0-2)	0 (0-1)	NA
Ileal histologic LD score	0 (0-2)	0 (0-2)	NA
Duodenal mucosal score	6 (2-10)	2 (0-9)	NA
Duodenal inflammatory score	5 (2-8)	3 (1-8)	NA
Duodenal total WSAVA score	11 (5-18)	5 (1-13)	NA
Ileal mucosal score	4 (0-8)	0 (0-4)	NA
Ileal inflammatory score	3 (0-6)	2 (1-7)	NA
Ileal total WSAVA score	7 (0-14)	2 (1-11)	NA

^Total score for villous stunting, epithelial injury, crypt distension, lacteal dilation and mucosal fibrosis. *Total score for intraepithelial lymphocytes, lamina propria lymphocytes/plasma cells, lamina propria eosinophils, and lamina propria neutrophils. CE-PLE: chronic enteropathy with protein-losing enteropathy; CE-N: chronic enteropathy with serum albumin concentration ≥2.5 g/dL; LD: lacteal dilation; WSAVA: World Small Animal Veterinary Association.

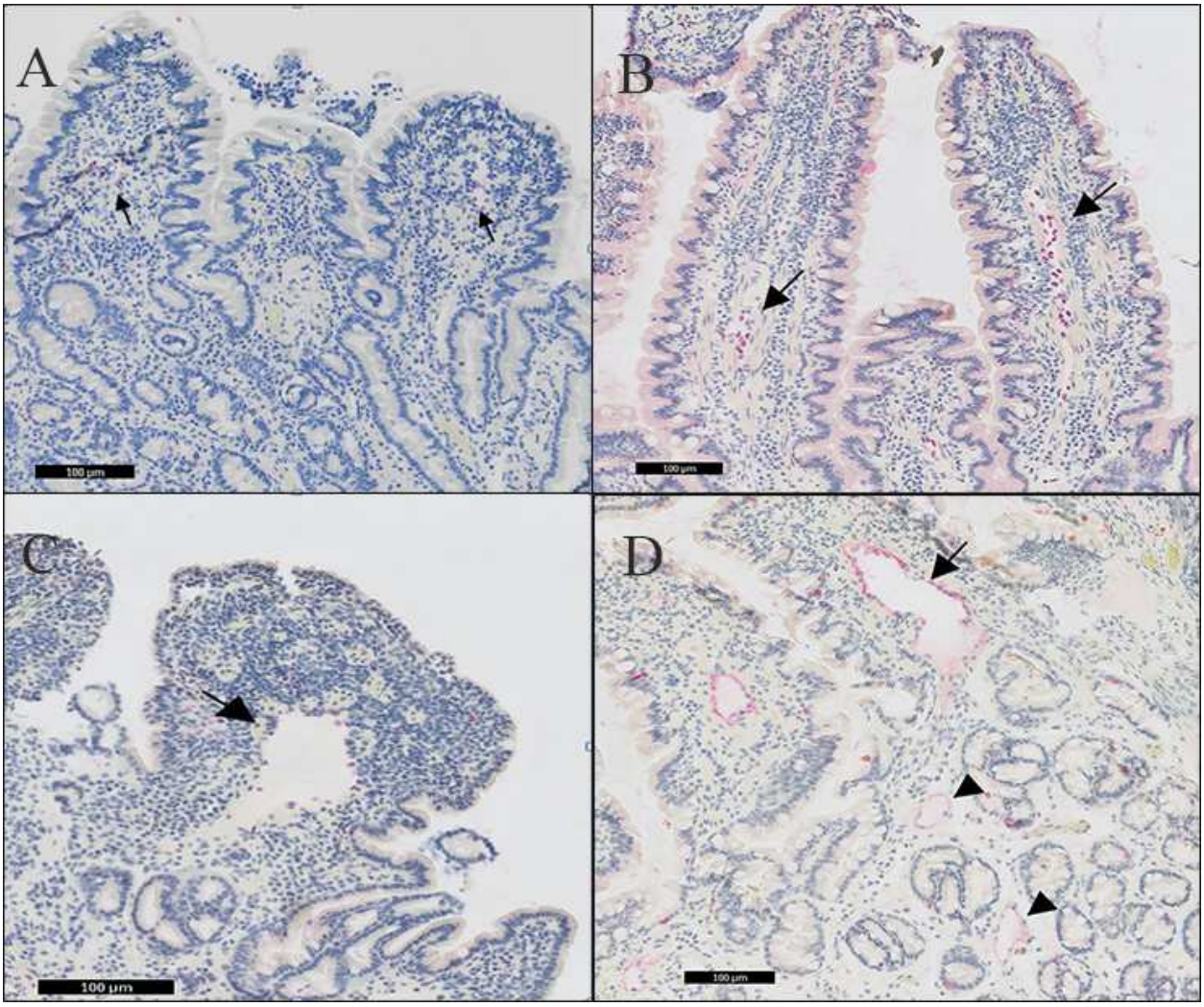

Figure 5.1 Immunolabeled villous lymphatics of dogs with CE and healthy control dog**.** (A) Duodenal villi from healthy control dog. Arrows show nuclear immunoreactivity of lymphatic endothelial cells along villous lacteals. Prox-1 IHC. (B) Duodenal villi from dog with CE and serum albumin concentration of 3.4 g/dL. Prox-1 IHC. (C) Duodenal villi from dog with CE and serum albumin concentration of 1.7 g/dL and central villous lymphatic dilation (arrow; lacteal dilation score=2) Prox-1 IHC. Severe lamina proprial lymphoplasmacytic inflammation is also visible. (D) Ileal villi from dog with CE, serum albumin concentration 1.9 g/dL, and central villous lymphatic dilation (arrow; lacteal dilation score=2). Cytoplasmic immunoreactivity of lymphatic endothelial cells shown with LYVE-1 IHC. Mucosal lymphangiectasia can also be seen (arrowheads).

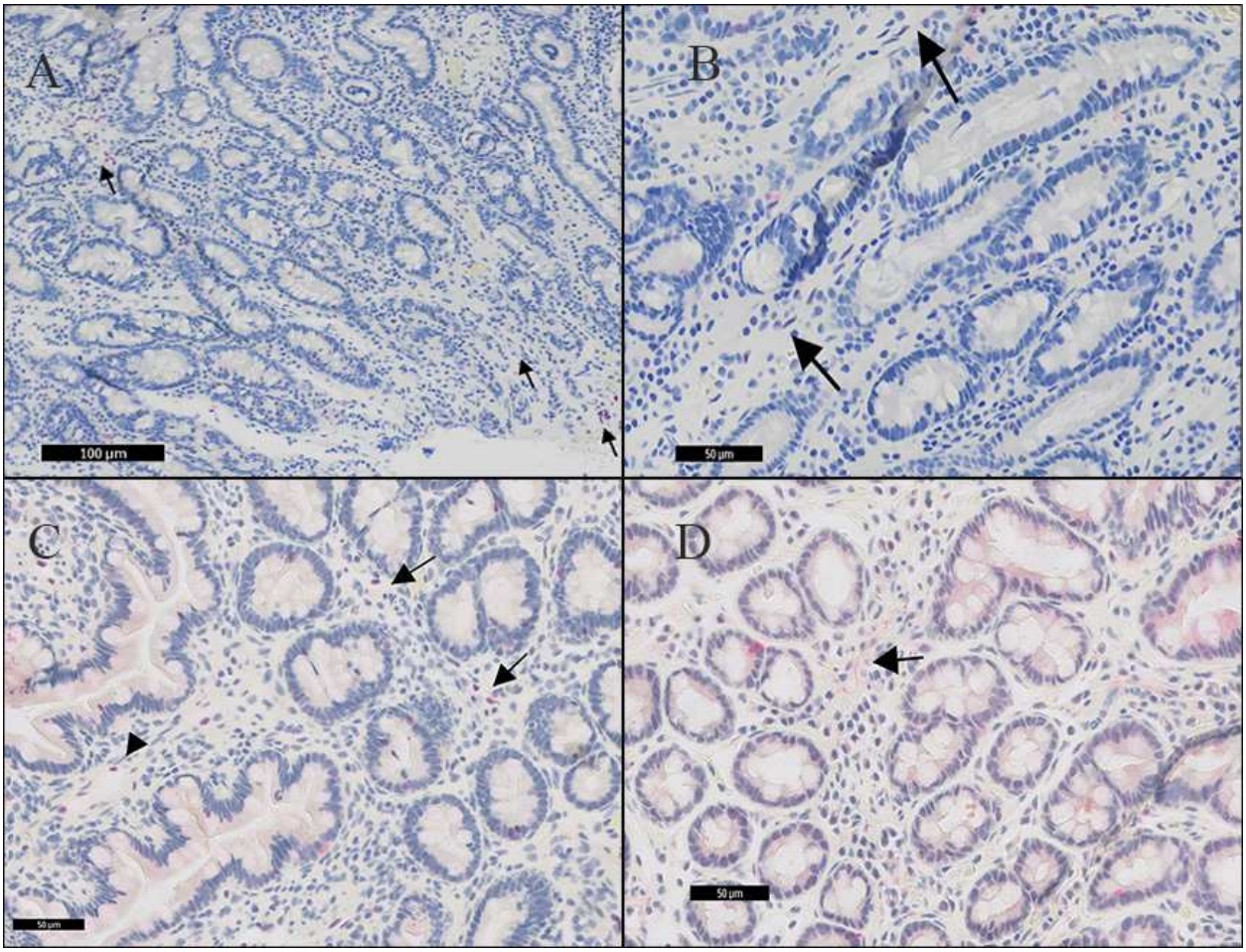

Figure 5.2 Immunolabeled mucosal lymphatics of dogs with CE and healthy control dogs. (A) Arrows show villous and mucosal nuclear immunoreactivity of lymphatic endothelial cells in duodenum of healthy control dog. Prox-1 IHC. (B) Mucosal nuclear immunoreactivity of lymphatic endothelial cells in duodenum healthy control dog at 20x. Prox-1 IHC. (C) Ileal mucosal lymphatics (arrows) in a dog with CE and serum albumin concentration 3.6 g/dL. Immunolabeled villous lymphatic (arrowhead) also shown to show orientation of lymphatics. Prox-1 IHC. (D) Cytoplasmic immunoreactivity of lymphatic endothelial cells (arrow) shown with LYVE-1 IHC in the ileum of a dog with CE and serum albumin concentration 3.5 g/dL.

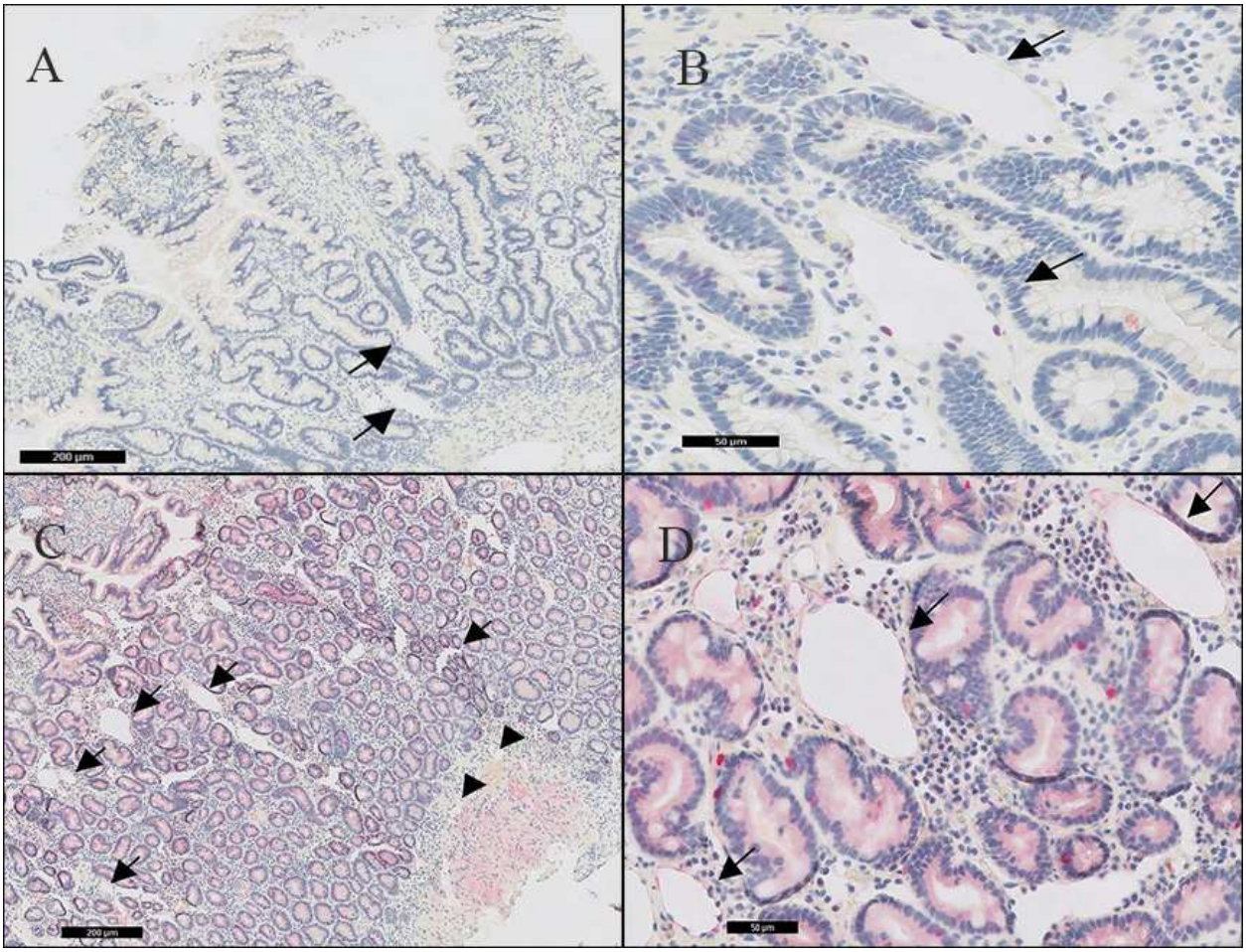

Figure 5.3 Immunolabeled mucosal lymphatics of dogs with CE-PLE. (A&B) Mucosal lymphangiectasia (arrows) in the ileum of a dog with CE and serum albumin concentration 1.5 g/dL at low (A) and high (B) power. Prox-1 IHC. (C) Mucosal lymphangiectasia (arrows) in the duodenum of a dog with serum albumin concentration 1.6 g/dL. Lymphatic dilation in the submucosa is also observed (arrowheads). LYVE-1 IHC. (D) 20x view of duodenal mucosal lymphangiectasia (arrows) from C. LYVE-1 IHC.

Table 5.2 Intestinal villous and mucosal lymphatic scores for dogs with chronic enteropathy with and without protein-losing enteropathy (duodenal and ileal) and healthy controls (duodenal only)

Variable	Group	Median	IQR	Min	Max	Spearman's Correlation Score^	P-value*
Duodenal VLW (um)	CE-PLE	34.6	20.5	15	53.9	-0.2533	0.2323
	CE-N	24.9	14.9	16.4	63.4		
	Healthy	12.2	3.3	11.4	15.6		
Duodenal MLW (um)	CE-PLE	21.8	16.9	9.6	41.5	-0.3777	0.0688
	CE-N	11.6	8.2	7.1	39.5		
	Healthy	6.7	1.5	5.7	7.3		
Duodenal MLEC	CE-PLE	25.6	11.4	13.1	37.8	-0.4359	0.0332
	CE-N	16.8	14.1	5.2	30.2		
	Healthy	2.5	0.9	2.1	3.1		
Ileal VLW (um)	CE-PLE	22.9	24.1	11	51.2	-0.4644	0.0223
	CE-N	15.3	6	10.9	39.8		
Ileal MLW (um)	CE-PLE	28.1	22.25	5	51	-0.6514	0.0006
	CE-N	12.8	7.4	9	25.7		
Ileal MLEC	CE-PLE	18.5	22.6	6	41.5	-0.5677	0.0038
	CE-N	10.6	6.6	4.8	16.1		

*P-value as assessed by Spearman correlation. ^Spearman correlation performed with data from clinical cases only. VLW: villous lacteal width; MLW: mucosal lacteal width; MLEC: number of mucosal lymphatic endothelial cells; CE-PLE: chronic enteropathy with protein-losing enteropathy; CE-N: chronic enteropathy with serum albumin concentration ≥2.5 g/dL; IQR: interquartile range.

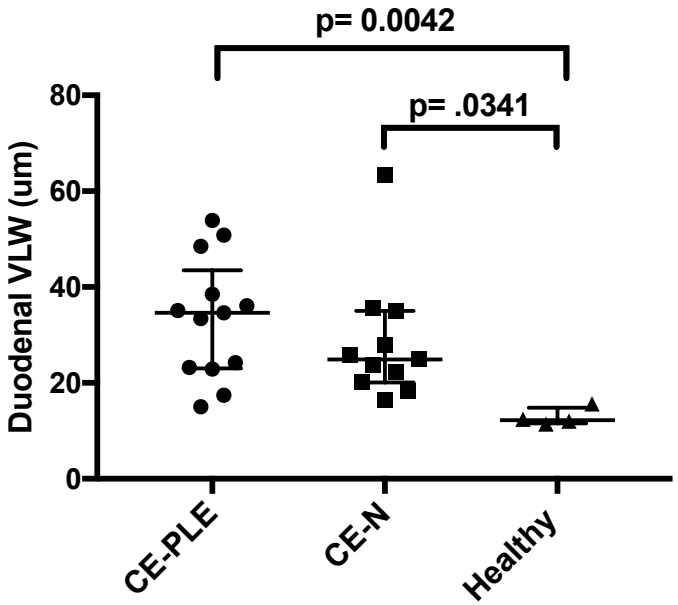

Figure 5.4 Scatter dot plot of duodenal villous lacteal width (um) in dogs with CE with and without PLE and healthy controls. Horizontal bar represents median. Interquartile range shown. CE-PLE= dogs with chronic enteropathy and serum albumin concentrations <2.5 g/dL; CE-N= dogs with chronic enteropathy and serum albumin concentrations ≥ 2.5 g/dL. VLW= villous lacteal width.

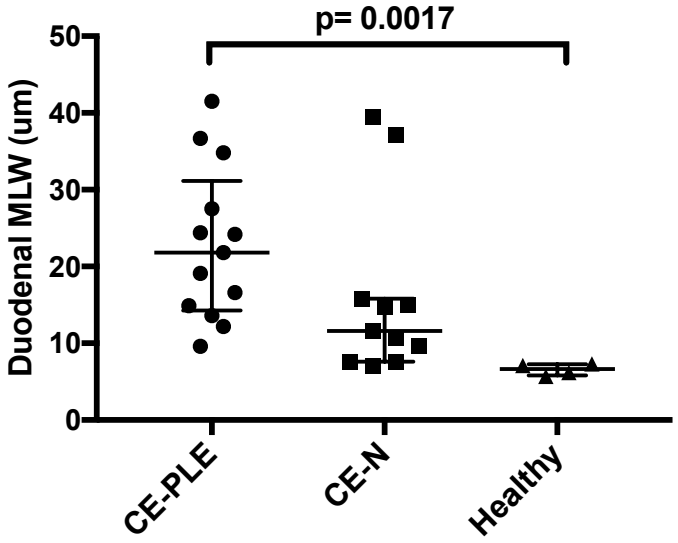

Figure 5.5 Scatter dot plot of duodenal mucosal lacteal width (um) in dogs with CE with and without PLE and healthy controls. Horizontal bar represents median. Interquartile range shown. CE-PLE= dogs with chronic enteropathy and serum albumin concentrations <2.5 g/dL; CE-N= dogs with chronic enteropathy and serum albumin concentrations ≥ 2.5 g/dL. MLW= proprial mucosal lacteal width.

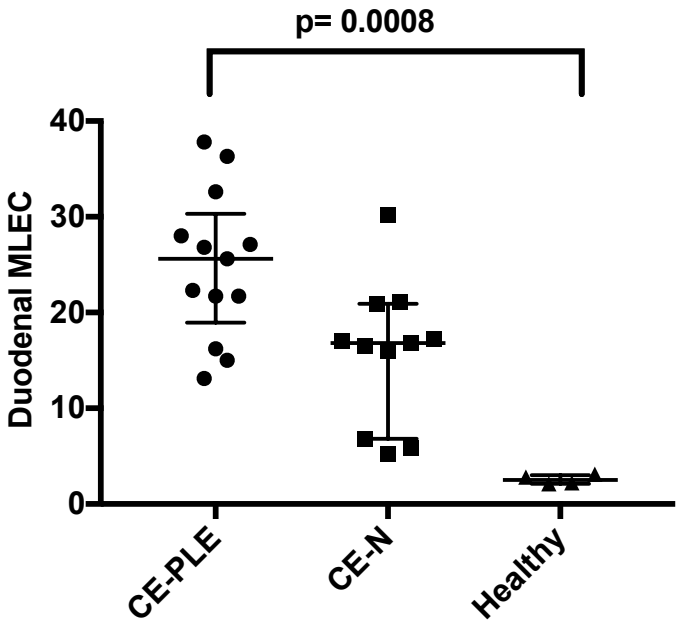

Figure 5.6 Scatter dot plot showing the number of duodenal mucosal lymphatic endothelial cells per 20x field in dogs with CE with and without PLE and healthy controls. Horizontal bar represents median. Interquartile range shown. CE-PLE= dogs with chronic enteropathy and serum albumin concentrations <2.5 g/dL; CE-N= dogs with chronic enteropathy and serum albumin concentrations ≥ 2.5 g/dL. MLEC= number of mucosal lymphatic endothelial cells.

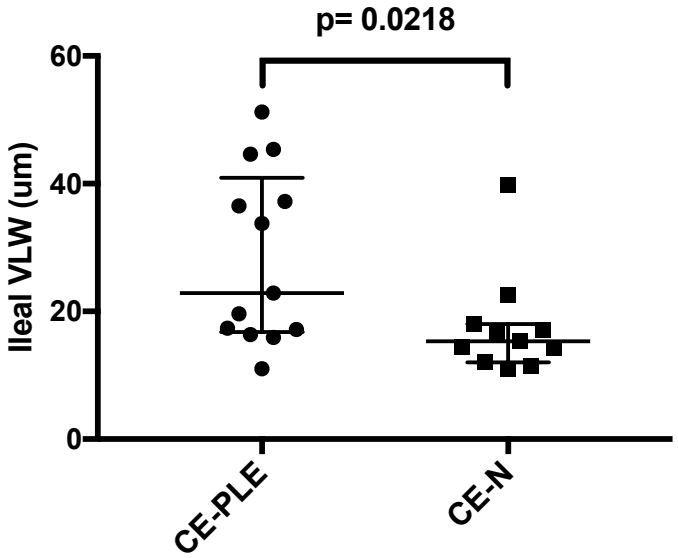

Figure 5.7 Scatter dot plot of ileal villous lacteal width (um) in dogs with CE with and without PLE. Horizontal bar represents median. Interquartile range shown. CE-PLE= dogs with chronic enteropathy and serum albumin concentrations <2.5 g/dL; CE-N= dogs with chronic enteropathy and serum albumin concentrations ≥ 2.5 g/dL. VLW= villous lacteal width.

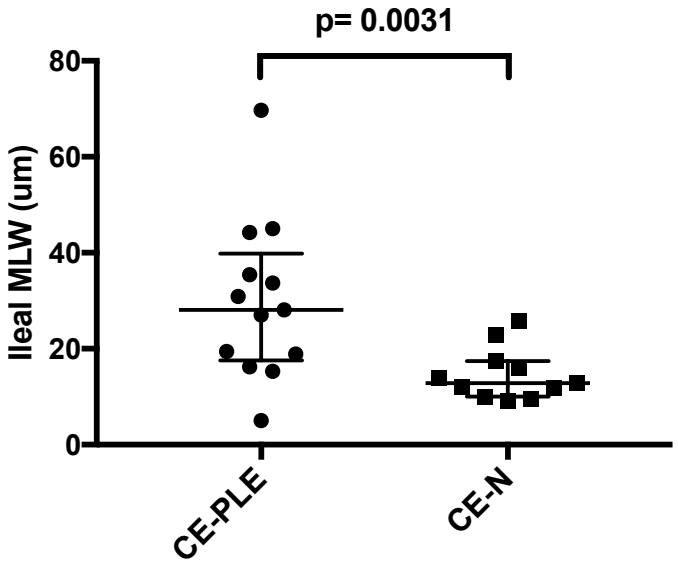

Figure 5.8 Scatter dot plot of ileal mucosal lacteal width (um) in dogs with CE with and without PLE. Horizontal bar represents median. Interquartile range shown. CE-PLE= dogs with chronic enteropathy and serum albumin concentrations <2.5 g/dL; CE-N= dogs with chronic enteropathy and serum albumin concentrations ≥ 2.5 g/dL. MLW= proprial mucosal lacteal width.

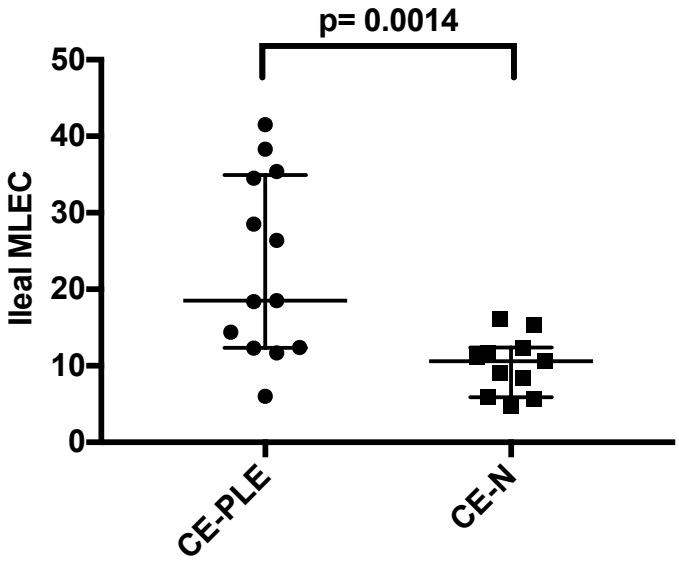

Figure 5.9 Scatter dot plot showing the number of ileal mucosal lymphatic endothelial cells per 20x field in dogs with CE with and without PLE. Horizontal bar represents median. Interquartile range shown. CE-PLE= dogs with chronic enteropathy and serum albumin concentrations <2.5 g/dL; CE-N= dogs with chronic enteropathy and serum albumin concentrations ≥ 2.5 g/dL. MLEC= number of mucosal lymphatic endothelial cells.

5.5 Discussion

In this study, we utilized LEC-specific markers to evaluate the intestinal lymphatic vasculature in dogs with CE with and without protein-losing enteropathy. In our group of 24 dogs with CE, serum albumin concentrations were correlated with the number of mucosal LEC in the duodenum and ileum and with villous and mucosal lacteal width in the ileum. Further, the villous and mucosal lacteals in the ileum of dogs with CE with PLE were wider than dogs with CE and serum albumin concentrations ≥2.5 g/dL. For both the duodenum and the ileum, villous lacteal dilation scores calculated with the use of IHC were correlated with lacteal dilation scores as traditionally assessed via routine H&E staining. In addition, ileal endoscopic lacteal dilation scores were correlated with lacteal dilation scores calculated with the use of IHC.

The most striking finding in this study was the presence of apparent mucosal lymphangiectasia of the intestine of dogs with CE, in particular in the ileum of several dogs with CE and albumin concentrations <2.5 g/dL. In addition, in some of these dogs with apparently dilated mucosal lymphatics, villous lymphatics were not concurrently dilated and these dogs had not been diagnosed with lymphangiectasia on routine histopathologic examination. The appearance of the distended mucosal lymphatics in the ileum of these dogs is similar to what has been identified in humans with Crohn's disease,[16] an idiopathic chronic inflammatory intestinal disease most commonly found in the ileum.[17] In humans with CD, lymphangiectasia has also been identified in the submucosa, muscularis propria and subserosa.[15,16,27] In addition, granuloma-obstructed lymphatics have long been recognized in the ileum of patients with CD.[28] Notably, endoscopically obtained biopsies often fail to recognize granuloma-obstructed lymphatics in patients with CD, as granulomas are found more commonly in the deeper layers of the intestine.[16] Therefore, it is possible that full thickness intestinal biopsies with LEC

immunolabeling may identify more abnormalities in the lymphatic vasculature of dogs with CE than are currently seen.

Mucosal lymphangiectasia was also identified in the duodenum of some dogs with CE, including in two dogs with serum albumin concentrations ≥2.5 g/dL. Mucosal lacteal width in the duodenum was not significantly correlated with serum albumin concentration, but approached statistical significance. An increased sample size may have detected a statistical difference. It is possible that lymphatic abnormalities are more easily detected in the ileum due to the fact that lymphatic nodules are most abundant in this location.[29] Nonetheless, the fact that we found differences between the lymphatic abnormalities in the segments of intestine in individual dogs lends additional support to the recommendation for obtaining ileal samples in cases of CE, particularly in dogs with PLE.

Increased lymphatic vessel density has been recognized in both the inflamed and non-inflamed ileal mucosa of patients with CD when compared with healthy controls.[30] We set out to quantify lymphatic vessel density in our study by counting LEC numbers in the mucosa of dogs with CE and healthy controls. Dogs with CE and concurrent PLE had more lymphatic endothelial cells in their duodenal mucosa compared to healthy controls, and more lymphatic endothelial cells in their ileal mucosa when compared to dogs with CE and serum albumin concentrations ≥2.5 g/dL. Dogs with CE and serum albumin concentrations ≥2.5 g/dL trended towards an increased number of LEC's in their duodenal mucosa when compared to healthy controls, but no significant difference was found ($p=0.0996$). An increased lymphatic density in the intestine has been identified in association with human IBD in multiple studies.[17,18,30-32] It is suspected to be the result of lymphangiogenesis, or the formation of new lymphatic vessels, which is considered rare and tightly regulated in adulthood.[30] The cause of this

lymphangiogenesis in humans with IBD is not well understood. Several inflammatory conditions are known to increase the expression of certain lymphangiogenic factors, such as vascular endothelial growth factor D (VEGF-D) and interleukin growth factor 2 (IGF-2), among others.[33, 34] Additionally, the expression of lymphangiogenic factors is dependent on pro-inflammatory cytokines.[35] Therefore, it may be that lymphangiogenesis occurs in response to the inflammatory conditions in the intestine of patients with CD, and may develop in an attempt to improve transport of intestinal immune and inflammatory cells. It has also been suggested that lymphangiogenesis may occur to compensate for lymphatic insufficiency that develops as a result of lymphatic obstruction or lymphatic pump failure in cases of IBD.[27] Based on this work, we suspect that a similar phenomenon of lymphatic proliferation occurs in dogs with CE. Quantification of lymphatic vessel densities in a larger population of dogs with CE and healthy controls would be an important.

Whether the lymphatic abnormalities are the cause or consequence of disease in humans with CD is debated among gastroenterologists and pathologists.[30] Several experimental models of lymphatic obstruction or insufficiency have demonstrated the importance of functional lymphatics to intestinal health.[36-40] In one such study, the ablation of lymphatics resulted in mucosal barrier injury, allowing for the invasion of intestinal pathogens and subsequent development of intestinal inflammation.[38] These authors suggested that intestinal inflammation could begin with injury to the lymphatics. Regardless of whether the lymphatic abnormalities are a cause or consequence of disease, they likely represent an important component of the disease process. In addition to their role in the transport of intestinal immune and inflammatory cells, the lymphatic vasculature is responsible for the regulation of the pressure of interstitial fluid in tissues, and the transport of excess fluid back to the circulation. Furthermore, lymphatic vessels

are the main route of absorption of fat, cholesterol, fat-soluble vitamins, and gut hormones.[41] Therefore, the obstruction and/or dysfunction of the lymphatic vasculature should have significant consequences and the recognition of lymphatic abnormalities is likely important to the management of dogs with CE.

A second objective of this study was to explore how the use of LEC-specific markers correlated to traditional methods of identifying lymphangiectasia in cases of canine CE. Lymphangiectasia is traditionally identified in intestinal biopsies of dogs by estimating or exactly quantifying the width of central villous lacteals in relation to the width of the villus they occupy.[6,7] Gross endoscopic evaluation of lacteal dilation is also used, and has been shown to have good sensitivity, but poor specificity for the diagnosis of intestinal lymphangiectasia.[7] In our study, the use of LEC markers for the measurement of duodenal and ileal villous lacteals in relation to the villus width correlated well with blinded lacteal dilation scores as assigned by a board-certified veterinary pathologist. The IHC derived lacteal dilation scores were also, not surprisingly, correlated to the villous lacteal width in both the duodenum and ileum. However, these scores were not correlated with the number of LEC in the intestinal mucosa, or to the mucosal lacteal width, suggesting that the traditional methods of assessing lymphangiectasia are not adequate for evaluation of lymphangiectasia in the mucosa of dogs. Without the use of LEC-specific markers, the lymphatics in the mucosa could easily be confused with edema, an error that we found to be easily avoided with the use of LEC IHC markers. Finally, endoscopic lacteal dilation scores in both the duodenum and ileum were also correlated to villous lacteal dilation scores in their respective tissues as assessed by IHC, suggesting that endoscopic examination can have a role in identifying villous lymphangiectasia in cases of CE.

Although response to therapy and outcomes were not evaluated in this study, follow-up information is available for a number of dogs in this study. Six dogs in the study had an average ileal mucosal lymphatic width >30 um, all of whom had serum albumin concentrations <2.5 g/dL. Blinded ileal lacteal dilation scores were 0 in 4/6 of these dogs. Of these dogs, 3 were euthanized due to their disease, one was euthanized > 12 months following diagnosis of CE-PLE due to splenic hemangiosarcoma, and 2 are alive at the time of publication. Of the 3 dogs that were euthanized, 2 were treated with traditional therapies including commercial gastrointestinal diets and glucocorticoids for <2 weeks following their diagnosis with no significant clinical improvement noted. One dog that was euthanized was initially glucocorticoid-responsive but then relapsed when glucocorticoids were tapered and her owner chose not to pursue further therapy. Interestingly, all 3 dogs with ileal mucosal width >30 um had persistent clinical signs despite treatment with glucocorticoids, immunosuppressives, vitamin supplementation, supportive care and commercially available hydrolyzed and low-fat diets. All 3 of these dogs ultimately had an apparent clinical response once switched to a veterinary nutritionist formulated home cooked diet, formulated to be lower in fat (10-15 % by metabolizable energy [ME]) than the commercially available diets. Although anecdotal, and only a few cases, this population of dogs may represent a subset of dogs with CE-PLE that have significant abnormalities to their lymphatic vasculature that require the administration of a diet that is lower in fat than what is commercially available.

This study has several limitations. First, and importantly, ileal biopsies from healthy control dogs were not available for this study. Further studies of the lymphatic vasculature in cases of canine CE should include the evaluation of ileal samples from healthy control dogs. We also recognize that 4 healthy controls are likely not representative of the duodenum of all healthy

dogs and an increased number of controls would have strengthened our study considerably. In addition, while a lot of effort was made to standardize the evaluation of the intestinal lymphatic vasculature in these cases, the sectioning of intestinal tissue, in particular endoscopic biopsies, cannot be entirely uniform. Therefore it is certainly possible that in some cases the lymphatics were less or more visible due to the angle of sectioning, and this may have affected the results. In 2 clinical cases (one ileal sample from hypoalbuminemic dog, one duodenal sample from normoalbuminemic dog) and 2 control cases, only 5 measurable mucosal lymphatics could be definitively identified, therefore only 5 lymphatic's were used in the analysis compared to 10 in all other cases, which may have affected the results. Further, despite the use of a team of a blinded veterinary pathologist and veterinary pathologist in training to score histopathologic lesions in the intestine via established guidelines, histopathologic evaluation of the intestine in dogs is known to be subjective with significant interobserver variation.[42,43] While endoscopic scores were performed by a single investigator, the investigator was not blinded to the case information, so this may have affected the endoscopic score results. Moreover, the determination of lacteal dilation scores via H&E were determined by the pathologist and pathologist-in-training, while lacteal dilation scores via IHC were determined by a different evaluator. Although these scores were found to be well correlated in both the duodenum and ileum, ideally a single investigator would have performed both evaluations. A final limitation is that 2 dogs in the study did not have hypoadrenocorticism definitively excluded prior to their enrollment in the study. Both dogs had previously not had their clinical signs improve with glucocorticoid therapy so hypoadrenocorticism was considered unlikely, however ideally it would have been excluded definitively.

In conclusion, the use of LEC IHC markers allowed for the identification of both villous and mucosal intestinal lymphangiectasia in cases of CE. Several abnormalities of the intestinal lymphatic vasculature were correlated with serum albumin, and/or were more likely to be present in the intestine of dogs with CE and concurrent PLE when compared to the duodenum of healthy control dogs or the ileum of CE dogs with a serum albumin concentration ≥2.5 g/dL. The most notable and novel finding was the discovery of apparently distended mucosal intestinal lymphatics, the most striking of which was seen in the ileum of dogs with CE and concurrent PLE. This study or similar studies need to be repeated in a larger group of dogs with CE with and without concurrent PLE. The identification of lymphangiectasia and possible lymphatic obstruction deeper in intestinal biopsies is an important discovery, as it will impact the therapeutic management of these cases. This may suggest that evaluation of the villous lacteals alone in cases of CE is underestimating abnormalities to the lymphatic vasculature. Furthermore, the development of therapies targeting the abnormalities of the intestinal lymphatic vasculature in patients with CD is underway,[44] and may have future application to dogs if these findings are repeatable. Therefore, after the evaluation of additional cases and repeatability of these findings, the use of LEC markers to evaluate the intestinal lymphatic vasculature may be considered an important addition to the routine evaluation of intestinal biopsy samples of dogs with CE.

REFERENCES

1. Allenspach K, Wieland B, Grone A, et al. Chronic enteropathies in dogs: Evaluation of risk factors for negative outcome. J Vet Intern Med 2007;21:700–708.
2. Jergens AE, Moore FM, Haynes JS, et al. Idiopathic inflammatory bowel disease in dogs and cats: 84 Cases (1987–1990). J Am Vet Med Assoc 1992;201:1603–1608.
3. German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. J Vet Intern Med 2003;17:8–20.
4. Craven M, Simpson JW, Ridyard AE, et al. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). J Small Anim Pract 2004;45:336-342.
5. Dossin, Olivier, and Rachel Lavoué. Protein-losing enteropathies in dogs. Vet Clin: Small Anim Pract 2011;41:399-418.
6. Okanishi H, Yoshioka R, Kagawa Y, et al. The clinical efficacy of dietary fat restriction in treatment of dogs with intestinal lymphangiectasia. J Vet Intern Med 2014;28:809-817.
7. Larson RN, Ginn JA, Bell CM, et al. Duodenal endoscopic findings and histopathologic confirmation of intestinal lymphangiectasia in dogs. J Vet Intern Med 2012;26:1087–1092.
8. Rossi G, Cerquetella M, Antonelli E, et al. The importance of histologic parameters of lacteal involvement in cases of canine lymphoplasmacytic enteritis. Gastroenterol Hepatol Bed Bench 2015;8:33.
9. Wennogle SA, Priestnall SL, Webb CB. Histopathologic characteristics of intestinal biopsy samples from dogs with chronic inflammatory enteropathy with and without hypoalbuminemia. J Vet Intern Med 2017;31:371-376.
10. Moser K, Mitze S, Teske E, et al. Correlation of clinical, diagnostic and histopathological parameters in dogs with chronic lymphocytic-plasmacytic enteropathy. Tierärztliche Praxis K: Kleintiere/Heimtiere. 2018;46:15-20.
11. Van Kruiningen HJ, Colombel JF. The forgotten role of lymphangitis in Crohn's disease. Gut. 2008;57:1-4.
12. Alexander JS, Chaitanya GV, Grisham MB, et al. Emerging roles of lymphatics in inflammatory bowel disease. Ann NY Acad Sci 2010;1207:E75-85.
13. von der Weid PY, Rehal S, Ferraz JG. Role of the lymphatic system in the pathogenesis of Crohn's disease. Cur Op Gastro 2011;27:335-341.
14. Cerquetella M, Spaterna A, Laus F, et al. Inflammatory bowel disease in the dog: differences and similarities with humans. World J of Gastroenterology 2010;16:1050-1056.
15. Van Kruiningen HJ, Hayes AW, Colombel JF. Granulomas obstruct lymphatics in all layers of the intestine in Crohn's disease. Apmis 2014;122:1125-1129.
16. Sura R, Colombel JF, Van Kruiningen HJ. Lymphatics, tertiary lymphoid organs and the granulomas of Crohn's disease: an immunohistochemical study. Aliment Pharmacol Ther 2011;33:930-939.
17. Li Y, Ge Y, Gong J, et al. Mesenteric Lymphatic Vessel Density Is Associated with Disease Behavior and Postoperative Recurrence in Crohn's Disease. J Gastroi Surg 2018 Jul 24:1-8.
18. Pedica F, Ligorio C, Tonelli P, et al. Lymphangiogenesis in Crohn's disease: an immunohistochemical study using monoclonal antibody D2-40. Virchows Archiv 2008;452:57-63.

19. Oliver G, Sosa-Pineda B, Geisendorf S, et al. Prox 1, a prospero-related homeobox gene expressed during mouse development. Mech Dev 1993;44:3-16.
20. Banerji S, Ni J, Wang SX, et al. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. J Cell Bio 1999;144:789-801.
21. Halsey CH, Worley DR, Curran K, et al. The use of novel lymphatic endothelial cell-specific immunohistochemical markers to differentiate cutaneous angiosarcomas in dogs. Vet Comp Onc 2016;14:236-244.
22. Jergens AE, Schrreiner CA, Frank DE, et al. A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med 2003;17:291-297.
23. Slovak JE, Wang C, Sun Y, et al. Development and validation of an endoscopic activity score for canine inflammatory bowel disease. Vet J 2015;203:290-295.
24. Washabau RJ, Day MJ, Willard MD, et al. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med 2010;24:10–26.
25. Ramzan NN, Leighton JA, Heigh RI, et al. Clinical significance of granuloma in Crohn's disease. Inflamm Bowel Dis 2002;8:168–173.
26. Stange EF, Travis SP, Vermeire S, et al. European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. Gut 2006; 55: i1-i15.
27. Alexander JS, Ganta VC, Jordan PA, et al. Gastrointestinal lymphatics in health and disease. Pathophysiology 2010;17:315-335.
28. Van Kruiningen HJ, Colombel JF. The forgotten role of lymphangitis in Crohn's disease. Gut. 2008;57:1-4.
29. Jung C, Hugot JP, Barreau F. Peyer's patches: the immune sensors of the intestine. Intern J Inflamm 2010.
30. Rahier JF, De Beauce S, Dubuquoy L, et al. Increased lymphatic vessel density and lymphangiogenesis in inflammatory bowel disease. Aliment Pharmacol Ther 2011;34:533-543.
31. Geleff S, Schoppmann SF, Oberhuber G. Increase in podoplanin-expressing intestinal lymphatic vessels in inflammatory bowel disease. Virchows Archiv 2003;442:231-237.
32. Fogt, F, Pascha, TL, Zhang, PJ, et al. Proliferation of D2-40-expressing intestinal lymphatic vessels in the lamina propria in inflammatory bowel disease. Int. J. Mol. Med 2004;13:211–214.
33. Mouta C, Heroult M. Inflammatory triggers of lymphangiogenesis. Lymphat Res Biol 2003; 1: 201–18.
34. Ristimaki A, Narko K, Enholm B, et al. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor- C. J Biol Chem 1998; 273: 8413–8.
35. Polzer K, Baeten D, Soleiman A, et al. Tumour necrosis factor blockade increases lymphangiogenesis in murine and human arthritic joints. Ann Rheum Dis 2008; 67: 1610–6.
36. Kalima T. Experimental lymphatic obstruction in the ileum. Ann Chir Gyanec Fenn 1970;59:187–201.
37. Kalima T, Saloniemi H, Rahko T. Experimental regional enteritis in pigs. Scand J Gastroenterol 1976;11:353–362.

38. Jang JY, Koh YJ, Lee SH, et al. Conditional ablation of LYVE-1+ cells unveils defensive roles of lymphatic vessels in intestine and lymph node. Blood 2013.
39. Davis RB, Kechele DO, Blakeney ES, et al. Lymphatic deletion of calcitonin receptor–like receptor exacerbates intestinal inflammation. JCI insight 2017;2.
40. Rehal S, Stephens M, Roizes S, et al. Acute small intestinal inflammation results in persistent lymphatic alterations. Am J Phys-Gastro Liv Path 2017;314:G408-417.
41. Miller MJ, McDole JR, Newberry RD. Microanatomy of the intestinal lymphatic system. Ann NY Acad Sci 2010;1207:21-28.
42. Jergens AE, Evans RB, Ackermann M, et al. Design of a simplified histopathologic model for gastrointestinal inflammation in dogs. Vet Pathol 2014;51:946–950.
43. Willard MD, Jergens AE, Duncan RB, et al. Interobserver variation among histopathologic evaluations of intestinal tissues from dogs and cats. J Am Vet Med Assoc 2002;220:1177–1182.
44. D'Aiessio S, Tacconi C, Danese S. Targeting lymphatics in inflammatory bowel disease. Oncotarget 2015;6:34047.

CHAPTER 6: EVALUATION OF APPETITE SCORES, ALPHA-TOCOPHEROL, RETINOL, SERUM PROTEINS, AND MARKERS OF SYSTEMIC AND INTESTINAL INFLAMMATION IN DOGS WITH CHRONIC ENTEROPATHY AND LOW OR NORMAL SERUM 25(OH)D CONCENTRATIONS

6.1 Overview

Background: The mechanism of hypovitaminosis D in dogs with chronic enteropathy (CE) is unknown.

Objectives: To improve understanding of the pathogenesis of hypovitaminosis D in dogs with CE by evaluating variables associated with intake of vitamin D, concentrations of other-fat soluble vitamins, concentrations of vitamin D serum binding proteins, and markers of systemic and intestinal inflammation in dogs with CE and low or normal serum 25(OH)D concentrations.

Animals: 15 dogs with CE and low serum 25-hydroxyvitamin D (25[OH]D) concentrations and 15 dogs with CE and normal serum 25(OH)D concentrations.

Methods: Prospective cohort study. Clinical and clinicopathologic variables were compared between groups. Correlations between serum 25(OH)D and histopathologic variables were assessed.

Results: Higher CCECAI scores ($p=0.0032$), lower serum α-tocopherol ($p=0.0007$), cholesterol ($p< 0.0001$), and albumin ($p< 0.0001$) concentrations and higher serum CRP ($p=0.0041$) concentrations were more likely in CE dogs with low serum 25(OH)D concentrations when compared to CE dogs with normal 25(OH)D concentrations. Serum concentrations of vitamin D binding protein (VDBP) were not different between groups ($p= 0.9105$). Duodenal and combined duodenal/ileal morphologic ($p=0.0016$ and $p=0.0003$, respectively) and overall WSAVA scores ($p=0.0003$ and $p=0.0024$, respectively) were correlated with serum 25(OH)D.

Conclusions and Clinical Importance: The pathogenesis of hypovitaminosis D in dogs with CE is likely multifactorial. Fat malabsorption deserves further study in dogs with low vitamin D and CE. Loss of VDBP may not be a significant mechanism in dogs with low vitamin D and CE.

6.2 Introduction

Decreased serum 25-hydroxyvitamin D (25[OH]D) concentrations have been well documented in dogs with chronic enteropathy (CE) and protein-losing enteropathy (PLE)[1-3] and have been associated with a poor outcome.[4] Similarly, children and adults with IBD, namely Crohn's disease (CD), are more likely to be vitamin D deficient than healthy controls.[5-8] When compared to patients with IBD and normal vitamin D levels, humans with IBD and vitamin D deficiency experience poorer quality of life[9] and increased risk for hospitalization and surgery.[10]

Proposed mechanisms for vitamin D deficiency in human patients with IBD include reduced exposure to sunlight, reduced oral vitamin D intake, malabsorption, loss of vitamin D through the intestinal tract, and an active inflammatory state.[11] Oral intake of vitamin D has not been consistently correlated with hypovitaminosis D in human patients with IBD.[11] One group of researchers found an approximately 30% reduction in absorption of oral vitamin D2 in patients with CD when compared to healthy controls[12], but in other studies intestinal absorption of vitamin D was found to be normal in most patients with IBD.[13,14] A systemic review and meta-analysis found that in addition to vitamin D, levels of other fat-soluble vitamins (A, E, and K) are decreased in CD patients compared to healthy controls.[15] Direct loss of vitamin D has been suggested as a mechanism, but a very small amount (<1%) of vitamin D circulates unbound. Vitamin D metabolites are primarily transported through circulation bound to vitamin D binding protein (VDBP [85-90%]), and to a lesser degree albumin (10-15%).[16] A recent study found that

VDBP levels were decreased in a cohort of children with IBD compared to healthy controls.[17] In another study serum 25(OH)D was positively correlated with albumin in a group of 130 children and young adults with IBD.[18]

In dogs with CE, the mechanism of low serum vitamin D is unknown.[1] Reduced dietary intake of vitamin D may contribute to low serum vitamin D levels in dogs, however one study found that dogs with reduced appetite and non-gastrointestinal illness had higher serum 25(OH)D levels than dogs with CE, suggesting that poor appetite does not always lead to decreased serum vitamin D levels.[2] In 33 dogs with CE, a relationship between serum albumin and serum 25(OH)D was found.[2] VDBP levels and serum levels of other fat-soluble vitamins have not previously been reported in dogs with hypovitaminosis D and CE. Finally, low vitamin D status has been associated with markers of systemic and intestinal inflammation in the dog.[19]

Investigation into the relationship of clinical, clinicopathologic and histopathologic variables with 25(OH)D concentrations in a group of dogs with CE may lead to a better understanding of why vitamin D decreases and improve our ability to detect and treat low serum vitamin D levels. Therefore, the objective of this study was to evaluate variables associated with intake of vitamin D, concentrations of other-fat soluble vitamins, concentrations of vitamin D serum binding proteins, and markers of systemic and intestinal inflammation in dogs with chronic enteropathy and low or normal serum 25(OH)D concentrations.

6.3 Materials and Methods

6.3.1 Study Population

Dogs presenting to CSU Veterinary Teaching Hospital for evaluation of chronic gastrointestinal signs of greater than 3 weeks duration were eligible for inclusion in the study.

Expressed written consent was obtained from the owners of each of the study participants. Dogs were eligible for inclusion if diagnostic evaluation consisting of hematology, biochemical profile, and abdominal ultrasonography excluded non-gastrointestinal illness, and histopathologic evidence of small intestinal disease characterized by inflammatory infiltrates and morphologic changes was present. Dogs with histopathologic evidence of intestinal neoplasia were excluded. Urinalysis +/- urine protein: creatinine ratio and fasting and post-prandial bile acids were performed to exclude other causes of hypoalbuminemia in dogs with serum albumin <2.5 g/dL. The feces of all dogs were screened for helminthes (fecal floatation), *Giardia* (IFA*),* and *Cryptosporidium* (IFA)*,* with no parasites detected in any case. All dogs had exocrine pancreatic insufficiency excluded by fasted serum trypsin-like immunoreactivity >5.0 ng/ml and hypoadrenocorticism excluded by basal cortisol >2 ug/ml or normal response to ACTH stimulation. Dogs being administered vitamin D or calcium supplementation were excluded. Additionally, dogs that had consumed a diet for >72 hours in the preceding three weeks that was not formulated to meet Association of American Feed Control Officials vitamin requirements (vitamin A 5000 IU/kg dry matter basis [DMB]; vitamin D 500 IU/kg DMB; vitamin E 50 IU/kg DMB) were not eligible for inclusion. Dogs being administered glucocorticoids for >7 days prior to enrollment were excluded from analysis.

Age, breed, sex, body weight (kilograms [kg]), and duration of illness (DOI) were recorded. Six and twelve month outcomes (alive, euthanized/died) were also recorded, where available.

6.3.2 Measurement of Ionized Calcium (iCa), Parathyroid Hormone (PTH), and Serum 25(OH)D Concentrations

Serum was collected from all dogs at the time of initial evaluation and processed within 30 minutes of collection. In the majority of cases, serum was shipped on dry ice the same day as collection to the Michigan State University's Diagnostic Center for Population and Animal Health (DACPAH)[a] for completion of Vitamin D Panel (measurement of 25[OH]D, ionized calcium [iCa], and parathyroid hormone [PTH]). In a small number of cases serum was stored at -80°C after processing and before being shipped on dry ice to DACPAH for evaluation of 25(OH)D, iCa, and PTH. Serum 25(OH)D, iCa, and PTH have previously been shown to be stable under these conditions.[20,21] 25(OH)D was measured using a commercially available radioimmunoassay kit and ionized calcium concentrations were measured using an ion specific electrode. PTH was measured with an immunoradiometric assay, as previously described.[22]

6.3.3 Clinical Activity, Appetite, and Body Condition Scores

At the time of initial evaluation, owners were asked to score appetite, activity level, vomiting, fecal consistence, fecal frequency and weight loss in order to determine a canine inflammatory bowel disease activity index (CIBDAI)[23] score for each dog. The owners were also asked to score pruritus on a scale of 0-3 to aid in determining the canine chronic enteropathy clinical activity index (CCECAI).[24] Following the results of the biochemical profile (serum albumin) and abdominal ultrasound (peritoneal effusion), a CCECAI was calculated for each dog. Appetite scores were also recorded in isolation. Appetite score was given as 0-3 where

[a] MSU Diagnostic Center for Population and Animal Health, Meridian Charter Township, MI, USA

0=normal, 1=mildly decreased, 2=moderately decreased, and 3=severely decreased. Body condition score (BCS; scale 0-10)[25] was assigned for each dog by a single evaluator.

6.3.4 Measurement of Serum Alpha-Tocopherol, Retinol and Serum Cholesterol

Serum vitamin alpha-tocopherol (Vitamin E) and retinol (Vitamin A) concentrations were batch measured on stored, aliquoted frozen serum samples by high-performance liquid chromatography. Samples had been frozen immediately following collection and processing, stored at -80°C, and thawed before analysis. Alpha-tocopherol and retinol are known to be stable in serum for months to years.[26,27] Because of its role as a precursor to vitamin D, serum cholesterol concentrations were also recorded.

6.3.5 Markers of Systemic and Intestinal Inflammation

Plasma fibrinogen and serum C-reactive protein levels were available for many of the dogs in the study as markers of systemic inflammation. Quantitative plasma fibrinogen level was obtained at the time of collection via the Clauss method[b]. Serum CRP concentration was determined via a commercially available enzyme-linked immunosorbent assay (ELISA)[c]. All dogs underwent gastroduodenoscopy and ileocolonoscopy for evaluation of the intestinal mucosa and procurement of tissue samples. Histopathologic evaluation of intestinal tissue from clinical cases (duodenum +/- ileum) was performed by a board-certified veterinary pathologist and pathologist-in-training blinded to clinical data, clinicopathologic information, and groups. Biopsy samples were assessed as adequate for evaluation, and both evaluators reviewed duodenal and ileal tissues and reached a consensus for the presence and degree of morphologic

[b] Tcoag TriniCLOT™ PT Excel, Bray, Wicklow, Ireland
[c] Tridelta Development Limited™, Maynooth, County Kildare, Ireland

criteria (villous stunting, epithelial injury, crypt distension, lacteal dilation, mucosal fibrosis) and inflammatory criteria (intraepithelial lymphocytes, lamina propria eosinophils, lamina propria lymphocytes/plasma cells, lamina propria neutrophils) based on WSAVA guidelines.[28] For the degree of each change, the following scores were applied based on established criteria: 0 = normal, 1 = mild, 2 = moderate, 3 = marked. Individual scores were recorded, and then the total morphologic score and total inflammatory score per section of intestine were recorded in isolation, and then summed for a total WSAVA score per section of intestine. Combined duodenal and ileal total morphologic score, inflammatory score, and total WSAVA scores were also recorded. Because vitamin D is a fat-soluble vitamin, lacteal dilation (LD) scores for each section of the intestine, and a total LD score were also recorded.

6.3.6 Measurement of Serum Proteins

Serum albumin was assessed routinely through the Colorado State University Veterinary Diagnostic Laboratory with a Cobas Integra[d] biochemistry analyzer. Serum VDBP concentrations were measured using a human-specific ELISA[e], validated for measurement in the dog. The ELISA kit uses mouse monoclonal anti-human highly purified human Gc globulin (VDBP) as capture antibody (Immunogen™, Waltham, MA) and goat anti-human synthetic peptide ERGRDYEKNKVCK (corresponding to amino acids 18-30 of human vitamin D binding protein (near the N terminal) as detection antibody (Immunogen™, Waltham, MA). Serum samples had been frozen immediately following collection, stored at -80°C, and thawed before analysis. Samples were stored for 2-18 months prior to analysis; length of time of sample storage

[d] Roche Diagnostics Limited™, West Sussex, UK
[e] My BioSource Inc.™, San Diego, CA, USA

prior to analysis was recorded. Analysis and calculation of results were performed according to manufacturer instructions.

6.3.7 Statistical Analysis

Statistical analysis was performed using GraphPad Prism scientific statistic software (Graph Pad Prism, GraphPad Software, Inc, San Diego, CA). For comparisons, groups were defined as dogs with low serum 25(OH)D concentrations and dogs with normal serum 25(OH)D concentrations. Dogs were considered low in vitamin D if serum concentration of 25(OH)D was returned as subnormal (<109 nmol/L) as defined by an established reference interval (109-423 nmol/L), and normal if serum 25(OH)D concentration fell within the reference interval. Fischer's exact test was used to determine if there were differences between groups for sex. Mann-Whitney *U* was performed to determine if there were differences between groups for age, body weight, and duration of illness. Categorical data (outcome at 6 months, outcome at 12 months) was compared with Fischer's exact test.

The distribution of data was assessed by the Shapiro-Wilk test. Normally distributed (parametric) clinical and clinicopathologic variables were compared with a *t*-test. Non-normally distributed (non-parametric) clinicopathologic variables were compared with Mann-Whitney *U* test. Bonferroni correction was applied to data to account for multiple testing. Results are reported as mean ± standard deviation (SD) for the parametric variables and the median and range (min-max) for non-parametric variables. A multiple logistic regression analysis was performed using those variables found to be significantly associated with 25(OH)D based on Mann-Whitney *U* and *t*-test. Specifically, serum 25(OH)D (decreased or normal) was the response variable and the variables serum albumin, serum alpha-tocopherol, serum cholesterol

and CCECAI were used as predictors. Serum CRP was not included in the model due to missing values. Backwards elimination was then used to reduce the number of predictors in the logistic regression model.

A Spearman (rank-based) correlation was performed to evaluate for correlations between vitamin D concentration and iCa, PTH, and histopathologic scores. Following Spearman, a Bonferroni correction was performed to account for multiple testing. Because of the unknown effects of storage, a Pearson correlation was performed to evaluate for correlation between serum sample storage time at -80°C and VDBP concentration. Statistical significance for all statistical comparisons was set at $p < 0.05$.

6.4 Results

Thirty dogs with CE were enrolled, 15 of which had low serum 25(OH)D concentrations. The other 15 dogs had serum 25(OH)D levels within the reference interval (Figure 6.1). Breeds in the low 25(OH)D group included Bernese mountain dog (2), mixed breed dog (2), Yorkshire terrier (2), and one each of the following: Australian shepherd, English bulldog, German shepherd dog, Jack Russell terrier, Labrador retriever, Pembroke Welsh corgi, pug, and Rottweiler. Breeds in the normal 25(OH)D group included mixed breed dog (6), Bernese mountain dog (2), Labrador retriever(2) and one each of the following: Cavalier King Charles spaniel, German shepherd dog, German shorthaired pointer, Great Pyrenees, and Siberian husky. Groups were not different in regards to age, sex, body weight, or duration of illness. Median age of low 25(OH)D dogs was 7 years (1-10 years); median age of normal 25(OH)D dogs was 5 years (1-12 years). Five dogs in each group were spayed females; the remaining dogs were neutered males. Median body weight was 19 kg (3-47 kg) in the low 25(OH)D group and 22 kg

(6-46 kg) in the normal 25(OH)D group. Median duration of illness was 3 months (1-11 months) in the low 25(OH)D group, and 6 months (1-24 months) in the normal 25(OH)D group. Two dogs in each group had received glucocorticoids for less than 7 days prior to enrollment.

Summary statistics and p-values are presented in Table 6.1 for clinical scores, BCS, fat-soluble vitamins, cholesterol, serum proteins, and serum inflammatory markers. In brief, dogs with low serum 25(OH) had higher CCECAI scores ($p=0.0032$), lower serum α-tocopherol ($p=0.0007$; Figure 6.2), lower serum albumin ($p<0.0001$; Figure 6.3), lower serum cholesterol ($p<0.0001$; Figure 6.4) and higher serum CRP ($p=0.0041$; Figure 6.5) when compared to dogs with normal 25(OH)D. Serum concentrations of retinol (Figure 6.6) and VDBP (Figure 6.7) were not different between groups ($p=0.3646$ and $p=0.9105$, respectively). After backwards elimination (using alpha = 0.05), only albumin remained significant in the multiple regression analysis model (OR = 0.022, p-value = 0.002). The odds ratio (OR) represents the odds of decreased serum 25(OH)D corresponding to a one-unit increase in serum albumin.

Serum PTH was negatively correlated (r value -0.6827) with serum 25(OH)D ($p<0.0001$). Serum iCa was positively correlated (r value 0.7343) with 25(OH)D ($p<0.0001$). Gastroduodenoscopy and ileocolonoscopy was attempted in all dogs. The ileum could not be successfully biopsied in 2 dogs in the low vitamin D group and 2 dogs in the normal vitamin D group, so ileal tissue was not available for evaluation in those cases. Summary statistics and p-values for histopathologic variables are presented in Table 6.2. In brief, serum 25(OH)D concentrations were correlated with total morphologic scores for duodenum ($p=0.0016$) and for duodenum + ileum($p=0.0003$) and with overall WSAVA scores for duodenum ($p=0.0003$) and for duodenum+ ileum ($p=0.0024$). Serum VDBP concentration was not correlated with length of time the sample was stored ($p=0.539$).

Six month outcome data (alive or dead/euthanized) was available for all 30 dogs, twelve month outcome data was available for n=11 dogs in the low 25(OH)D group and n=13 dogs in the normal 25(OH)D dog. One dog was lost to follow-up and the remainder of the dogs were diagnosed <1 year prior. Six and twelve month outcomes (alive or dead/euthanized) were not different between groups (p=0.6513 and p=0.3783, respectively). Six and 12 month outcomes were also evaluated specifically for dogs with serum albumin concentration <2.5 g/DL, and were also not different between groups (p=0.125 and p=0.1923, respectively).

Table 6.1 Clinical scores, BCS, and serum fat-soluble vitamins, cholesterol, proteins, and inflammatory markers in dogs with CE and low or normal 25(OH)D concentrations

Variable	Reference Interval	Low 25(OH)D (n=15) Median (range) or Mean ±SD	Normal 25(OH)D (n=15) Median (range) or Mean ±SD	P-value^
25(OH)D (nmol/L)	109-423	15 (6-58)	195 (117-370)	
CIBDAI	0-15	9.1 ±3.96	6.7 ±3.09	0.0744
CCECAI	0-24	12.3 ±4.13	7.7 ±3.54	**0.0032**
Appetite Score	0-3	1 (0-3)	1 (0-3)	0.8483
BCS	0-9	3.7 ±1.16	3.9 ±1.36	0.7746
α-tocopherol (ug/ml)	5-24	11 (3.3-25)	23 (5.1-56)	0.**0007**
Retinol (ug/ml)	0.3-1	4± 2.54	4.7± 1.42	0.3646
Cholesterol (mG/dL)	130-300	80 (53-120)	198 (97-447)	**<0 .0001**
Albumin (g/dL)	3-4.3	1.6 (0.9-2.9)	3.3 (1.7-3.9)	**< 0.0001**[%]
VDBP (ug/ml)	–	183 (132-299)	193 (119-718)	0.9105
Fibrinogen (mG/dL)[*]	123-210	333 (201-835)	232 (146-523)	0.0301
CRP (ug/ml)[#]	0-7.6	43.1 (9.5-60)	2.5 (0.3-60)	**0.0041**

^P-value as assessed by Mann-Whitney *U* for non-parametric variables (data presented as median (range) and *t*-test for parametric variables (data presented as mean ±SD). Significance set at p< .0045 [%] Retained significance in multivariate analysis. [*]Data available from 10 dogs per group. [#]Data available from 13 dogs per group. BCS: body condition score; CE: chronic enteropathy; CIBDAI: canine inflammatory bowel disease activity index; CCECAI: canine chronic enteropathy clinical activity index; VDBP: vitamin D binding protein; CRP: C-reactive protein.

Table 6.2 Histopathology scores in dogs with CE and low or normal 25(OH)D concentrations

Variable	Group	Med	IQR	Min	Max	Spearman's Correlation Score	P-value*
LD- duodenum	Low 25(OH)D	1	1	0	2	-0.2842	0.128
	Normal 25(OH)D	0	1	0	2		
LD- ileum^	Low 25(OH)D	1	2	0	3	-0.5015	0.0091
	Normal 25(OH)D	0	0	0	2		
LD- duodenum + ileum^	Low 25(OH)D	3	3	0	3	-0.3787	0.0564
	Normal 25(OH)D	0	1	0	4		
Morphologic score#- duodenum	Low 25(OH)D	3	5	0	10	-0.5516	**0.0016**
	Normal 25(OH)D	3	5	0	10		
Morphologic score#- ileum^	Low 25(OH)D	2	3	0	6	-0.3903	0.0487
	Normal 25(OH)D	1	4	0	8		
Morphologic score#- duodenum+ ileum^	Low 25(OH)D	10	3	3	13	-0.6505	**0.0003**
	Normal 25(OH)D	3	6	0	18		
Inflammatory score$-duodenum	Low 25(OH)D	5	3	2	8	-0.5108	0.0039
	Normal 25(OH)D	4	2	1	8		
Inflammatory score$-ileum^	Low 25(OH)D	2	3	0	5	0.0066	0.9744
	Normal 25(OH)D	2	4.5	0	7		
Inflammatory score$-duodenum + ileum^	Low 25(OH)D	6	5.5	3	12	-0.2872	0.1549
	Normal 25(OH)D	5	6.5	2	15		
Overall WSAVA score- duodenum	Low 25(OH)D	11	4	2	15	-0.613	**0.0003**
	Normal 25(OH)D	7	5	1	18		
Overall WSAVA score- ileum^	Low 25(OH)D	5	4.5	1	11	-0.2199	0.2805
	Normal 25(OH)D	3	8	0	14		
Overall WSAVA score- duodenum + ileum^	Low 25(OH)D	16	10	8	23	-0.5685	**0.0024**
	Normal 25(OH)D	9	13.5	2	32		

*P-value as assessed by Spearman correlation. Significance set at p< .0036. ^Scores available for 13 dogs per group. # Total score for villous stunting, epithelial injury, crypt distension, lacteal dilation and mucosal fibrosis. $ Total score for intraepithelial lymphocytes, lamina propria lymphocytes/plasma cells, lamina propria eosinophils, and lamina propria neutrophils. Low 25(OH)D: low 25-hydroxyvitamin D (<109 nmol/L); Normal 25(OH)D: normal 25-hydroxyvitamin D (109-423 nmol/L); LD: lacteal dilation; WSAVA: World Small Animal Veterinary Association; IQR: interquartile range

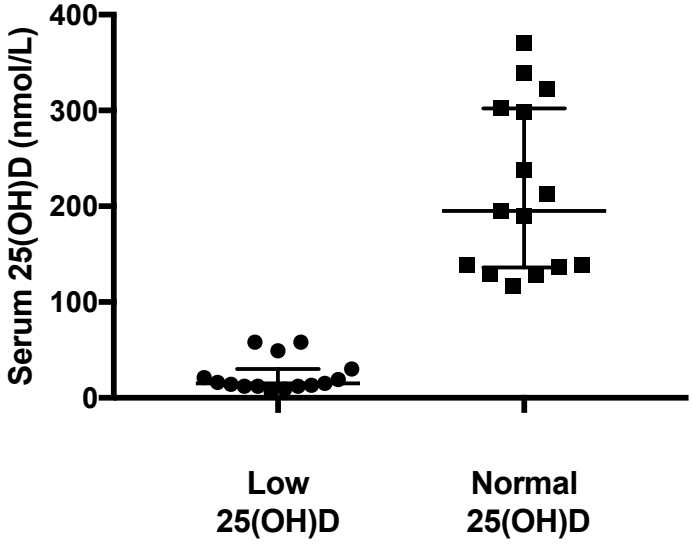

Figure 6.1 Scatter dot plot of serum 25(OH)D in dogs with CE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown.

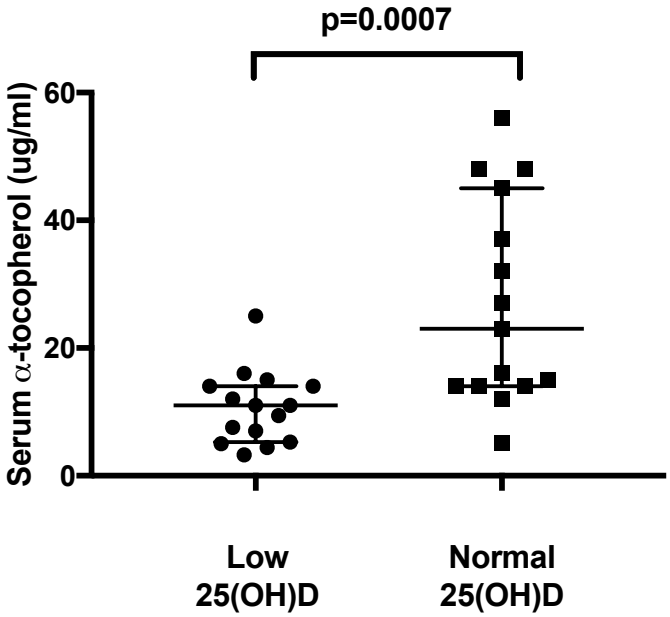

Figure 6.2 Scatter dot plot of serum α-tocopherol in dogs with CE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown.

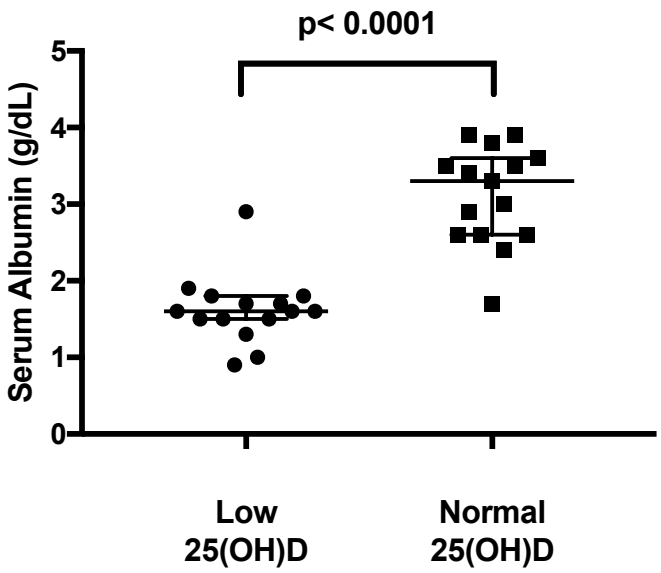

Figure 6.3 Scatter dot plot of serum albumin in dogs with CE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown.

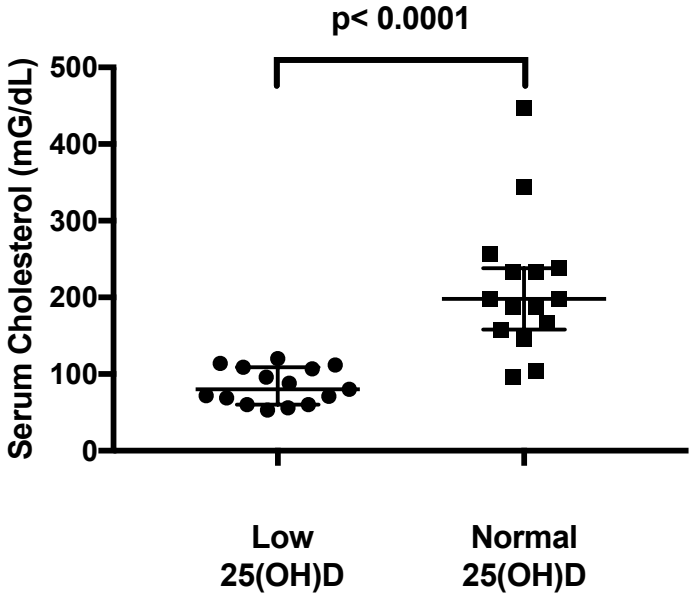

Figure 6.4 Scatter dot plot of serum cholesterol in dogs with CE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown.

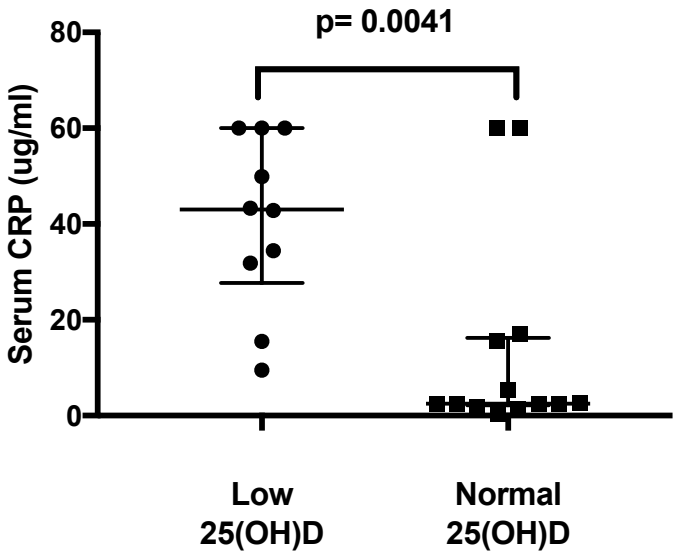

Figure 6.5 Scatter dot plot of serum C-reactive protein in dogs with CE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown.

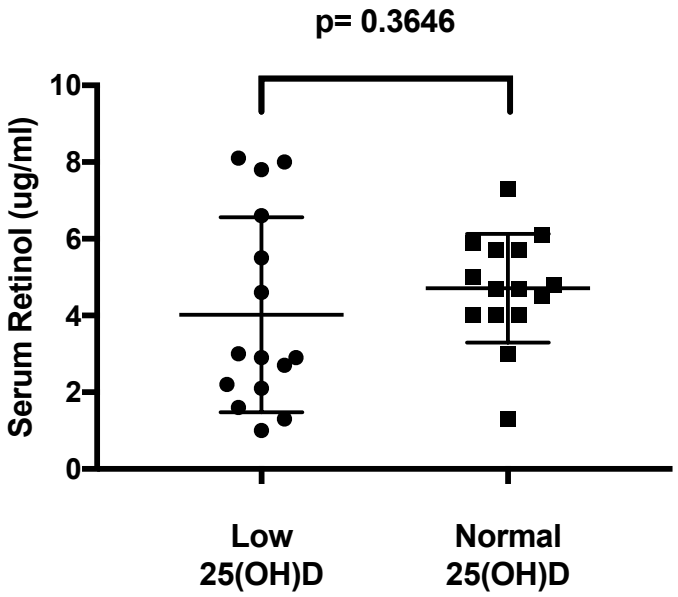

Figure 6.6 Scatter dot plot of serum retinol in dogs with CE with and without low serum 25(OH)D. Horizontal bar represents mean. Standard deviation shown.

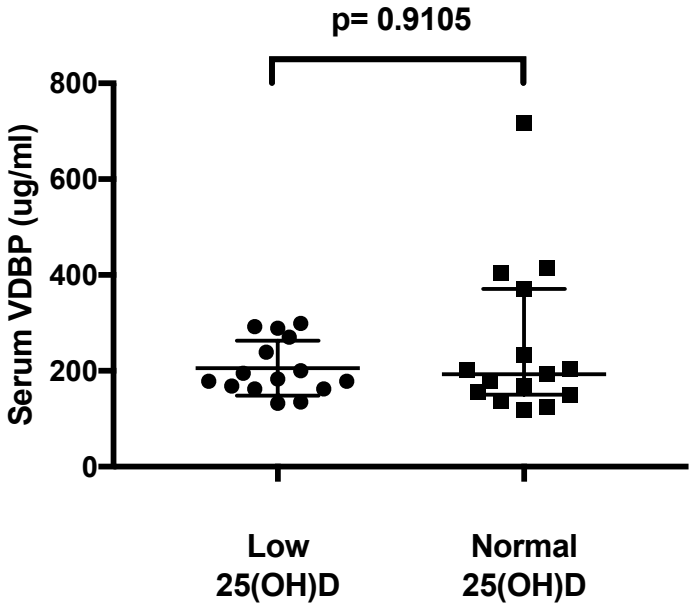

Figure 6.7 Scatter dot plot of serum vitamin D binding protein in dogs with CE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown.

6.5 Discussion

In our study, CCECAI scores and CRP concentrations were higher, while serum α-tocopherol, cholesterol and albumin concentrations were lower in dogs with CE and low serum 25(OH)D concentrations when compared to dogs with CE and normal serum 25(OH)D concentrations. In addition, duodenal, combined duodenal/ileal morphologic scores, and overall WSAVA scores were correlated with serum 25(OH)D levels in dogs with CE.

CCECAI scores, but not CIBDAI scores, were found to be significantly higher in CE dogs with low vitamin D status when compared to CE dogs with normal vitamin D status. Clinical scores have been variably associated with vitamin D status in dogs with CE and PLE.[1,4] In our study, both CIBDAI and CCECAI scores trended higher in low 25(OH)D dogs, but given

the association between low vitamin D status and hypoalbuminemia in this group of dogs, we suspect that the contribution of serum albumin to the CCECAI score is likely the reason that only the CCECAI score was different between groups. Appetite scores were also evaluated in isolation. In a previous study, serum 25(OH)D concentration was measured in healthy dogs, hospitalized dogs with non-gastrointestinal illness, and 33 dogs with idiopathic IBD with and without hypoalbuminemia. In this study, IBD dogs with hypovitaminosis D were more likely to have poor appetite when compared to dogs without hypovitaminosis D, but hospitalized dogs with non-gastrointestinal illness and poor appetite had higher serum 25(OH)D levels when compared to the dogs with IBD.[2] A recent study found serum 25(OH)D levels to be lower in critically ill dogs compared to healthy control dogs. Appetite scores or nutritional intake were not recorded for the dogs in this retrospective study, so the effect of intake could not be evaluated.[29] In our study, appetite scores were not different between groups. In fact, the owners of an equal number of dogs in each group (n=6) described their dogs as having a normal appetite (appetite score 0). This would suggest that poor oral intake of vitamin D is unlikely to be the sole explanation for why some dogs with CE have low vitamin D and others do not, however precise quantification of dietary vitamin D intake would be needed to definitively make this conclusion.

Alpha-tocopherol is the most biologically important of the eight fat-soluble chemically related compounds that make up Vitamin E. Vitamin E is an antioxidant typically abundant in plasma and somatic cells with an important role in stabilizing lipid membranes.[30] Symptomatic vitamin E deficiency is rare in humans and results from either a primary defect in the alpha-tocopherol transport protein or an acquired defect due to various conditions that result in fat malabsorption, including Crohn's disease.[15,31] Retinol is the most biologically active form of vitamin A and has functions in reproduction, bone growth, maintenance of epithelial tissue, and

vision. Vitamin A also plays a protective role against oxidative stress and free radical formation.[30] Deficiency of vitamin A is uncommon in the United States, but is a significant problem in developing countries if nutritional intake is inadequate. Other causes of vitamin A deficiency include alcoholic liver disease, conditions resulting in fat malabsorption, and iron deficiency.[32,33] Serum cholesterol and α- tocopherol (vitamin E) concentrations were lower in low 25(OH)D CE dogs. Additionally, based on the laboratory-established reference interval (RI) two dogs in the low 25(OH)D group had subnormal vitamin E with serum concentrations <5 ug/ml. Neither dog displayed overt clinical signs associated with vitamin E deficiency. Serum retinol concentrations fell within or above the normal reference interval (RI) for all dogs in this study, and were not different between CE dogs with low serum 25(OH)D levels compared to CE dogs with normal serum 25(OH)D levels. In humans with CD, measurement of hepatic vitamin A stores using the relative dose response test was demonstrated to be more sensitive for the detection of vitamin A deficiency compared to serum retinol measurement.[32] Therefore, it is possible that measurement of hepatic vitamin A stores may have been a more accurate way to assess vitamin A levels in our patients.

Intestinal absorption of dietary cholesterol and fat-soluble vitamins (including A, D, E, and K) require emulsification, hydrolysis and micellization as well as adequate concentrations of pancreatic lipase and bile acids in the intestinal lumen.[34] Bile salt micelles facilitate the transfer of cholesterol and fat-soluble vitamins into the enterocyte, where they are incorporated into chylomicrons within the endoplasmic reticulum of the cell. Following this, chylomicrons are secreted into the lymphatic system, which is the major route of transport of cholesterol and fat-soluble vitamins from the intestine to the blood.[35] Therefore, dietary fat malabsorption due to lack of bile salts, lymphatic dysfunction or other causes may be an explanation for the decreased

serum concentrations of cholesterol and α- tocopherol in dogs with low serum 25(OH)D and CE. It is important to note that in humans cholesterol is fundamental to the endogenous synthesis of vitamin D following sunlight exposure.[36] However, because endogenous synthesis of vitamin D is thought to be limited in the dog[37], it is unlikely that hypocholesterolemia associated with PLE[38] was the direct cause of low serum 25(OH)D in our cases. To further investigate the possibility of fat malabsorption in dogs with CE and low serum 25(OH)D, a coefficient of fat absorption test of stool, or malabsorption blood test could be performed.[39] Body condition scores were not different between groups in our study. Although BCS has shown good correlation with more direct body fat measuring methods such as dual-energy x-ray absorptiometry[40], it is still based on palpation and visual inspection, making it open to subjective variation. In addition, BCS may be a better measure of subcutaneous fat than visceral fat.[41]

Several of the dogs in our study had serum α-tocopherol and retinol concentrations above the laboratory-established reference interval. This finding is similar to a recent study evaluating lipid-soluble vitamins in dogs with exocrine pancreatic insufficiency,[42] which included healthy control animals. This finding deserves further study.

A relationship between serum albumin and 25(OH)D levels has previously been demonstrated in humans[18] and dogs[2] with IBD. In our study dogs, CE dogs with low levels of serum 25(OH)D had serum albumin concentrations lower than CE dogs with normal levels of serum 25(OH)D. In fact, only 1/15 dogs (7%) with serum 25(OH)D levels below the laboratory-established RI for 25(OH)D had a serum albumin concentration above 2.0 g/dL. Since vitamin D and its metabolites are largely bound to VDBP in circulation, and VDBP belongs to the same family of binding proteins as albumin,[17] it had been postulated that loss of VDBP is likely a mechanism for decreased 25(OH)D levels in human and canine patients with hypoalbuminemia

secondary to IBD.[2,18] However, VDBP concentrations have only been studied in one cohort of children with IBD. This study found that serum VDBP concentrations were lower in children with IBD when compared to healthy controls, but no correlation between serum VDBP and serum 25(OH)D or albumin was found.[17] As far as we know, serum VDBP concentrations have not previously been measured in dogs with CE. We found that VDBP concentrations were similar between the low 25(OH)D and normal 25(OH)D groups. Because dogs with hypoalbuminemia were largely separated by serum 25(OH)D levels, VDBP is likely not correlated with albumin in our group of dogs, similar to the study performed in children. It is important to note however, that since we do not have an established RI for serum VDBP concentration in dogs, we do not know how these values compare to what would be found in a population of healthy dogs. Loss of serum albumin may be directly related to decreased 25(OH)D levels in dogs with CE and hypoalbuminemia, since vitamin D and its metabolites circulate in part bound to albumin. Only about 10-15% of vitamin D and its metabolites circulate bound to albumin, but albumin is thought to be the most affected protein in PLE, due to its slow turnover compared to other serum proteins.[17] An additional or alternative explanation would be that the relationship between serum albumin and serum 25(OH)D is not causal, but representative of an underlying pathophysiology that results in the decrease of both. There is general consensus that albumin moves unidirectionally from the blood to the tissue, and there is only minute diffusion of albumin from tissue to the blood. Lymphatic return of albumin is required to balance the one-way loss from blood to tissue.[43] Therefore if intestinal lymphatic abnormalities are present, albumin may not be adequately returned to the blood resulting in hypoalbuminemia over a prolonged period of time.

Low serum 25(OH)D concentrations were correlated to higher scores for morphologic changes to the intestine. In a previous study, total WSAVA scores were found to be negatively associated with serum 25(OH)D concentrations in dogs with CE.[19] Generally, less attention has been paid to the morphologic changes of the intestine when compared to inflammatory changes in dogs with CE, despite the fact that morphologic changes have been correlated to clinicopathologic markers and clinical severity.[44] In this study, we elected to evaluate the morphologic and inflammatory changes in isolation, and together. In this group of dogs morphologic changes to the duodenum consisting of villous stunting, epithelial injury, crypt distension, lacteal dilation, and mucosal fibrosis, were negatively associated with serum 25(OH)D levels. In addition, when duodenal and ileal morphologic scores were summed, a significant negative correlation was also appreciated. Lacteal dilation was also evaluated in isolation and ileal lacteal dilation scores approached but did not reach statistical significance. It is important to note that the histopathologic interpretation of lacteal dilation has limitations, particularly in endoscopic samples. Lymphangiectasia can be segmental, and in some cases, confined to deeper layers of the intestine that may not be sampled in endoscopic biopsies.[45]

The importance of morphologic changes to the intestine in cases of CE has been gaining attention in part due to studies that have revealed their significance with regards to clinical scores,[46] hypoalbuminemia[46,47] and response to therapy.[48] Recently, a group of dogs with food-responsive enteropathy were demonstrated to have improvement in the ultrastructural lesions in their intestine that correlated with clinical improvement, despite a lack of significant improvement in the cellular infiltrates in their intestine.[48] In our study dogs, duodenal inflammatory scores also approached statistical significance, and total WSAVA scores in the duodenum and combined duodenal/ileal were negatively correlated with serum 25(OH)D

concentrations. Further, serum CRP concentrations were higher in dogs with decreased 25(OH)D concentrations, suggesting systemic inflammation may play in a role in the development of hypovitaminosis D in dogs. Both systemic and intestinal inflammation may play a role in the development of hypovitaminosis D in dogs with CE. However, this work would suggest that the morphologic changes also play a role, adding to the growing evidence of the importance of morphologic changes to the intestine in cases of CE.

Contrary to previous investigations[1,4], our study dogs with decreased 25(OH)D concentrations were not more likely to die or be euthanized due to their disease when compared to dogs with normal 25(OH)D concentrations. The reason for the discrepancy between our study and others is not known, but we feel it is important to highlight that dogs with hypovitaminosis D and CE can have good outcomes.

Finally, as expected 25(OH)D concentrations were positively correlated with serum ionized calcium concentrations and negatively correlated with serum PTH concentrations. Ten dogs in in the low 25(OH)D group had ionized hypocalcemia defined by serum iCa concentrations <1.25 mmol/L. Apparent nutritional secondary hyperparathyroidism was diagnosed in ten dogs with hypovitaminosis D based on serum PTH concentrations >5.8 pmol/L. This is the appropriate physiologic response to a vitamin D deficient state,[49] but it was not present in all dogs.

This study has several limitations. First, the ileum was unable to be biopsied in two dogs in each group. Since pathology can differ between sections of intestine[50,51] this may have influenced our results. Also, despite the use of a blinded pathologist and a pathologist-in-training to come to a consensus score using WSAVA standards, histopathologic interpretation of the intestine is still considered relatively subjective[52] and interobserver variation exists.[53] Serum

CRP and fibrinogen levels were not measured in all dogs, as the study protocol was amended to include these variables after several dogs had already been enrolled. This may have affected our results with these variables. Since a reference interval has not been established for serum VDBP in healthy dogs, no conclusions can be drawn about the levels of VDBP in dogs with CE compared to healthy controls. The inclusion of a robust healthy control group, particularly for serum fat-soluble vitamins as well as VDBP could have strengthened our results. Two dogs in each study had received glucocorticoids for <7 days prior to enrollment. While glucocorticoids are known to affect vitamin D metabolism at several different levels, studies have shown that short-term administration (<14 days) of glucocorticoids did not affect concentrations of serum 25(OH)D in humans.[54] While we cannot be certain vitamin D metabolism in dogs is equally unaffected, since 2 dogs were present in each group we do not expect this affected the results significantly. Finally, while radioimmunoassay for serum 25(OH)D concentration is the most widely used approach to measure whole body vitamin D status[5], liquid chromatography-mass spectrometry is considered by some to be a more accurate form of measurement.[4]

This study was intended to be an investigatory survey of multiple variables in a group of dogs with CE with and without decreased serum concentration of 25(OH)D in order to elucidate which variables warrant continued investigation. It is important to note that causal relationships cannot be inferred from this study. Further evaluation, particularly of serum concentrations of the other fat-soluble vitamins and VDBP, in a larger number of clinical cases as well as in healthy controls is necessary in order to be able to better understand the mechanism(s) of low serum 25(OH)D in dogs with CE. Based on our study dogs, intestinal fat malabsorption, potentially related to lymphatic dysfunction deserves further study in dogs with CE and low serum 25(OH)D. Based on this preliminary evaluation, loss of VDBP does not appear to be a major

mechanism of hypovitaminosis D, but this requires confirmation in a much larger group of dogs, as well as healthy controls. The influence of systemic and intestinal inflammation on vitamin D levels was identified as an important area of study in the mechanism of low serum 25(OH)D among dogs with CE.

REFERENCES

1. Titmarsh H, Gow AG, Kilpatrick S, et al. Association of vitamin D status and clinical outcome in dogs with a chronic enteropathy. J Vet Intern Med 2015;29:1473-1478.
2. Gow AG, Else R, Evans H, et al. Hypovitaminosis D in dogs with inflammatory bowel disease and hypoalbuminaemia. J Sm Anim Pract 2011;52:411-418.
3. Mellanby RJ, Mellor PJ, Roulois A, et al. Hypocalcaemia associated with low serum vitamin d metabolite concentrations in two dogs with protein‐losing enteropathies. J Sm Anim Pract 2005;46:345-51.
4. Allenspach K, Rizzo J, Jergens AE, et al. Hypovitaminosis D is associated with negative outcome in dogs with protein losing enteropathy: a retrospective study of 43 cases. BMC Vet Res 2017;13:96.
5. Nielsen OH, Rejnmark L, Moss AC. Role of Vitamin D in the natural history of inflammatory bowel disease. J Crohns Col 2018;12:742-752.
6. Del Pinto R, Pietropaoli D, Chandar AK, et al. Association between inflammatory bowel disease and vitamin D deficiency: a systematic review and metaanalysis. Inflamm Bowel Dis 2015; 21:2708–2717.
7. Sadeghian M, Saneei P, Siassi F, et al. Vitamin D status in relation to Crohn's disease: meta-analysis of observational studies. Nutrition 2016;32:505–14.
8. Suibhne TN, Cox G, Healy M, O'Morain C, O'Sullivan M. Vitamin D deficiency in Crohn's disease: prevalence, risk factors and supplement use in an outpatient setting. J Crohns Colitis 2012;6:182–188.
9. Ulitsky A, Ananthakrishnan AN, Naik A, et al. Vitamin D deficiency in patients with inflammatory bowel disease: association with disease activity and quality of life. JPEN J Parenter Enteral Nutr 2011;35:308–316.
10. Ananthakrishnan AN, Cagan A, Gainer VS, et al. Normalization of plasma 25-hydroxy vitamin D is associated with reduced risk of surgery in Crohn's disease. Inflamm Bowel Dis 2013;19:1921–1927.
11. Ardesia M, Ferlazzo G, Fries W. Vitamin D and inflammatory bowel disease. BioMed research international 2015;470805.
12. Farraye FA, Nimitphong H, Stucchi A, et al. Use of a novel vitamin D bioavailability test demonstrates that vitamin D absorption is decreased in patients with quiescent Crohn's disease. Inflamm Bow Dis 2011;17:2116-2221.
13. Lo CW, Paris PW, Clemens TL, et al. Vitamin D absorption in healthy subjects and in patients with intestinal malabsorption syndromes. Am J Clin Nutr 1985; 42:644–649.
14. Vogelsang H, Schofl R, Tillinger W, et al. 25-hydroxyvitamin D absorption in patients with Crohn's disease and with pancreatic insufficiency. Wien Klin Wochenschr. 1997; 109:678–682.
15. Fabisiak N, Fabisiak A, Watala C, et al. Fat-soluble vitamin deficiencies and inflammatory bowel disease. J Clin Gastro 2017;51:878-889.
16. Garg M, Lubel JS, Sparrow MP, et al. Vitamin D and inflammatory bowel disease–established concepts and future directions. Aliment Pharmacol Ther 2012;36:324-344.
17. Strisciuglio C, Cenni S, Giugliano FP, et al. The role of inflammation on Vitamin D levels in a cohort of pediatric patients with inflammatory bowel disease. J Ped Nutr Gastro 2018.

18. Pappa HM, Gordon CM, Saslowsky TM, et al. Vitamin D status in children and young adults with inflammatory bowel disease. Pediatrics 2006;118:1950-1961.
19. Titmarsh HF, Gow AG, Kilpatrick S, et al. Low vitamin D status is associated with systemic and gastrointestinal inflammation in dogs with a chronic enteropathy. PloS one 2015;10:e0137377.
20. Agborsangaya C, Toriola AT, Grankvist K, et al. The effects of storage time and sampling season on the stability of serum 25-hydroxy vitamin D and androstenedione. Nutr Cancer. 2010;62:51–57.
21. Cavalier E, Delanaye P, Hubert P, et al. Estimation of the stability of parathyroid hormone when stored at− 80° C for a long period. Clin J Am Soc Nephro 2009:CJN-03970609.
22. Parker VJ, Harjes LM, Dembek K, et al. Association of vitamin D metabolites with parathyroid hormone, fibroblast growth factor‐23, calcium, and phosphorus in dogs with various stages of chronic kidney disease. J Vet Intern Med 2017;31:791-79
23. Laflamme, D. R. P. C. Development and validation of a body condition score system for dogs. Canine Pract. 1997;22:10-15.
24. Jergens AE,Schrreiner CA, Frank DE, et al. A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med 2003;17:291-297.
25. Allenspach K, Wieland A, Grone A, et al. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med 2007;21:700-708.
26. Comstock GW, Alberg AJ, Helzlsouer KJ. Reported effects of long-term freezer storage on concentrations of retinol, beta-carotene, and alpha-tocopherol in serum or plasma summarized. Clin Chem 1993;39:1075-1078.
27. Jezequel-Cuer M, Le Moel D, Covi G, et al. Stability of alpha-tocopherol: pre-analytical conditions in its determination in blood samples. Ann Biol Clin 1994;52:271-276.
28. Washabau RJ, Day MJ, Willard MD, et al. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. J Vet Intern Med 2010;24:10–26.
29. Jaffey JA, Backus RC, McDaniel KM, et al. Serum vitamin D concentrations in hospitalized critically ill dogs. PloS one 2018;13:e0194062.
30. Case LP, Daristotle L, Hayek, MG, et al. Canine and Feline Nutrition. 3rd edition. Mosby Elsevier 2011: 27-35.
31. Aslam A, Misbah SA, Talbot K, et al. Vitamin E deficiency induced neurological disease in common variable immunodeficiency: two cases and a review of the literature of vitamin E deficiency. Clin Immuno 2004;112:24-29.
32. Soares-Mota M, Silva TA, Gomes LM,et al. High prevalence of vitamin A deficiency in Crohn's disease patients according to serum retinol levels and the relative dose-response test. WJG; 2015;21:1614-1620.
33. Wiseman EM, Bar-El Dadon S, Reifen R. The vicious cycle of vitamin A deficiency: A review. Cri Rev Food Sci 2017;57:3703-3714.
34. Iqbal J, Hussain MM. Intestinal lipid absorption. Am J Phys-Endo Met 2009;296:E1183-94.
35. Thompson GR. Lipid related consequences of intestinal malabsorption. Gut 1989;30:29-34.
36. Wacker M, Holick MF. Sunlight and Vitamin D: A global perspective for health. Dermato-endocrinology 2013;5:51-108.
37. Weidner N, Verbrugghe A. Current knowledge of vitamin D in dogs. Crit Rev Food Sci Nutr 2017;57:3850-3859.
38. Dossin, Olivier, and Rachel Lavoué. Protein-losing enteropathies in dogs. Vet Clin: Small

Anim Pract 2011;41:399-418.
39. Mascarenhas MR, Mondick J, Barrett JS, et al. Malabsorption blood test: Assessing fat absorption in patients with cystic fibrosis and pancreatic insufficiency. J Clin Pharm 2015;55:854-865.
40. Lauten SD, Cox NR, Brawner WR Jr, et al. Use of dual 247 energy x-ray absorptiometry for noninvasive body composition measurements in 248 clinically normal dogs. Am. J. Vet. Res. 62: 1295–1301.
41. Kim D, Noh D, Oh T, et al. Body fat assessment by computed tomography and radiography in normal Beagle dogs. J Vet Med Sci 2018:18-0216.
42. Barko PC, Williams DA. Serum concentrations of lipid‐soluble vitamins in dogs with exocrine pancreatic insufficiency treated with pancreatic enzymes. Journal of veterinary internal medicine. 2018.
43. Levitt DG, Levitt MD. Human serum albumin homeostasis: a new look at the roles of synthesis, catabolism, renal and gastrointestinal excretion, and the clinical value of serum albumin measurements. Int J Gen Med 2016;9:229-255.
44. Jergens AE, Willard MD, Allenspach K. Maximizing the diagnostic utility of endoscopic biopsy in dogs and cats with gastrointestinal disease. Vet J 2016;214:50-60.
45. Larson RN, Ginn JA, Bell CM, et al. Duodenal endoscopic findings and histopathologic confirmation of intestinal lymphangiectasia in dogs. J Vet Intern Med 2012;26:1087–1092.
46. Moser K, Mitze S, Teske E, et al. Correlation of clinical, diagnostic and histopathological parameters in dogs with chronic lymphocytic-plasmacytic enteropathy. Tierärztliche Praxis K: Kleintiere/Heimtiere 2018;46:15-20.
47. Wennogle SA, Priestnall SL, Webb CB. Histopathologic characteristics of intestinal biopsy samples from dogs with chronic inflammatory enteropathy with and without hypoalbuminemia. J Vet Intern Med 2017;31:371-376.
48. Walker D, Knuchel‐Takano A, McCutchan A, et al. A comprehensive pathological survey of duodenal biopsies from dogs with diet‐responsive chronic enteropathy. J Vet Intern Med 2013;27:862-874.
49. Dhupa N, Proulx J. Hypocalcemia and hypomagnesemia. Vet Clin NA Sm Anim Pract 1998;28:587-608.
50. Procoli F, Motskula PF, Keyte SV, et al. Comparison of histopathologic findings in duodenal and ileal endoscopic biopsies in dogs with chronic small intestinal enteropathies. J Vet Intern Med 2013;27:268–274.
51. Casamian-Sorrosal D, Willard MD, Murray JK, et al. Comparison of histopathologic findings in biopsies from the duodenum and ileum of dogs with enteropathy. J Vet Intern Med 2010;24:80–83.
52. Jergens AE, Evans RB, Ackermann M, et al. Design of a simplified histopathologic model for gastrointestinal inflammation in dogs. Vet Pathol 2014;51:946–950.
53. Willard MD, Jergens AE, Duncan RB, et al. Interobserver variation among histopathologic evaluations of intestinal tissues from dogs and cats. J Am Vet Med Assoc 2002;220:1177–1182.
54. Hahn TJ, Halstead LR, Baran DT. Effects of short term glucocorticoid administration on intestinal calcium absorption and circulating vitamin D metabolite concentrations in man. TJ Clin Endo Meta 1981;52:111-115.

CHAPTER 7: CONCLUDING REMARKS AND FUTURE DIRECTIONS

7.1 Significance of Work

The goal of the research presented in this dissertation was to explore clinical, clinicopathologic, histopathologic, and immunohistochemical features of dogs with idiopathic chronic enteropathies, with a specific emphasis on gaining an improved understanding of the etiopathogenesis of the development of protein-losing enteropathy in cases of CE. As many as 20% of dogs with CE develop protein-losing enteropathy[1,2], and idiopathic chronic enteropathy is considered the most common cause of chronic gastrointestinal signs in the dog.[3] Therefore, the results of this work have potential application to a significant population of dogs with chronic gastrointestinal signs. It was my hope that some aspects of this research could impact these populations immediately, as well as inspire further investigations that may impact dogs with CE and PLE in the future. In chapter 3, we retrospectively examined histopathologic features of dogs with CE with and without PLE. In chapter 4, we set out to prospectively evaluate whether a novel dietary approach could result in clinical improvement in dogs with CE and PLE who were not responding well to traditional therapy with glucocorticoids. In chapter 5, we utilized lymphatic endothelial cell (LEC) markers and immunohistochemistry to evaluate the intestinal lymphatic vasculature in a group of dogs with CE. Finally, in chapter 6 we aimed to improve our understanding of the pathogenesis of low serum vitamin D in dogs with CE by evaluating a variety of variables we felt could be associated with the mechanisms of low serum vitamin D in this population.

While each of these chapters had a variety of unique findings and conclusions, the common theme was the exploration of the role of the intestinal lymphatic vasculature in cases of

CE and PLE in the dog. Based on the collective findings of this work, we can conclude that the intestinal lymphatic vasculature plays a larger role in these disease processes than has previously been appreciated and that the application of lymphatic endothelial cell IHC should be considered in select clinical cases. Based on the findings in chapter 5 we can recommend that ileal biopsies should be obtained for all dogs undergoing intestinal biopsy for the diagnostic investigation of chronic gastrointestinal signs, and in particular dogs with PLE. Regardless of the lymphatic findings on routine H&E evaluation, the implementation of a fat-restricted diet should be considered in dogs with CE and PLE failing traditional therapies. We can also conclude that the role of the lymphatic vasculature in cases of canine CE deserves further study. This study should include techniques for evaluating the structure and function of lymphatics, including study of the phenomenon of lymphangiogenesis. In addition, specific therapies to target abnormalities of the intestinal lymphatic vasculature, including the implementation of various fat-restricted diets, should be further investigated.

7.2 Future Directions

Since this body of work was initiated, the finding in chapter 3 of the correlation of serum albumin concentrations with lacteal dilation in cases of canine PLE has been corroborated in other studies.[4,5] However, the findings of chapter 4, 5, and 6 are relatively novel and repeatability of these findings in more dogs with CE and PLE would strengthen these findings. Therefore, follow-up studies to this body of work could include the application of LEC markers using immunohistochemical techniques to a larger population of dogs (retrospectively or prospectively). In a similar vein, the investigations carried out in chapter 6 should be completed in a larger population of dogs with CE and PLE.

In addition, as this body of work has included a variety of findings that suggest fat malabsorption may be a particular problem for certain dogs with CE, specific fat malabsorption studies could be considered as a follow-up to this work. While protocols for the measurement of fecal fat exist for the dog,[6] limited studies have been performed specifically evaluating fecal fat in dogs with chronic gastrointestinal signs.[7] Furthermore, a malabsorption blood test has been developed to assess fat absorption in humans with cystic fibrosis and pancreatic insufficiency.[8] This technique involves the administration of a fat-loading meal and subsequent measurements in plasma to determine the absorption of the components of that meal.[8,9] This type of investigation would likely be safe and simple to implement in dogs with CE and PLE, and would be an interesting follow-up to the work presented here.

This work provides a foundation for further studies investigating the intestinal lymphatic vasculature in cases of CE and PLE. In addition to repeat studies utilizing IHC to label and therefore better define lymphatic populations in intestinal biopsies, further studies should also explore lymphangiogenesis and alterations of lymphatic function in these populations. A number of different lymphatic regulatory molecules including VEGF-C & D, FoxC2, SOX18, and Prox-1 can be measured in intestinal biopsies with the use of real-time PCR[10]. Upregulation of these molecules suggest proliferating lymphatics and would further support the theory of lymphangiogenesis as a consequence of inflammation. Lymphangiogenesis is generally thought to have a beneficial effect by controlling tissue edema and promoting leukocyte, bacterial antigen and inflammatory chemokine clearance. Whether these changes occur as a consequence of inflammation and/or as a response to dysfunctional lymphatic vasculature is unknown[11]. Therefore, lymphatic function studies it would be important to establish whether the lymphatic populations in these patients are functional. Lymphatic function studies are difficult to perform

and often invasive. The contractile function of the lymphatic vasculature in guinea pigs with trinitrobenzenesulfonic acid (TNBS)-induced ileitis was found to be abnormal by surgically isolating lymphatic vessels and measuring their contractile activity[12]. These types of studies would not be suitable for clinical patients. Therefore, other techniques to study lymphatic function must be considered. Recently, CT lymphangiography (CTL) was used to detect intestinal and mesenteric lymphangiectasia as well as lymphangiomas in humans with primary intestinal lymphangiectasia. Lymph fluid reflux and intraabdominal lymphatic leakages were also detected. In some cases, CTL was better than histology for the detection of intestinal lymphangiectasia[13]. This technique could be applied to dogs with CE with and without PLE to better understand intestinal lymphatic architecture and function.

Finally, based on this work we need to develop a better understanding of the specific therapies that may lead to clinical improvement in cases of CE with hypovitaminosis D and/or lymphatic abnormalities detected on routine H&E histopathologic exam or with the help of LEC immunolabeling. Some of this understanding may come from prospective clinical trials utilizing therapies to treat hypovitaminosis D or therapies to better address intestinal lymphatic disease in cases of CE. As we do not yet have a gold-standard protocol for the treatment of low serum 25(OH)D in dogs,[14] much work is needed in this arena. We also lack a definitive recommendation on the amount of dietary fat-restriction needed for the treatment of lymphangiectasia in dogs, and we lack a true understanding of how else to address lymphatic abnormalities in canine patients. Therefore, opportunities for continued study in these areas of canine gastroenterology are plentiful, and the work described in this dissertation provides a solid foundation for further research.

REFERENCES

1. Craven M, Simpson JW, Ridyard AE, et al. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). J Small Anim Pract 2004;45:336-342.
2. Allenspach K, Wieland A, Grone A, et al. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. J Vet Intern Med 2007;21:700-708.
3. Volkmann M, Steiner JM, Fosgate GT, et al. Chronic diarrhea in dogs–retrospective study in 136 cases. J Vet Intern Med 2017;31:1043-1055.
4. Rossi G, Cerquetella M, Antonelli E, et al. The importance of histologic parameters of lacteal involvement in cases of canine lymphoplasmacytic enteritis. Gastroenterol Hepatol Bed Bench 2015;8:33.
5. Moser K, Mitze S, Teske E, et al. Correlation of clinical, diagnostic and histopathological parameters in dogs with chronic lymphocytic-plasmacytic enteropathy. Tierärztliche Praxis K: Kleintiere/Heimtiere. 2018;46:15-20.
6. Piccione G, Fazio F, Giudice E, et al. Blood lipids, fecal fat and chymotrypsin excretion in the dog: influence of age, body weight and sex. J Vet Med Sci 2004;66:59-62.
7. Burrows CF, Merritt AM, Chiapella AM. Determination of fecal fat and trypsin output in the evaluation of chronic canine diarrhea. J Am Vet Med Assoc 1979;174:62-66.
8. Mascarenhas MR, Mondick J, Barrett JS, et al. Malabsorption blood test: Assessing fat absorption in patients with cystic fibrosis and pancreatic insufficiency. J Clin Pharm 2015;55:854-865.
9. Nikaki K, Gupte GL. Assessment of intestinal malabsorption. Best Pract Res Clin Gastro 2016;30:225-235.
10. Rahier JF, De Beauce S, Dubuquoy L, et al. Increased lymphatic vessel density and lymphangiogenesis in inflammatory bowel disease. Aliment Pharmacol Ther 2011;34:533-543.
11. Alexander JS, Ganta VC, Jordan PA, et al. Gastrointestinal lymphatics in health and disease. Pathophysiology 2010;17:315-335.
12. Wu TF, Carati CJ, MacNaughton WK, et al. Contractile activity of lymphatic vessels is altered in the TNBS model of guinea pig ileitis. Amer J Phys-Gastro Liver Phys 2006 ;291:G566-74.
13. Dong J, Xin J, Shen W, et al. CT Lymphangiography (CTL) in primary intestinal lymphangiectasia (PIL): a comparative study with intraoperative enteroscopy (IOE). Acad Rad 2018; Published online.
14. Weidner N, Verbrugghe A. Current knowledge of vitamin D in dogs. Crit Rev Food Sci Nutr 2017;57:3850-3859.