


STANDARD ARTICLE

Lymphatic endothelial cell immunohistochemical markers for evaluation of the intestinal lymphatic vasculature in dogs with chronic inflammatory enteropathy

Sara A. Wennogle¹  | Simon L. Priestnall² | Alejandro Suárez-Bonnet²  |
Sirikul Soontarak¹ | Craig B. Webb¹

¹Department of Clinical Sciences, College of Veterinary Medicine, Colorado State University, Fort Collins, Colorado

²Department of Pathobiology and Population Sciences, Royal Veterinary College, Hatfield, United Kingdom

Correspondence

Sara A. Wennogle, Department of Clinical Sciences, College of Veterinary Medicine, Colorado State University Veterinary Teaching Hospital, 300 West Drake Road, Fort Collins, CO 80525.

Email: sara.wennogle@colostate.edu

Funding information

Naniboujou Legacy Fund; Rocky's Research Fund; Royal Canin

Abstract

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

Objectives: To utilize LEC markers to identify and measure intestinal lymphatic vasculature in endoscopic biopsy samples of CIE dogs. To evaluate whether measured lymphatic vasculature variables correlate with serum albumin concentrations.

Animals: Twenty-four dogs with CIE; $n = 13$, serum albumin concentration <2.5 g/dL (CIE-protein-losing enteropathy [PLE]), $n = 11$, serum albumin concentration ≥ 2.5 g/dL (CIE-N).

Methods: Prospective study. Lymphatic endothelial cell immunolabeling with Prox-1 and LYVE-1 performed on endoscopic biopsy samples from 24 dogs with CIE. Duodenal and ileal villous lacteal width (VLW) and proprial mucosal lacteal width (MLW) were determined for each case and analyzed for correlation with serum albumin concentration. Lacteal dilatation scores using routine H&E histopathology were assessed for correlation with immunohistochemistry (IHC)-calculated VLW and MLW.

Results: Lower serum albumin concentrations were correlated with increased VLW ($\rho = -.4644$; $P = .02$) and MLW ($\rho = -.6514$; $P < .001$) in the ileum. Lymphatic endothelial cell IHC identified presumptive proprial mucosal lymphangiectasia in some dogs that was not recognized with routine H&E staining. Lacteal dilatation scores were correlated with VLW in duodenum ($\rho = .4634$; $P = .02$) and ileum ($\rho = .5292$; $P = .008$), but did not correlate with MLW.

Abbreviations: CCECAI, canine chronic enteropathy clinical activity index; CD, Crohn's disease; CIE, chronic inflammatory enteropathy; CIE-N, chronic inflammatory enteropathy with serum albumin concentrations ≥ 2.5 g/dL; CIE-PLE, chronic inflammatory enteropathy with protein-losing enteropathy; IHC, immunohistochemistry; IL, intestinal lymphangiectasia; LD, lacteal dilatation; LEC, lymphatic endothelial cell; LYVE-1, lymphatic vascular endothelial hyaluronin acid receptor; MLW, mucosal lacteal width; PLE, protein-losing enteropathy; Prox-1, human prospero homeobox; VLW, villus lacteal width.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2019 The Authors. *Journal of Veterinary Internal Medicine* published by Wiley Periodicals, Inc. on behalf of the American College of Veterinary Internal Medicine.

Conclusions and Clinical Importance: Lymphatic endothelial cell immunolabeling identified presumptive proprial mucosal lymphangiectasia in CIE dogs, particularly in the ileum of hypoalbuminemic dogs. Routine evaluation of villous lacteals likely underestimates abnormalities of the lymphatic vasculature in dogs with CIE.

KEYWORDS

canine, enteropathy, immunolabeling, lacteals

1 | INTRODUCTION

Chronic inflammatory enteropathy (CIE) refers to conditions of the intestinal tract that are characterized by the presence of gastrointestinal signs of at least 3 weeks' duration, histologic evidence of intestinal inflammation, and the exclusion of neoplastic, infectious, and extra-gastrointestinal causes of gastrointestinal signs.¹⁻³ Intestinal lymphangiectasia (IL) is a disorder of dilated lymphatic vasculature at any level of the intestinal lymphatic system. Although it can be a primary condition, it also can occur secondary to a variety of other intestinal disorders, including CIE. When it occurs secondary to CIE, it is presumably a result of increased lymphatic pressure associated with inflammatory infiltrates in the intestine, and may result in hypoalbuminemia, or protein-losing enteropathy (PLE), because of direct loss of protein-rich lymph, lymphatic dysfunction, or both.⁴⁻⁶

Several recent studies have found a relationship between serum albumin concentrations and IL in cases of idiopathic CIE in the dog, however lymphatic abnormalities have been inconsistently reported.⁷⁻⁹ Lymphangiectasia can be underappreciated on routine histopathologic examination of the intestine, because it can have a segmental distribution and, in some cases, be confined to deeper layers of the intestine that may not be sampled endoscopically.^{5,6} Lymphatic abnormalities can be similarly confined to the deeper layers of the intestine in cases of Crohn's disease (CD),¹⁰⁻¹² a type of inflammatory bowel disease in humans characterized by idiopathic inflammation that can be localized to the ileum or found diffusely throughout the small intestine.¹³ In some cases of CD, dilated lymphatics are not identified in the superficial mucosa, but rather found in the deeper mucosa, submucosa, and muscularis layers of the intestine, as well as in the mesentery.^{14,15} In humans with CD, immunolabeling with lymphatic endothelial cell (LEC)-specific markers is superior to standard microscopy for identifying abnormalities of the lymphatic vasculature, including lymphangiectasia, and obstructed lymphatics.¹⁵

Lymphatic endothelial cells are derived from venous progenitor cells and express various antigens that distinguish them from blood vessel endothelial cells.¹⁶ Lymphatic endothelial cell markers are numerous and include human prospero homeobox (Prox-1), a nuclear transcription factor,¹⁷ and lymphatic vascular endothelial hyaluronic acid receptor (LYVE-1).¹⁸ Prox-1 and LYVE-1 immunolabeling previously has been used to differentiate types of angiosarcomas in the dog,¹⁹ but to our knowledge has not been used to evaluate the intestinal lymphatic vasculature in dogs with CIE.

Our objective was to utilize the LEC markers Prox-1 and LYVE-1 to identify and evaluate the intestinal lymphatic vasculature in endoscopic biopsy samples of dogs with CIE, and to determine whether abnormalities associated with the lymphatic vasculature were related to serum albumin concentrations in dogs with idiopathic CIE.

2 | MATERIALS AND METHODS

2.1 | Study population

Client-owned dogs presented to the Colorado State University Veterinary Teaching Hospital for evaluation of chronic gastrointestinal signs (eg, decreased appetite, vomiting, diarrhea, and weight loss) of >3 weeks' duration were recruited for participation in the study. To be eligible for inclusion, dogs were required to have had routine fecal screening with no parasites detected, no evidence of clinically relevant non-gastrointestinal illness, as assessed by routine hematology and serum biochemical profile, and a histopathologic diagnosis of inflammatory enteritis for which no distinct cause could be identified. Dogs had to have had duodenal and ileal biopsies performed. Dogs with histopathologic evidence of intestinal neoplasia were excluded. To be eligible for the study, exclusion of exocrine pancreatic insufficiency as a cause of clinical signs with a fasted serum canine trypsin-like immunoreactivity concentration >5.0 ng/mL was required. Hypoalbuminemic dogs (serum albumin concentration <2.5 g/dL) also were required to have no clinically relevant proteinuria (negative urine dipstick test result or urine protein:creatinine ratio <0.5) and no evidence of clinically relevant hepatic disease based on normal fasted and postprandial bile acid concentrations or normal synthetic liver function and enzyme activity on routine serum biochemistry profile.

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. After the results of the serum biochemical profile (serum albumin concentration) and abdominal ultrasound examination (peritoneal effusion), and using the owner's score, a canine chronic enteropathy clinical activity index (CCEAI)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.

2.2 | Histopathologic evaluation

Twelve duodenal and 5 ileal biopsy samples were obtained from each dog for histopathologic evaluation. Histopathologic evaluation of endoscopically obtained intestinal tissue from CIE dogs was performed by a board-certified veterinary pathologist (S.L.P.) and pathologist-in-training (A.S.-B.) blinded to clinical data and clinicopathologic information. Biopsy samples were assessed as adequate for evaluation, and both pathologists evaluated duodenal and ileal tissues and reached a consensus for the presence and severity of morphologic criteria (villous stunting, epithelial injury, crypt distension, lacteal dilatation [LD], and mucosal fibrosis) and inflammatory criteria (intraepithelial lymphocytes, lamina propria eosinophils, lamina propria lymphocytes or plasma cells, and lamina propria neutrophils) based on World Small Animal Veterinary Association (WSAVA) guidelines.²⁰ For the severity of each change, the following scores were applied based on established criteria: 0 = normal, 1 = mild, 2 = moderate, and 3 = marked. Scores for LD were based on the most severely affected villus in each case. If the lacteal occupied 0%-25% of the villus width, a score of 0 was given, 25%-50% was a score of 1, 50%-75% was a score of 2, and >75% of the width of the villus resulted in a score of 3.

2.3 | Immunohistochemistry

All immunohistochemical (IHC) labeling was performed using a Leica Bond III immunostainer (Leica Biosystems Inc, California, Illinois). Formalin-fixed paraffin-embedded tissues were sectioned at 5 μ m and mounted on positively charged slides for IHC. Dewaxing and epitope retrieval were performed using the Bond III instrument. Epitope retrieval was performed using the ER1 solution (Leica Biosystems Inc), a pH 6.0 citrate buffer. Antibodies against Prox-1 (rabbit polyclonal; Angiobio [Delmar, California]) and LYVE-1 (rabbit polyclonal; Abcam [Cambridge, Massachusetts]) 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 sections then were incubated for 20 minutes 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 irrelevant primary antibodies were used as negative controls.

2.4 | Immunohistochemical evaluation

The Prox-1 and LYVE-1 IHC slides as well as negative control slides were digitally scanned using Philips Ultra Fast Scanner slide (Philips IntelliSite Pathology Solution, Philips Electronics, Amsterdam, the Netherlands) and analyzed with the use of Philips Image Management System viewer (version 2.4.1.2; Philips IntelliSite Pathology Solution, Philips Electronics) by a single evaluator (S.W.), who was blinded to the case data. To be counted or measured as a lymphatic vessel, a visible lumen was required in addition to immunolabeling with the LEC markers. Lymphatic vessels

were measured using LYVE-1 immunolabeling. The Prox-1 slides subsequently were evaluated to verify the structures as lymphatic vessels. On 4 \times , 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 (μ m) was taken as the distance from 1 side of the lacteal to the other, measured perpendicular to the midline of the lacteal. Next, well-oriented areas of the propria mucosa were evaluated at 4 \times for immunolabeling. In 10 distinct 20 \times fields in the propria mucosa, lymphatic vessels were identified and lacteal width (μ m) was measured 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 20 \times field. If >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. In addition, the viewing trail feature was used to ensure that all areas of the slide were assessed. For both VLW and MLW, the mean of the 10 measurements was recorded for each tissue in each case. In 2/24 cases, only 5 definitively measurable mucosal lymphatics could be identified, and therefore those cases had MLW scores calculated as the mean of the 5 measurements, rather than 10.

2.5 | 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 were not normally distributed. Spearman (rank-based) correlation was used to evaluate relationships between the lymphatic variables and serum albumin concentration in each section of intestine. Spearman (rank-based) correlation also was performed to assess the relationship between the routine LD score as assessed by the blinded pathologists based on WSAVA guidelines and the VLW and MLW in each section of the intestine. For Spearman, a statistically significant correlation score of (+/-) 0.3-0.5 was considered a weak correlation, (+/-) 0.5-0.7 a moderate correlation, and (+/-) 0.7-1.0 a strong correlation.²¹ All statistical analysis was performed using GraphPad Prism scientific statistic software (Graph Pad Prism, GraphPad Software, Inc, San Diego, California). Statistical significance for all statistical comparisons was set at $P < .05$.

3 | RESULTS

Thirty dogs were screened for inclusion in the study. All dogs were fasted for a minimum of 24 hours before endoscopic examination and biopsies. After routine histopathologic evaluation, 1 dog was diagnosed with intestinal lymphoma and therefore was excluded from the study. Ileal biopsy samples were not obtained in 2 dogs, which prompted exclusion from the study. In 3 additional cases, the quality of the endoscopic biopsy samples was considered inadequate for IHC evaluation of LEC markers because of either the presence of only villus tips, or inability to accurately examine the lymphatic vasculature because of tissue

orientation. Therefore, 24 clinical cases were included in the final data analysis. Twenty-two dogs had a basal serum cortisol concentration $>2 \mu\text{g/mL}$ or normal response to ACTH, ruling out hypoadrenocorticism as a cause of their clinical signs. 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 before endoscopic examination.

Of the 24 cases, 13 dogs had serum albumin concentration $<2.5 \text{ g/dL}$, which was defined as chronic inflammatory enteropathy with PLE (CIE-PLE), and 11 dogs had serum albumin concentration $\geq 2.5 \text{ g/dL}$ (CIE-N). Breeds with CIE-PLE included Bernese Mountain Dog (2), Labrador Retriever (2), mixed breed dog (2), and 1 each of the following: Australian Shepherd, English Bulldog, Great Pyrenees, Pembroke Welsh Corgi, Pug, Rottweiler, and Yorkshire Terrier. Breeds with CIE-N included mixed breed dog (3), and 1 each of Australian Terrier, Bernese Mountain Dog, Brittany Spaniel, Cavalier King Charles Spaniel, German Shepherd dog, German Shorthaired Pointer, Labrador Retriever, and Siberian Husky. Dogs with CIE-PLE consisted of 7 neutered males and 5 spayed females, and dogs with CIE-N consisted of 8 neutered males and 3 spayed females. Additional descriptive statistics of interest are presented in Table 1. Age and sex were not different between dogs with CIE-PLE and dogs with CIE-N.

TABLE 1 Selected descriptive statistics for dogs with chronic inflammatory enteropathy with (CIE-PLE) and without (CIE-N) protein-losing enteropathy

Variable	CIE-PLE (n = 13) median (range)	CIE-N (n = 11) median (range)
Age	7 (1-10)	4 (1-12)
Body weight (kg)	24 (5-42)	21 (6-43)
CCECAI	11 (5-19)	8 (4-11)
Duration of illness (mo)	3 (1-12)	6 (2-24)
Serum albumin concentration (g/dL)	1.7 (0.9-2.4)	3.3 (2.6-3.9)
Duodenal histologic LD score	1 (0-2)	0 (0-1)
Ileal histologic LD score	0 (0-2)	0 (0-2)
Duodenal mucosal score ^a	6 (2-10)	2 (0-9)
Duodenal inflammatory score ^b	5 (2-8)	3 (1-8)
Duodenal total WSAVA score	11 (5-18)	5 (1-13)
Ileal mucosal score ^a	4 (0-8)	0 (0-4)
Ileal inflammatory score ^b	3 (0-6)	2 (1-7)
Ileal total WSAVA score	7 (0-14)	2 (1-11)

Notes: LD, mucosal, and inflammatory histologic scores reported in this table were obtained by blinded evaluation of H&E samples. Abbreviations: CIE-N, chronic inflammatory enteropathy with serum albumin concentration $\geq 2.5 \text{ g/dL}$; CIE-PLE, chronic inflammatory enteropathy with protein-losing enteropathy (serum albumin $<2.5 \text{ g/dL}$); LD: lacteal dilatation; WSAVA: World Small Animal Veterinary Association. ^aTotal score for villus stunting, epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis.

^bTotal score for intraepithelial lymphocytes, lamina propria lymphocytes/plasma cells, lamina propria eosinophils, and lamina propria neutrophils.

The 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. Human prospero homeobox has been reported to be variably expressed in enteroendocrine epithelial cells in the crypts.²² In our cases, Prox-1 labeling was visible in some individual crypt epithelial cells but was easily differentiated from lymphatic vessel endothelial labeling. Although LYVE-1 is expressed by some macrophages in the intestine,²³ this staining was easily distinguishable from lymphatic vessel endothelial labeling. Lymphatic endothelial cell labeling was not observed on any of the negative control slides. Examples of immunolabeled villus lymphatics are shown in Figure 1. Examples of immunolabeled proprial mucosal lymphatics are shown in Figure 2. Villus lacteal width in the ileum was weakly negatively ($\rho = -.4644$) correlated with serum albumin concentration ($P = .02$). Proprial MLW in the ileum was moderately negatively ($\rho = -.6514$) correlated with serum albumin concentration ($P < .001$).²¹ Summary statistics and correlation data for lymphatic variables with serum albumin concentration are presented in Table 2.

Routine H&E LD scores as determined by blinded board-certified pathologist and pathologist-in-training were assessed for correlation to the width of the villus and proprial mucosal lymphatics (VLW and MLW, respectively) as determined by IHC for each tissue section. Duodenal LD was weakly positively correlated with duodenal VLW ($\rho = .4634$; $P = .02$) but not correlated with duodenal MLW ($\rho = .2767$; $P = .19$). Ileal LD was moderately positively correlated with ileal VLW ($\rho = .5292$; $P = .008$) but not correlated with ileal MLW ($\rho = .3889$; $P = .06$).

4 | DISCUSSION

We utilized LEC-specific markers to evaluate the intestinal lymphatic vasculature in dogs with CIE with and without PLE, as defined by serum albumin concentration $<2.5 \text{ g/dL}$. Lymphatic endothelial cell IHC was successful in labeling the lymphatic vasculature in dogs with CIE, and identified apparently dilated lymphatics in the proprial mucosa that were not identified as lymphatics by routine H&E assessment for lymphangiectasia. In addition, in our 24 dogs with CIE, serum albumin concentrations were negatively correlated with villus and MLW in the ileum. For both the duodenum and the ileum, average VLW as determined by use of LEC IHC was correlated with LD scores as traditionally assessed using routine H&E staining. Proprial MLW as determined by the use of IHC was not correlated with LD scores as traditionally assessed by routine H&E staining in either section of intestine.

The most striking finding of our study was the presence of apparent proprial mucosal lymphangiectasia in the intestine of dogs with CIE, in particular in the ileum of several dogs with CE and serum albumin concentrations $<2.5 \text{ g/dL}$. In some of these dogs with apparently dilated proprial mucosal lymphatics, villus lymphatics were not concurrently dilated and these dogs had not been diagnosed with lymphangiectasia on routine histopathologic examination. The appearance of the distended proprial mucosal lymphatics in the ileum of these dogs may be similar to what has been identified in humans with CD,¹⁵ an idiopathic

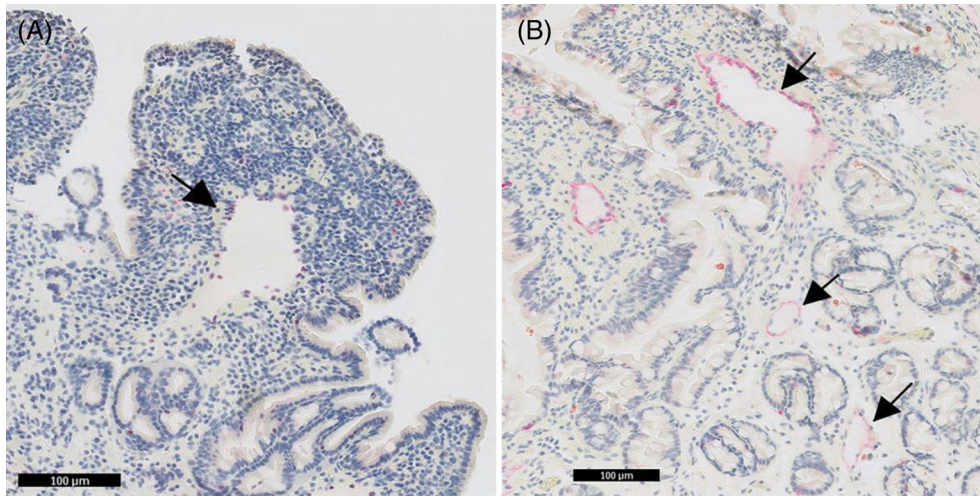


FIGURE 1 Immunolabeled villous lymphatics of dogs with chronic inflammatory enteropathy (CIE). A, Duodenal villi from dog with CE and serum albumin concentration of 1.7 g/dL and central villous lymphatic dilation (arrow; lacteal dilation score = 2); human prospero homeobox immunohistochemistry (IHC). Severe lamina propria lymphoplasmacytic inflammation is also visible. B, Ileal villi from dog with CIE, 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 lymphatic vascular endothelial hyaluronic acid receptor IHC. Apparent proprial mucosal lymphangiectasia can also be seen (arrows)

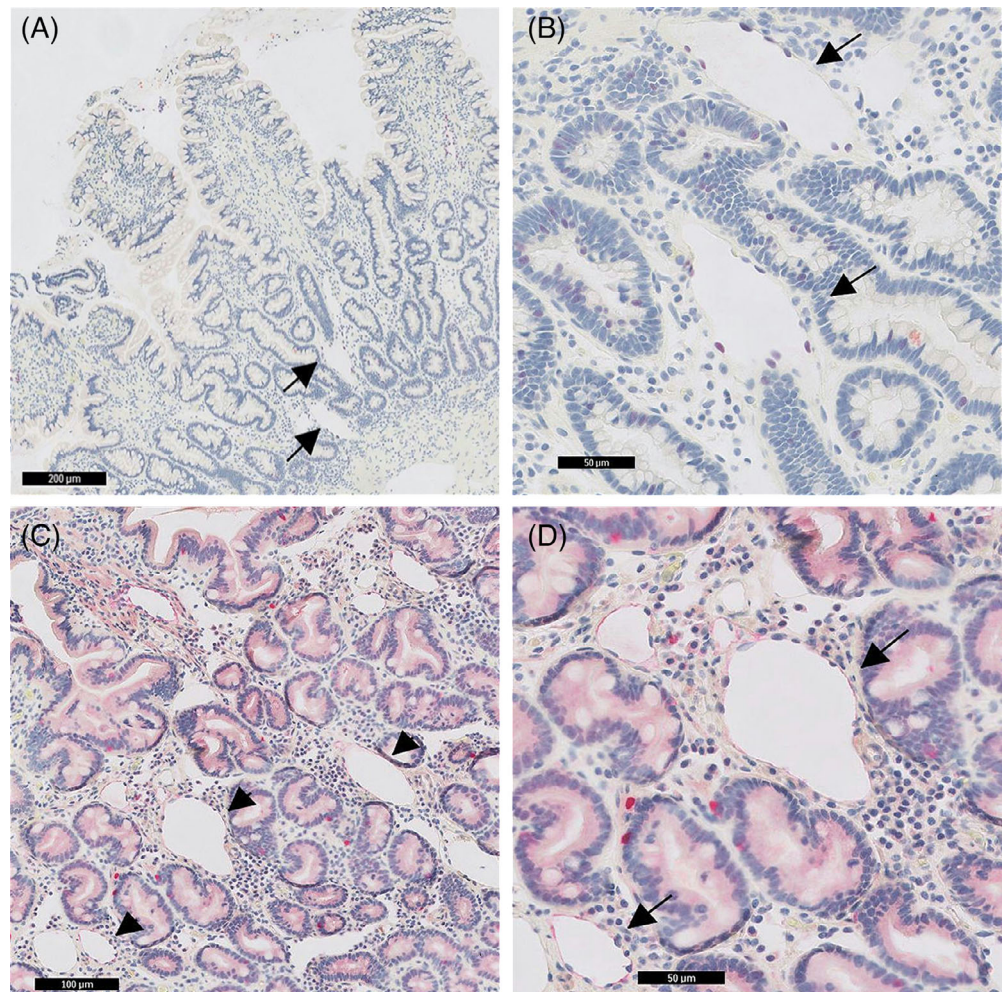


FIGURE 2 Immunolabeled proprial mucosal lymphatics of dogs with CIE-PLE (A-D). Apparent proprial mucosal lymphangiectasia (arrows) in the ileum of a dog with CE and serum albumin concentration 1.5 g/dL at 4× (A) and 20× (B) power; human prospero homeobox immunohistochemistry (IHC). Apparent proprial mucosal lymphangiectasia (arrowheads and arrows) in the duodenum of a dog with serum albumin concentration 1.6 g/dL at 4× (C) and 20× (D) power; lymphatic vascular endothelial hyaluronic acid receptor IHC

TABLE 2 Intestinal villous and mucosal lymphatic scores for dogs with chronic inflammatory enteropathy with (CIE-PLE) and without (CIE-N) protein-losing enteropathy

Variable	Group	Median	IQR	Min	Max	Spearman's Correlation Score ^a	P-value [*]
Duodenal VLW (μm)	CIE-PLE	34.6	20.5	15	53.9	-.2533	.23
	CIE-N	24.9	14.9	16.4	63.4		
Duodenal MLW (μm)	CIE-PLE	21.8	16.9	9.6	41.5	-.3777	.07
	CIE-N	11.6	8.2	7.1	39.5		
Ileal VLW (μm)	CIE-PLE	22.9	24.1	11	51.2	-.4644	.02
	CIE-N	15.3	6	10.9	39.8		
Ileal MLW (μm)	CIE-PLE	28.1	22.25	5	51	-.6514	<.001
	CIE-N	12.8	7.4	9	25.7		

Abbreviations: CIE-N, chronic inflammatory enteropathy with serum albumin concentration ≥ 2.5 g/dL; CIE-PLE, chronic inflammatory enteropathy with protein-losing enteropathy (serum albumin < 2.5 g/dL); MLW, mucosal lacteal width; IQR, interquartile range; VLW, villus lacteal width.

^aSpearman correlation performed between lacteal variables and serum albumin.

^{*}P-value as assessed by Spearman correlation.

chronic inflammatory intestinal disease most commonly found in the ileum.¹³ In humans with CD, lymphangiectasia also has been identified in the submucosa, muscularis propria and subserosa.^{14,15,24} Therefore, it is possible that full thickness intestinal biopsy samples with LEC immunolabeling may identify more abnormalities in the lymphatic vasculature of dogs with CIE than are currently seen in endoscopic biopsy samples.

Presumptive proprial mucosal lymphangiectasia also was identified in the duodenum of some dogs with CIE, including in 2 dogs with serum albumin concentrations ≥ 2.5 g/dL. Proprial MLW in the duodenum was not significantly correlated with serum albumin concentration. It is possible that with an increased sample size, a statistical difference may have been detected. A previous study comparing histologic findings in the duodenum versus ileum in a group of dogs with chronic small intestinal enteropathies found hypoalbuminemia to be correlated with ileal LD, but not with duodenal LD.²⁵ The albumin correlation results of our study also suggest that lymphatic abnormalities can differ among sections of the intestine, lending additional support to the recommendation to always obtain ileal samples in the diagnostic evaluation of dogs with CIE.

In many cases, it is unknown whether lymphatic abnormalities are a cause or consequence of intestinal disease. Regardless, 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 regulation of the pressure of interstitial fluid in tissues, and transport of excess fluid back to the circulation. Furthermore, lymphatic vessels are the main route of absorption of fat, cholesterol, fat-soluble vitamins, and gastrointestinal hormones.^{24,26} Obstruction or dysfunction of the lymphatic vasculature or both should have important consequences, and the recognition of lymphatic abnormalities is likely important to the management of dogs with CIE. Although response to treatment and outcomes were not evaluated in our study, follow-up information was available for several dogs. Six dogs in the study had an average ileal mucosal lymphatic width > 30 μm , all of which had serum albumin concentrations < 2.5 g/dL. Blinded ileal LD scores were 0 in 4/6 of these

dogs. Of these dogs, 3 were euthanized as a consequence of their disease, 1 was euthanized because of unrelated disease, and 2 were alive at the time of publication. Of the 3 dogs that were euthanized because of their disease, 2 received traditional treatments including commercial gastrointestinal diets and glucocorticoids for > 2 weeks after their diagnosis with no clinically relevant improvement noted. The third dog that was euthanized initially was glucocorticoid-responsive but then relapsed when glucocorticoids were tapered and the owner chose not to pursue further treatment. The other 3 dogs had persistent clinical signs despite treatment with glucocorticoids, immunosuppressive drugs, 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) than the commercially available diets. Two of those dogs were alive at the time of publication; the third was euthanized because of splenic hemangiosarcoma 12 months after response to the home-cooked diet. Although anecdotal, and only a small number of cases, this population of dogs may represent a subset of dogs with CIE that have important abnormalities of their lymphatic vasculature that require the administration of a diet that is lower in fat than what is commercially available.

Our study had some limitations. First, biopsy samples from healthy control dogs were not available, and we cannot accurately determine normal lymphatic width in the propria mucosa of the intestine. Further studies of the lymphatic vasculature in dogs with CIE ideally should include evaluation of samples from healthy control dogs. In addition, although effort was made to standardize evaluation of the intestinal lymphatic vasculature, the sectioning of intestinal tissue, in particular endoscopically obtained biopsy samples, cannot be entirely uniform. It is possible that in some cases the lymphatics were less or more visible because of the angle of sectioning, and this may have affected the results. In 2 cases (1 ileal sample from a hypoalbuminemic dog and 1 duodenal sample from a normoalbuminemic dog), only 5 measurable mucosal lymphatics could be definitively identified, compared to 10 in all other cases, which may have affected the results. Despite the use of a team of

a blinded veterinary pathologist and veterinary pathologist-in-training to score histopathologic lesions in the intestine using established guidelines, histopathologic evaluation of the intestine in dogs is known to be subjective with significant interobserver variation.^{27,28} A final limitation is that 2 dogs in the study did not have hypoadrenocorticism definitively excluded before their enrollment in the study. Both dogs had previously been treated with glucocorticoid treatment without clinical improvement and hypoadrenocorticism therefore was considered unlikely. Ideally, it would have been excluded definitively.

In conclusion, the use of LEC IHC allowed for identification of both villus and apparent proprial mucosal IL in dogs with CIE. Several abnormalities of the intestinal lymphatic vasculature were correlated with lower serum albumin concentrations. The most notable finding was the discovery of apparently distended proprial mucosal intestinal lymphatics using LEC IHC, the most striking of which was seen in the ileum of dogs with CIE and concurrent PLE. This apparent proprial mucosal lymphangiectasia had not been recognized on routine H&E evaluation of the lymphatics. Routine LD scoring using H&E did not correlate with the changes in the proprial mucosal lymphatics, which suggests that evaluation of the villus lacteals alone can underestimate abnormalities to the lymphatic vasculature in dogs with CIE. This finding should be assessed in a larger group of dogs with CIE with and without concurrent hypoalbuminemia because the identification of lymphangiectasia deeper in intestinal biopsy samples will impact the therapeutic management of these cases.

ACKNOWLEDGMENTS

The authors acknowledge Brendan Podell and CSU Veterinary Diagnostic Lab's Biopsy and Histopathology service and Ethos Diagnostic Science/STAT Veterinary Laboratory for their technical support. This work was performed with the support of Royal Canin as well as the Naniboujou Research Legacy.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The Clinical Review Board at Colorado State University approved all procedures.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Sara A. Wennogle  <https://orcid.org/0000-0002-6486-3644>

Alejandro Suárez-Bonnet  <https://orcid.org/0000-0003-0296-5896>

REFERENCES

- 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.
- Dandrieux JR. Inflammatory bowel disease versus chronic enteropathy in dogs: are they one and the same? *J Small Anim Pract.* 2016;57:589-599.
- German AJ, Hall EJ, Day MJ. Chronic intestinal inflammation and intestinal disease in dogs. *J Vet Intern Med.* 2003;17:8-20.
- Dossin O, Lavoué R. Protein-losing enteropathies in dogs. *Vet Clin North Am Small Anim Pract.* 2011;41:399-418.
- Okanishi H, Yoshioka R, Kagawa Y, Watari T. The clinical efficacy of dietary fat restriction in treatment of dogs with intestinal lymphangiectasia. *J Vet Intern Med.* 2014;28:809-817.
- Larson RN, Ginn JA, Bell CM, Davis MJ, Foy DS. Duodenal endoscopic findings and histopathologic confirmation of intestinal lymphangiectasia in dogs. *J Vet Intern Med.* 2012;26:1087-1092.
- 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.
- 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.
- Moser K, Mitze S, Teske E, et al. Correlation of clinical, diagnostic and histopathological parameters in dogs with chronic lymphocytic-plasmacytic enteropathy. *Tierarztl Prax Ausg K Kleintiere Heimtiere Tierarztl Prax Ausg K Kleintiere Heimtiere.* 2018;46:15-20.
- Van Kruiningen HJ, Colombel JF. The forgotten role of lymphangitis in Crohn's disease. *Gut.* 2008;57:1-4.
- Alexander JS, Chaitanya GV, Grisham MB, Boktor M. Emerging roles of lymphatics in inflammatory bowel disease. *Ann N Y Acad Sci.* 2010;1207:E75-E85.
- von der Weid PY, Rehal S, Ferraz JG. Role of the lymphatic system in the pathogenesis of Crohn's disease. *Curr Opin Gastroenterol.* 2011;27:335-341.
- Cerquetella M, Spaterna A, Laus F, et al. Inflammatory bowel disease in the dog: differences and similarities with humans. *World J Gastroenterol.* 2010;16:1050-1056.
- 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.
- 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.
- Pedica F, Ligorio C, Tonelli P, Bartolini S, Baccharini P. Lymphangiogenesis in Crohn's disease: an immunohistochemical study using monoclonal antibody D2-40. *Virchows Arch.* 2008;452:57-63.
- 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.
- 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.
- 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.

20. 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.
21. Mukaka MM. A guide to appropriate use of correlation coefficient in medical research. *Mal Med J.* 2012;24:69-71.
22. Petrova TV, Nykänen A, Normén C, et al. Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype. *Cancer Cell.* 2008;13:407-419.
23. Kim KE, Sung HK, Koh GY. Lymphatic development in mouse small intestine. *Dev Dyn.* 2007;236:2020-2025.
24. Alexander JS, Ganta VC, Jordan PA, Witte MH. Gastrointestinal lymphatics in health and disease. *Pathophysiology.* 2010;17:315-335.
25. Procoli F, Mötsküla PF, Keyte SV, Priestnall S, Allenspach K. 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.
26. Miller MJ, McDole JR, Newberry RD. Microanatomy of the intestinal lymphatic system. *Ann N Y Acad Sci.* 2010;1207:21-28.
27. 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.
28. 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.

How to cite this article: Wennogle SA, Priestnall SL, Suárez-Bonnet A, Soontarak S, Webb CB. Lymphatic endothelial cell immunohistochemical markers for evaluation of the intestinal lymphatic vasculature in dogs with chronic inflammatory enteropathy. *J Vet Intern Med.* 2019;1-8. <https://doi.org/10.1111/jvim.15545>