Received: 18 March 2019 | Accepted: 20 August 2019

DOI: 10.1111/jvim.15614

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Comparison of clinical, clinicopathologic, and histologic variables in dogs with chronic inflammatory enteropathy and low or normal serum 25-hydroxycholecalciferol concentrations

Sara A. Wennogle[1] | Simon L. Priestnall[2] | Alejandro Suárez-Bonnet[2] | Craig B. Webb[1]

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

[2]Department 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, Fort Collins, CO 80523.
Email: sara.wennogle@colostate.edu

Funding information
Naniboujou Legacy Foundation; Rockys Research Fund

Abstract

Background: The cause of low serum vitamin D concentrations in dogs with chronic inflammatory enteropathy (CIE) is not well understood.

Objective: Improve understanding of pathogenesis of low serum vitamin D concentrations in dogs with CIE by comparing several clinical, clinicopathologic, and histologic variables between CIE dogs with low and normal serum 25-hydroxyvitamin D concentrations (25[OH]D).

Animals: Fifteen dogs with CIE and low serum 25[OH]D concentrations; 15 dogs with CIE 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 concentration and histopathologic variables were assessed.

Results: Dogs with CIE and low serum 25(OH)D concentrations had higher canine chronic enteropathy clinical activity index scores ($P = .003$), lower serum α-tocopherol ($P < .001$), cholesterol ($P < .001$), and albumin ($P < .001$) concentrations and higher serum C-reactive protein ($P = .004$) concentrations compared to CIE dogs with normal serum 25(OH)D concentrations. Serum concentrations of vitamin D-binding protein (VDBP) were not different between groups ($P = .91$). Duodenal morphologic and inflammatory histopathological scores ($P = .002$ and $P = .004$, respectively) and total histopathological scores in duodenum and combined duodenum and ileum negatively correlated with serum 25(OH)D concentration.

Conclusions and Clinical Importance: The pathogenesis of low serum vitamin D concentrations in dogs with CIE is likely multifactorial. Fat malabsorption deserves further study in dogs with low serum vitamin D concentration and CIE.

Abbreviations: 25[OH]D, serum 25-hydroxyvitamin D; CCECAI, canine chronic enteropathy clinical activity index; CE, chronic enteropathy; CIE, chronic inflammatory enteropathy; CD, Crohn's disease; CRP, C reactive protein; CSU, Colorado State University; DACPAH, Michigan State University's Diagnostic Center for Population and Animal Health; DMB, dry matter basis; DOI, duration of illness; IBD, inflammatory bowel disease; LD, lacteal dilatation; OR, odds ratio; PLE, protein-losing enteropathy; RI, reference interval; VDBP, vitamin D-binding protein; WSAVA, World Small Animal Veterinary Association.

Loss of VDBP does not appear to be an important cause of low serum vitamin D concentration in dogs with CIE.

KEYWORDS
gastroenterology, gastrointestinal tract, inflammatory disease, vitamin D

1 | INTRODUCTION

Decreased serum 25-hydroxyvitamin D (25[OH]D) concentrations have been well documented in dogs with chronic inflammatory enteropathy (CIE) and protein-losing enteropathy (PLE),[1-3] and have been associated with poor outcome.[4] Similarly, children and adults with inflammatory bowel disease (IBD), specifically Crohn's disease (CD), are more likely to be vitamin D deficient than healthy controls[5-8] and can 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 decreased exposure to sunlight, decreased PO vitamin D intake, malabsorption, loss of vitamin D through the intestinal tract, and the presence of 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% decrease in absorption of PO administered 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 systematic review and meta-analysis found that in addition to vitamin D, concentrations 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 vitamin D metabolites are primarily transported in the circulation bound to vitamin D-binding protein (VDBP [85%-90%]), and to a lesser extent albumin (10%-15%).[16] Concentrations of VDBP were decreased in a cohort of children with IBD compared to healthy controls,[17] and in another study, serum 25(OH)D concentration was positively correlated with serum albumin concentration in a group of 130 children and young adults with IBD.[18]

In dogs with CIE, the cause of low serum vitamin D concentration is not fully understood.[1] Decreased dietary intake of vitamin D may be a contributing factor, but 1 study found that dogs with decreased appetite and non-gastrointestinal illness had higher serum 25(OH)D concentrations than did dogs with chronic enteropathy (CE), suggesting that poor appetite does not always lead to decreased serum vitamin D concentrations.[2] In 33 dogs with CE, a relationship between serum albumin and serum 25(OH)D concentrations was found.[2] Additionally, markers of systemic and intestinal inflammation have been negatively associated with serum 25(OH)D concentrations in dogs with CE.[19] Serum concentration of VDBP and other fat-soluble vitamins have not been reported previously in dogs with hypovitaminosis D and CIE.

Determining the relationship of clinical, clinicopathologic, and histopathologic variables with serum 25(OH)D concentrations in dogs with CIE may lead to a better understanding of why vitamin D decreases in this cohort and improve our ability to detect and treat low serum vitamin D concentrations. Therefore, our objective 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 CE and low or normal serum 25(OH)D concentrations.

2 | MATERIALS AND METHODS

2.1 | Study population

Dogs presented to Colorado State University (CSU) Veterinary Teaching Hospital for evaluation of chronic gastrointestinal signs of >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, serum biochemical profile, and abdominal ultrasonography excluded non-gastrointestinal illness and histopathologic evidence of small intestinal disease characterized by inflammatory infiltrates and morphologic changes were present. Dogs with histopathologic evidence of intestinal neoplasia were excluded. Urinalysis with or without urine protein:creatinine ratio and fasting and postprandial bile acids were performed to exclude other causes of hypoalbuminemia in dogs with serum albumin concentration <3.0 g/dL. The feces of all dogs were screened for helminths (fecal floatation), *Giardia* (immunofluorescent antibody testing [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 serum cortisol concentration >2 μg/mL or normal response to ACTH stimulation. Dogs being treated with vitamin D or calcium supplementation were excluded. Additionally, dogs that had consumed a diet for >72 hours in the preceding 3 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 treated with glucocorticoids for >7 days before enrollment were excluded from analysis. Age, breed, sex, body weight (kg), and duration of illness (DOI) were recorded.

2.2 | Measurement of serum 25(OH)D concentrations, ionized calcium, and parathyroid hormone

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 on the day of collection to the Michigan State University's Diagnostic Center for Population and Animal Health (DACPAH; MSU Diagnostic Center for Population and Animal Health, Meridian Charter Township, Michigan) for completion of a Vitamin D panel (measurement of serum 25[OH]D, ionized calcium [iCa], and parathyroid hormone [PTH] concentrations). 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 concentrations. Serum 25(OH)D, iCa, and PTH concentrations previously have been shown to be stable under these conditions.[20,21] The 25(OH)D concentration was measured using a commercially available radioimmunoassay kit, and serum ionized calcium concentrations were measured using an ion-specific electrode. Serum PTH concentration was measured using an immunoradiometric assay, as previously described.[22]

2.3 | Clinical activity, appetite, and body condition scores

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. Applying the results of the serum biochemical profile (serum albumin concentration) and abdominal ultrasound examination (peritoneal effusion), and using the owner's scores, a canine chronic enteropathy clinical activity index (CCECAI) score was calculated.[23] Appetite scores also were recorded separately. Appetite score was assigned as 0-3, where 0 = normal, 1 = mildly decreased, 2 = moderately decreased, and 3 = severely decreased. Body condition score (BCS; scale 0-9) [24] was assigned for each dog by a single evaluator at the time of initial evaluation.

2.4 | Measurement of serum cholesterol, alpha-tocopherol, and retinol concentrations

Serum cholesterol concentrations were recorded as measured by the CSU Veterinary Diagnostic Laboratory using a Cobas Integra (Roche Diagnostics Limited, West Sussex, UK) biochemistry analyzer. Serum alpha-tocopherol (vitamin E) and retinol (vitamin A) concentrations were batch measured on stored, aliquoted frozen serum samples using high-performance liquid chromatography. Samples had been frozen immediately after collection and processing, stored at −80°C, and thawed before analysis. Alpha-tocopherol and retinol are known to be stable in serum for years.[25]

2.5 | Measurement of serum protein concentrations

Serum albumin concentration was measured routinely by the CSU Veterinary Diagnostic Laboratory using a Cobas Integra (Roche Diagnostics Limited) biochemistry analyzer. Serum VDBP concentrations were measured using a human-specific ELISA (My BioSource Inc, San Diego, California), validated for measurement in the dog for research by the kit manufacturer (see Supporting Information). The ELISA kit uses mouse monoclonal antihuman highly purified human Gc globulin (VDBP) as the capture antibody (Immunogen, Waltham, Massachusetts) and goat antihuman synthetic peptide ERGRDYEKNKVCK (corresponding to amino acids 18-30 of human VDBP [near the N terminal]) as the detection antibody (Immunogen). Serum samples had been frozen immediately after collection, stored at −80°C, and thawed before analysis. Samples were stored for 2-18 months before analysis; duration of time of sample storage before analysis was recorded. Analysis and calculation of results were performed according to manufacturer instructions.

2.6 | Markers of intestinal and systemic inflammation

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 or ileum or both) was performed by a board-certified veterinary pathologist (S.L.P.) and pathologist-in-training (A.S.-B.) 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 severity of morphologic criteria (villous stunting, epithelial injury, crypt distension, lacteal dilatation (LD), mucosal fibrosis) and inflammatory criteria (intraepithelial lymphocytes, lamina propria eosinophils, lamina propria lymphocytes and plasma cells, lamina propria neutrophils) based on the World Small Animal Veterinary Association (WSAVA) guidelines.[26] For the severity of each change, scores were applied based on established criteria: 0 = normal, 1 = mild, 2 = moderate, 3 = marked. Individual scores were recorded, and the total morphologic score and total inflammatory score per section of intestine were recorded separately and then summed for a total WSAVA score per section of intestine. Combined duodenal and ileal total WSAVA scores also were recorded. Because vitamin D is a fat-soluble vitamin, LD scores[26] for each section of the intestine also were recorded.

Plasma fibrinogen and serum C-reactive protein concentrations were available for many of the dogs in the study as markers of systemic inflammation. Quantitative plasma fibrinogen concentration was obtained at the time of collection using the Clauss method (Tcoag TriniCLOT PT Excel; Bray, Wicklow, Ireland). Serum C-reactive protein (CRP) concentration was determined using a commercially available ELISA (Tridelta Development Limited, Maynooth, County Kildare, Ireland).

2.7 | Statistical analysis

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 <109 nmol/L, as defined by an established reference interval (RI, 109-423 nmol/L), and normal if serum 25(OH)D concentration fell within the RI. Groups were assessed for differences before clinical and clinicopathological data analysis. To compare differences in sex and

group, Fisher's exact test was used. To compare differences in age, body weight, and DOI and group, the Mann-Whitney *U* was performed.

The distribution of clinical and clinicopathologic data was assessed by the Shapiro-Wilk test. Normally distributed (parametric) variables were compared using a *t*-test. Nonnormally distributed (nonparametric) clinicopathologic variables were compared using a Mann-Whitney *U* test. Bonferroni correction was applied to data to account for multiple testing. Results are reported as mean ± standard deviation for the parametric variables and median and range (minimum-maximum) for nonparametric variables. Variables found to be significantly associated with 25(OH)D based on the univariate Mann-Whitney *U* and *t*-test results were placed into a multiple logistic regression analysis. Specifically, serum 25(OH)D concentration (decreased or normal) was the response variable and the variables serum albumin, serum alpha-tocopherol, serum cholesterol concentration, and CCECAI were used as predictors. Serum CRP concentration was not included in the model because of missing results. Backward elimination then was used to decrease the number of predictors in the logistic regression model.

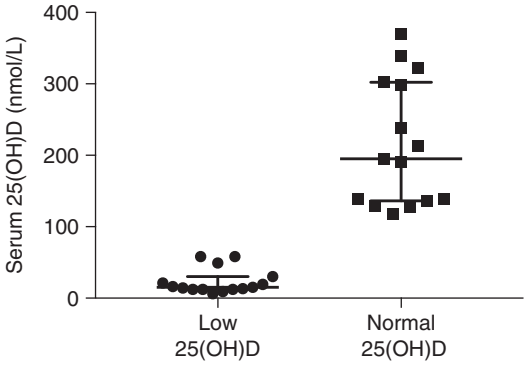

FIGURE 1 Scatter dot plot of serum 25(OH)D in dogs with CIE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown. CIE, chronic inflammatory enteropathy

To assess for correlations between vitamin D concentration and iCa, PTH, and histopathological scores, a Spearman (rank-based) test was performed. After Spearman testing, a Bonferroni correction was performed to account for multiple testing. Finally, 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. For Spearman and Pearson testing, 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.

Multiple logistic regression analysis was performed using R scientific statistical software (R Foundation for Statistical Computing; version 3.5.1). The remainder of the 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 $P < .05$ and adjusted after Bonferroni corrections.

3 | RESULTS

Thirty dogs with CIE were enrolled, 15 of which had low serum 25(OH)D concentrations. The other 15 dogs had serum 25(OH)D concentrations within the RI (Figure 1). Breeds in the low 25(OH)D group included Bernese Mountain Dog (2), mixed breed dog (2), Labrador Retriever (2),

TABLE 1 Clinical scores, BCS, and serum fat-soluble vitamins, cholesterol, proteins, and inflammatory markers in dogs with CIE 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)	
CCECAI	0-24	12.3 ± 4.13	7.7 ± 3.54	.003
Appetite score	0-3	1 (0-3)	1 (0-3)	.85
BCS	0-9	3.7 ± 1.16	3.9 ± 1.36	.78
α-Tocopherol (μg/mL)	5-24	11 (3.3-25)	23 (5.1-56)	<.001
Retinol (μg/mL)	0.3-1	4 ± 2.54	4.7 ± 1.42	.36
Cholesterol (mg/dL)	130-300	80 (53-120)	198 (97-447)	<.001
Albumin (g/dL)	3-4.3	1.6 (0.9-2.9)	3.3 (1.7-3.9)	<.001[a]
VDBP (μg/mL)	…	183 (132-299)	193 (119-718)	.91
Fibrinogen (mg/dL)[b]	123-210	333 (201-835)	232 (146-523)	.03
CRP (μg/mL)[c]	0-7.6	43.1 (9.5-60)	2.5 (0.3-60)	.004

Abbreviations: BCS, body condition score; CIE, chronic inflammatory enteropathy; CCECAI, canine chronic enteropathy clinical activity index; CRP, C-reactive protein; SD, standard deviation; VDBP, vitamin D-binding protein.

**P*-value as assessed by Mann-Whitney *U* test for nonparametric variables (data presented as median (range) and *t*-test for parametric variables (data presented as mean ± SD). Significance set at $P < .005$.

[a]Retained significance in multivariate analysis.

[b]Data available from 10 dogs per group.

[c]Data available from 13 dogs per group.

Yorkshire Terrier (2), and 1 each of the following: Australian Shepherd, English Bulldog, German Shepherd dog, Jack Russell Terrier, 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 1 each of the following: Cavalier King Charles Spaniel, German Shepherd dog, German Shorthaired Pointer, Great Pyrenees, and Siberian Husky. Groups were not different with regard to age, sex, body weight, or DOI. Median age of low 25(OH)D dogs was 7 years (range, 1-10 years) and normal 25(OH)D dogs was 5 years (range, 1-12 years). Five dogs in each group were spayed females; the remaining dogs were neutered males. Median body weight was 19 kg (range, 3-47 kg) in the low 25(OH)D group and 22 kg (range, 6-46 kg) in the normal 25(OH)D group. Median DOI was 3 months (range, 1-11 months) in the low 25(OH)D group and 6 months (range, 1-24 months) in the normal 25(OH)D group. Two dogs in each group had received glucocorticoids for <7 days before enrollment. At the time of enrollment, 6 of 15 dogs in the low 25(OH)D group and 7 of 15 dogs in the normal 25(OH)D group were being fed commercial over-the-counter diets formulated to meet Association of American Feed Control Officials vitamin requirements. The remaining 9 of 15 dogs in the

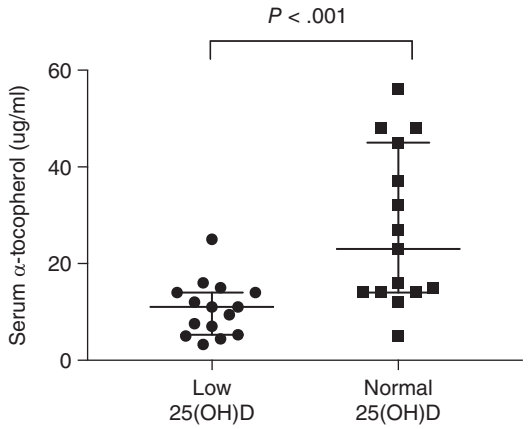

FIGURE 2 Scatter dot plot of serum α-tocopherol in dogs with CIE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown. CIE, chronic inflammatory enteropathy

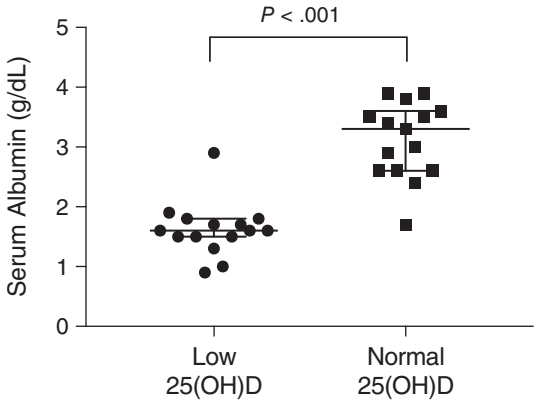

FIGURE 3 Scatter dot plot of serum albumin in dogs with CIE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown. CIE, chronic inflammatory enteropathy

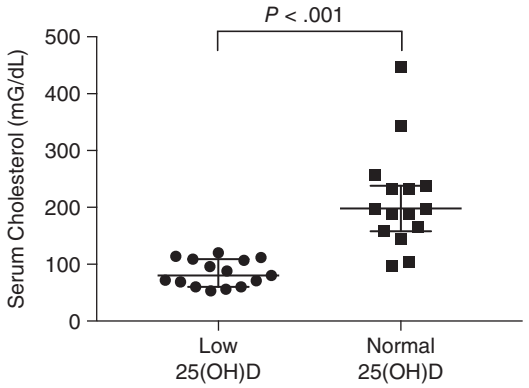

FIGURE 4 Scatter dot plot of serum cholesterol in dogs with CIE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown. CIE, chronic inflammatory enteropathy

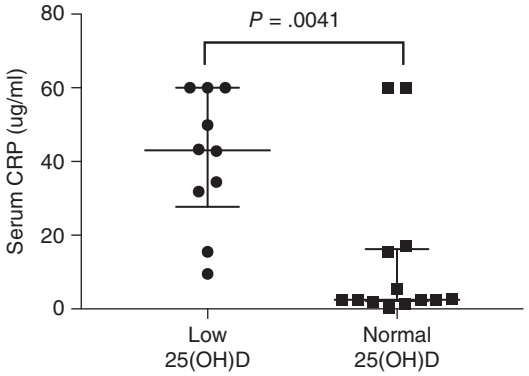

FIGURE 5 Scatter dot plot of serum C-reactive protein in dogs with CIE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown. CIE, chronic inflammatory enteropathy

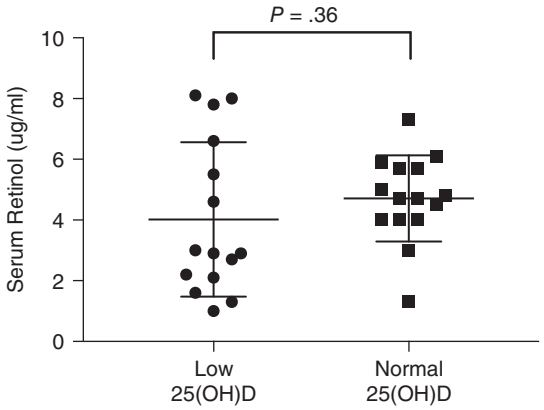

FIGURE 6 Scatter dot plot of serum retinol in dogs with CIE with and without low serum 25(OH)D. Horizontal bar represents mean. Standard deviation shown. CIE, chronic inflammatory enteropathy

low 25(OH)D group and 8 of 15 dogs in the normal 25(OH)D group were being fed various prescription veterinary gastrointestinal diets also formulated to meet Association of American Feed Control Officials vitamin requirements.

Summary statistics and *P*-values are presented in Table 1 for CCECAI, appetite score, BCS, fat-soluble vitamins, cholesterol, serum proteins, and serum inflammatory markers. In brief, dogs with low serum 25(OH) concentrations had higher CCECAI scores ($P = .003$), lower serum α-tocopherol concentrations ($P < .001$; Figure 2), lower serum albumin concentrations ($P < .001$; Figure 3), lower serum cholesterol concentrations ($P < .001$; Figure 4), and higher serum CRP concentrations ($P = .004$; Figure 5) when compared to dogs with normal serum 25(OH)D concentrations. Serum concentrations of retinol (Figure 6) and VDBP (Figure 7) were not different between groups ($P = .36$ and $P = .91$, respectively). After backward elimination (using alpha = .05), only albumin remained significant in the multiple regression analysis model (odds ratio [OR], 0.022; $P = .002$). The OR represents the odds of decreased serum 25(OH)D concentration corresponding to a 1 unit (g/dL) increase in serum albumin concentration.

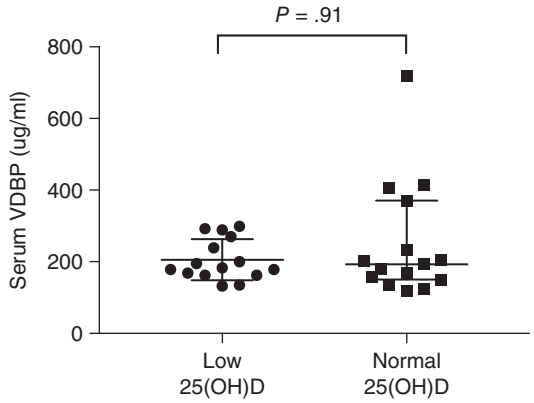

FIGURE 7 Scatter dot plot of serum vitamin D-binding protein in dogs with CIE with and without low serum 25(OH)D. Horizontal bar represents median. Interquartile range shown. CIE, chronic inflammatory enteropathy

Gastroduodenoscopy and ileocolonoscopy was attempted in all dogs. The ileum could not be successfully biopsied in 2 dogs in the low 25(OH)D group and 2 dogs in the normal 25(OH)D group, and thus ileal tissue was not available for evaluation in those cases. Summary statistics and *P*-values for histopathologic variables are presented in Table 2. In brief, a moderate negative correlation (rho value, −0.5516) was observed between serum 25(OH)D concentrations and total morphologic scores in the duodenum, and a moderate negative correlation (rho value, −0.5108) was observed between serum 25(OH)D concentrations and total inflammatory scores for the duodenum ($P = .002$ and $P = .004$, respectively). In addition, overall WSAVA scores for duodenum were moderately negatively correlated (rho value, −0.613; $P < .001$), and overall WSAVA scores for combined

TABLE 2 Histopathology scores in dogs with chronic inflammatory enteropathy and low or normal 25(OH)D concentrations

Variable	Group	Median	IQR	Min	Max	Spearman's correlation score	*P*-value*
LD—duodenum	Low 25(OH)D	1	1	0	2	−0.2842	.13
	Normal 25(OH)D	0	1	0	2		
LD—ileum[a]	Low 25(OH)D	1	2	0	3	−0.5015	.009
	Normal 25(OH)D	0	0	0	2		
Morphologic score[b]—duodenum	Low 25(OH)D	3	5	0	10	−0.5516	.002
	Normal 25(OH)D	3	5	0	10		
Morphologic score[b]—ileum[a]	Low 25(OH)D	2	3	0	6	−0.3903	.05
	Normal 25(OH)D	1	4	0	8		
Inflammatory score[c]—duodenum	Low 25(OH)D	5	3	2	8	−0.5108	.004
	Normal 25(OH)D	4	2	1	8		
Inflammatory score[c]—ileum[a]	Low 25(OH)D	2	3	0	5	0.0066	.97
	Normal 25(OH)D	2	4.5	0	7		
Overall WSAVA score—duodenum	Low 25(OH)D	11	4	2	15	−0.613	<.001
	Normal 25(OH)D	7	5	1	18		
Overall WSAVA score—ileum[a]	Low 25(OH)D	5	4.5	1	11	−0.2199	.28
	Normal 25(OH)D	3	8	0	14		
Overall WSAVA score—duodenum + ileum[a]	Low 25(OH)D	16	10	8	23	−0.5685	.002
	Normal 25(OH)D	9	13.5	2	32		

Abbreviations: IQR, interquartile range; 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 dilatation; WSAVA, World Small Animal Veterinary Association.

*P-value as assessed by Spearman correlation. Significance set at $P < .004$.

[a]Scores available for 13 dogs per group.

[b]Total score for villous stunting, epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis.

[c]Total score for intraepithelial lymphocytes, lamina propria lymphocytes/plasma cells, lamina propria eosinophils, and lamina propria neutrophils.

duodenum and ileum were moderately negatively correlated (rho value, −0.5685; $P = .002$) with serum 25(OH)D concentrations.

Serum PTH concentration was moderately negatively correlated (rho value, −0.6827) with serum 25(OH)D concentration ($P < .001$). Serum iCa concentration was strongly positively correlated (rho value, 0.7343) with serum 25(OH)D concentration ($P < .001$). Serum VDBP concentration was not correlated with the duration of time the sample was stored ($P = .54$).

4 | DISCUSSION

Although decreased serum vitamin D concentrations have been well documented in dogs with CE and PLE, the etiology has been relatively unexplored. In our study, we examined a variety of variables related to the proposed mechanisms of vitamin D deficiency in humans with IBD, which include lack of PO intake, malabsorption of fat, direct loss of vitamin D through the gastrointestinal tract, and an active inflammatory state resulting in decreased production of vitamin D. We found CCECAI scores and CRP concentrations were higher, whereas serum α-tocopherol, cholesterol, and albumin concentrations were lower in dogs with CIE and low serum 25(OH)D concentrations when compared to dogs with CIE and normal serum 25(OH)D concentrations. In addition, duodenal morphologic and inflammatory scores, and overall WSAVA scores in the duodenum and combined duodenum and ileum, were negatively correlated with serum 25(OH)D concentrations in dogs with CIE.

Higher CCECAI scores were observed in CIE dogs with low serum vitamin D concentrations when compared to CIE dogs with normal serum vitamin D concentrations. Similarly, humans with IBD and low serum vitamin D concentrations are reported to have worse pain, higher disease activity index scores, and lower quality of life scores when compared to IBD patients with normal serum vitamin D concentrations.[9,27] The appetite scores as a component of CCECAI were evaluated in isolation and 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 normal appetite (appetite score 0). This observation would suggest that poor PO intake of vitamin D is unlikely to be the sole explanation for why some dogs with CIE have low serum vitamin D concentrations and others do not, but precise quantification of dietary vitamin D intake would be needed to definitively make this conclusion.

Serum cholesterol and alpha-tocopherol (vitamin E) concentrations were lower in low 25(OH)D CIE dogs when compared to dogs with CIE and normal serum 25(OH)D concentrations. In addition, based on the laboratory-established RI, 2 dogs in the low 25(OH)D group had subnormal vitamin E with serum concentrations <5 μg/mL. Serum retinol (vitamin A) concentrations fell within or above the normal RI for all dogs in the study and were not different between CIE dogs with low serum 25(OH)D concentrations compared to CIE dogs with normal serum 25(OH)D concentrations.

The exact explanation for low serum cholesterol and vitamin E concentrations in dogs with CIE and low serum 25(OH)D concentrations is unknown. In humans, cholesterol is fundamental to the endogenous synthesis of vitamin D after sunlight exposure.[28] However, because endogenous synthesis of vitamin D is thought to be absent to limited in the dog,[29] it is unlikely that hypocholesterolemia associated with intestinal disease[30] was the direct cause of low serum 25(OH)D concentration in our cases. Intestinal absorption of dietary cholesterol and fat-soluble vitamins (including A, D, E, and K) requires emulsification, hydrolysis, and micellization as well as adequate concentrations of pancreatic lipase and bile acids in the intestinal lumen. Therefore, dietary fat malabsorption because of a lack of bile salts, lymphatic dysfunction, or other causes may be an explanation for the decreased serum concentrations of cholesterol and vitamin E in dogs with low serum 25(OH)D concentration and CIE.[31,32] To further investigate the possibility of fat malabsorption in dogs with CIE and low serum 25(OH)D concentration, a coefficient of fat absorption test of feces or malabsorption blood test[33] could be performed and compared between dogs with CIE and low serum 25(OH)D and normal serum 25(OH)D concentrations. Although serum vitamin A concentrations were not different between groups, measurement of hepatic vitamin A stores using the relative dose response test was demonstrated to be more sensitive than serum retinol concentration for the detection of vitamin A deficiency in humans with CD.[34] Therefore, it is possible that measurement of hepatic vitamin A stores may have been a more accurate way to assess vitamin A status in our patients. In addition to malabsorption, proposed mechanisms of fat-soluble vitamin deficiencies in humans with IBD include consumption of antioxidants by proinflammatory radicals, enteric loss, and decreased nutrient intake.[27,35]

Several of the dogs in our study had serum α-tocopherol and retinol concentrations above the laboratory-established RI. This finding is similar to a recent study evaluating lipid-soluble vitamins in dogs with exocrine pancreatic insufficiency[36] and deserves further study.

To investigate enteric loss as the cause of low serum vitamin D concentrations in dogs with CIE, we considered that vitamin D and its metabolites are largely bound to VDBP in circulation. A much lower proportion is bound to albumin in serum, and <1% of vitamin D circulates unbound.[16] Serum concentrations of VDBP only have been studied in 1 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 concentrations was found.[17] Although loss of VDBP has been proposed as a cause of decreased 25(OH)D concentrations in dogs with hypoalbuminemia secondary to IBD,[2] we are not aware of any previous study of serum VDBP concentrations in dogs with CIE. In our study, we found serum VDBP concentrations were similar between the low 25(OH)D and normal 25(OH)D groups. Because we do not have an established RI for serum VDBP concentration in dogs, we do not know how these results compare to what would be found in a population of healthy dogs. A relationship between serum albumin and 25(OH)D concentrations previously has been demonstrated in humans[18] and dogs[2] with IBD. In our study, CIE dogs with low serum 25(OH)D concentrations had serum albumin concentrations lower than CIE dogs with normal serum 25(OH)D concentrations. In fact, only 1 dog with serum 25(OH)D

concentrations below the laboratory-established RI for 25(OH)D had a serum albumin concentration >2.0 g/dL. Enteric loss of albumin may be directly related to decreased serum 25(OH)D concentrations in dogs with CIE and hypoalbuminemia. However, because about only 10%-15% of vitamin D and its metabolites circulate bound to albumin, an alternative explanation would be that the relationship between serum albumin and 25(OH)D concentrations is not causal but representative of underlying pathophysiology that results in the decrease of both, such as lymphatic dysfunction or alterations in intestinal permeability and absorption.

Finally, we evaluated markers of both intestinal and systemic inflammation to investigate the hypothesis of an active inflammatory state contributing to low serum vitamin D concentrations in dogs with CIE. Importantly, recent studies of dogs with CIE have determined the clinical relevance of morphologic changes in the intestine with regard to clinical scores,[37] hypoalbuminemia,[37,38] and response to treatment.[39] Therefore, we elected to evaluate the morphologic and inflammatory changes in the intestine separately and together. In a previous study, total WSAVA scores were negatively associated with serum 25(OH)D concentrations in dogs with CE.[19] In our study, low serum 25(OH)D concentrations were moderately correlated with higher scores for morphologic and inflammatory changes in the duodenum, as well as with overall WSAVA scores for the duodenum and combined duodenum and ileum. In our study, morphologic changes in the duodenum consisting of villous stunting, epithelial injury, crypt distension, LD, and mucosal fibrosis were moderately negatively correlated with serum 25(OH)D concentrations. Lacteal dilatation also was evaluated in isolation, and ileal LD scores showed a moderate negative correlation with serum 25(OH)D concentration that was not significant after correction for multiple testing. Histopathologic interpretation of LD 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 adequately sampled by endoscopic biopsies, and, therefore, lymphatic abnormalities may have been underestimated.[40] Duodenal inflammatory scores also were moderately negatively correlated with serum 25(OH)D concentrations, as well as total WSAVA scores in the duodenum and combined duodenal and ileum. Furthermore, serum CRP concentrations were higher in dogs with decreased 25(OH)D concentrations, suggesting that systemic inflammation may play in a role in the development of low serum vitamin D concentrations in dogs with CIE. We suspect that both systemic and intestinal inflammation may play a role in the development of low serum vitamin D concentrations in dogs with CIE. However, our study suggests that morphologic changes also play a role, adding to the growing evidence of the importance of morphologic changes in the intestine in cases of CIE.

As expected, serum 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 10 dogs with low serum vitamin D concentrations, based on serum PTH concentrations >5.8 pmol/L. This is the appropriate physiologic response to a vitamin D deficient state,[41] but it was not present in all dogs.

Our study had some limitations. First, the ileum was not biopsied in 2 dogs in each group. Because pathology can differ among sections of intestine,[42,43] 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 still is considered relatively subjective,[44] and interobserver variation exists.[45] Serum CRP and fibrinogen concentrations were not measured in all dogs, because the study protocol was amended to include these variables after several dogs had already been enrolled. This may have affected our results for these variables. Because an RI has not been established for serum VDBP concentrations in healthy dogs, no conclusions can be drawn about the VDBP concentrations in dogs with CIE compared to healthy controls. The inclusion of a robust healthy control group, particularly for serum concentrations of fat-soluble vitamins as well as VDBP could have strengthened our results. Additionally, although the ELISA kit for detection of VDBP in canine serum has been validated for research use in dogs based on high homology between human and canine VDBP sequences as well as positive reactivity with 35 healthy control canine samples, it is not approved for diagnostic purposes at this time. Two dogs in each group had received glucocorticoids for <7 days before enrollment. Although 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 serum concentrations of 25(OH)D in humans.[46] Although we cannot be certain that vitamin D metabolism in dogs is equally unaffected, because 2 dogs were present in each group, we do not expect this affected the results importantly. Finally, although 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]

The pathogenesis of low serum vitamin D concentrations in dogs with CIE is likely multifactorial. A better understanding of the etiology is desirable because it may improve our understanding of gastrointestinal disease in dogs and improve our ability to detect and treat dogs with low serum vitamin D concentrations and CIE. Moreover, vitamin D supports innate lymphoid cells, regulates tight junction expression, and plays a key role in gastrointestinal microbial health such that decreased serum vitamin D concentrations can negatively affect the intestinal immune system, impair gastrointestinal barrier function, and result in dysbiotic microbial communities.[47] Therefore, identification of decreased serum vitamin D concentrations in patients with CIE may be important for general gastrointestinal health. Based on our study, intestinal fat malabsorption, potentially related to lymphatic dysfunction or altered intestinal permeability, deserves further study in dogs with CIE and low serum 25(OH)D concentrations. Additionally, based on our preliminary evaluation, loss of VDBP does not appear to be a major mechanism of low serum vitamin D concentrations, but this requires confirmation in a larger group of dogs, including healthy controls. The effects of systemic and intestinal inflammation as well as morphologic changes in

the intestine on serum vitamin D concentrations were identified as important areas of study in the mechanism of low serum 25(OH)D concentrations among dogs with CIE. Causal relationships cannot be inferred from our study. Further evaluation, particularly of serum concentrations of the other fat-soluble vitamins and VDBP, in a larger number of affected dogs as well as in healthy controls is necessary to be able to better understand the cause of low serum 25(OH)D concentrations in dogs with CIE.

ACKNOWLEDGMENTS

The authors acknowledge the support of the Naniboujou Research Legacy Fund and Rocky's Research Fund in completion of this project.

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

Colorado State University Clinical Review Board approval.

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

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, Berry JL, Herrtage ME, Mellanby RJ. Hypovitaminosis D in dogs with inflammatory bowel disease and hypoalbuminaemia. *J Small 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 Small Anim Pract*. 2005;46:345-351.
4. Allenspach K, Rizzo J, Jergens AE, Chang YM. 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 Colitis*. 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, Esmaillzadeh A. Vitamin D status in relation to Crohn's disease: meta-analysis of observational studies. *Nutrition*. 2016;32:505-514.
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 Res Int*. 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 Bowel Dis*. 2011;17:2116-2221.
13. Lo CW, Paris PW, Clemens TL, Nolan J, Holick MF. 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, Fichna J. Fat-soluble vitamin deficiencies and inflammatory bowel disease. *J Clin Gastroenterol*. 2017;51:878-889.
16. Garg M, Lubel JS, Sparrow MP, Holt SG, Gibson PR. 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 Pediatr Gastroenterol Nutr*. 2018;67:501-506.
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 Nephrol*. 2009;4:1988-1992.
22. Parker VJ, Harjes LM, Dembek K, Young GS, Chew DJ, Toribio RE. 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;31:791-798.
23. 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.
24. Laflamme DRPC. Development and validation of a body condition score system for dogs. *Canine Pract*. 1997;22:10-15.
25. 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.
26. 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.
27. 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 Gastroenterol*. 2016;111:712-719.

28. Wacker M, Holick MF. Sunlight and vitamin D: a global perspective for health. *Dermatoendocrinol*. 2013;5:51-108.
29. Weidner N, Verbrugghe A. Current knowledge of vitamin D in dogs. *Crit Rev Food Sci Nutr*. 2017;57:3850-3859.
30. Dossin O, Lavoué R. Protein-losing enteropathies in dogs. *Vet Clin Small Anim Pract*. 2011;41:399-418.
31. Iqbal J, Hussain MM. Intestinal lipid absorption. *Am J Physiol Endocrinol Metab*. 2009;296:E1183-E1194.
32. Thompson GR. Lipid related consequences of intestinal malabsorption. *Gut*. 1989;30:29-34.
33. Mascarenhas MR, Mondick J, Barrett JS, Wilson M, Stallings VA, Schall JI. Malabsorption blood test: assessing fat absorption in patients with cystic fibrosis and pancreatic insufficiency. *J Clin Pharmacol*. 2015;55:854-865.
34. 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. *World J Gastroenterol*. 2015;21:1614-1620.
35. Bousvaros A, Zurakowski D, Duggan C, et al. Vitamin A and E serum levels in children and young adults with inflammatory bowel disease: effect of disease activity. *J Pediatr Gastroenterol Nutr*. 1998;26:129-135.
36. Barko PC, Williams DA. Serum concentrations of lipid-soluble vitamins in dogs with exocrine pancreatic insufficiency treated with pancreatic enzymes. *J Vet Intern Med*. 2018;32:1600-1608.
37. 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*. 2018;46:15-20.
38. 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.
39. 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.
40. 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.
41. Dhupa N, Proulx J. Hypocalcemia and hypomagnesemia. *Vet Clin North Am Small Anim Pract*. 1998;28:587-608.
42. 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.
43. Casamian-Sorrosal D, Willard MD, Murray JK, Hall EJ, Taylor SS, Day MJ. Comparison of histopathologic findings in biopsies from the duodenum and ileum of dogs with enteropathy. *J Vet Intern Med*. 2010;24:80-83.
44. 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.
45. 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.
46. Hahn TJ, Halstead LR, Baran DT. Effects of short term glucocorticoid administration on intestinal calcium absorption and circulating vitamin D metabolite concentrations in man. *J Clin Endocrinol Metab*. 1981;52:111-115.
47. Cantorna MT, Snyder L, Arora J. Vitamin a and vitamin D regulate the microbial complexity, barrier function, and the mucosal immune responses to ensure intestinal homeostasis. *Crit Rev Biochem Mol Biol*. 2019;8:1-9.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Wennogle SA, Priestnall SL, Suárez-Bonnet A, Webb CB. Comparison of clinical, clinicopathologic, and histologic variables in dogs with chronic inflammatory enteropathy and low or normal serum 25-hydroxycholecalciferol concentrations. *J Vet Intern Med*. 2019;1–10. https://doi.org/10.1111/jvim.15614