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Association of Vitamin D Status and Clinical Outcome in Dogs with a Chronic Enteropathy

H. Titmarsh, A.G. Gow, S. Kilpatrick, J. Sinclair, T. Hill, E. Milne, A. Philbey, J. Berry, I. Handel, and R.J. Mellanby

Background: Dogs with a chronic enteropathy (CE) have a lower vitamin D status, than do healthy dogs. Vitamin D status has been associated with a negative clinical outcome in humans with inflammatory bowel disease.

Objectives: To examine the relationship between serum 25 hydroxyvitamin D (25(OH)D) concentrations at diagnosis and clinical outcome in dogs with a CE.

Animals: Forty-one dogs diagnosed with CE admitted to the Royal Dick School of Veterinary Studies, Hospital for Small Animals between 2007 and 2013.

Methods: Retrospective review. Serum 25(OH)D concentrations were compared between dogs which were alive at follow up or had died because of non-CE-related reasons (survivors) and dogs which died or were euthanized due to their CE (non-survivors). A binary logistic regression analysis was performed to determine significant predictors of death in dogs with CE.

Results: Serum concentrations of 25(OH)D at the time a CE was diagnosed were significantly lower in nonsurvivors ($n = 15$) (median nonsurvivors 4.36 ng/mL, interquartile range 1.6–17.0 ng/mL), median survivors ($n = 26$) (24.9 ng/mL interquartile range 15.63–39.45 ng/mL, $P < .001$). Serum 25(OH)D concentration was a significant predictor of death in dogs with CE (odds ratio 1.08 [95% CI 1.02–1.18]).

Conclusions: Serum 25(OH)D concentrations at diagnosis are predictive of outcome in dogs with CE. The role of vitamin D in the initiation and outcome of chronic enteropathies in dogs is deserving of further study.

Key words: 25 (OH)D; Prognostic; Inflammatory bowel disease.

Chronic enteropathies in dogs are a major cause of morbidity and mortality.¹ A diagnosis of chronic enteropathy (CE) is made in dogs which have a several week history of gastrointestinal signs such as weight loss, vomiting, and diarrhea, and the absence of an underlying etiology based on diagnostic evaluation and the presence of an inflammatory infiltrate within gastrointestinal biopsies.² The pathogenesis of CE in dogs is considered to be multifactorial and includes factors such as abnormal mucosal immunity, disrupted epithe-

Abbreviations:

25(OH)D	25 hydroxyvitamin D
AIC	akaike information criteria
ALT	alanine transaminase
ALP	alkaline phosphatase
IBD	inflammatory bowel disease
CE	chronic enteropathy
CPA	Clinical Pathology Accreditation
CIBDAI	canine inflammatory bowel disease activity index
HPLC	high performance liquid chromatography
(TNF)- α	tumor necrosis factor alpha
VDR	vitamin D receptor
WSAVA	World Small Animal Veterinary Association

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Dogs were recruited from, and investigated at, the Hospital for Small Animals at the Royal (Dick) School of Veterinary Studies, The University of Edinburgh. Serum 25 hydroxyvitamin D measurement from cases recruited at the Hospital for Small Animals was performed at the Vitamin D research laboratory at the University of Manchester.

Previous presentations: These results have not been presented at any meetings.

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lial barrier function, altered intestinal microbial flora, environment, and genetics.^{2,3}

Dogs with CE have lower serum concentrations of 25 hydroxyvitamin D (25(OH)D), which is the vitamin D metabolite widely used to assess vitamin D status, than do healthy dogs and hospitalized dogs with nongastrointestinal illnesses.^{4,5} In addition, the severity of the clinical signs, as assessed by the canine inflammatory bowel activity index (CIBDAI), correlates with serum 25(OH)D concentrations in dogs with a CE.⁵ However, the prognostic significance of serum 25(OH)D concentrations has not been investigated in dogs with a CE.

In contrast, the relationship between vitamin D status and IBD has been extensively explored in human medicine. In human patients with IBD, notably ulcerative colitis and Crohn's disease, vitamin D deficiency is a frequent finding.^{6–9} Higher predicted vitamin D status has been associated with a reduced risk of developing Crohn's disease.¹⁰ Among people with IBD, 25(OH)D concentrations are related to disease severity scores and

patient quality of life.^{11–13} Low plasma 25(OH)D concentrations have also been associated with an increased risk of surgery and longer hospitalization periods in patients with either Crohn's disease or ulcerative colitis.¹⁴ In addition, normalization of 25(OH)D concentrations in patients with Crohn's disease has been associated with a reduction in the risks associated with surgery.¹⁴ Vitamin D status has also been linked to IBD treatment outcomes, as low pretreatment vitamin D status has been associated with reduced durability of response to anti-tumor necrosis factor (TNF)- α treatment.¹⁵ Studies have also demonstrated improvements in disease activity scores and quality of life scores in Crohn's disease patients supplemented with oral vitamin D.^{16,17}

The hypothesis of this study was that the vitamin D status would not be different in dogs that died or were euthanized because of the complications associated with their CE compared to dogs which were alive at follow up or had died because of diseases unrelated to their CE. The aim of this study was to measure serum concentrations of 25(OH)D, alongside a range of clinical and biochemical variables, in dogs with a confirmed diagnosis of CE and known clinical outcome.

Material and Methods

Study Population

The records of dogs referred to the Hospital for Small Animals, Royal Dick School of Veterinary Studies for investigation of chronic gastrointestinal disease (more than 3 weeks in duration) between 2007 and 2013 were retrospectively reviewed. Dogs were considered eligible for inclusion in the study if they had presenting clinical signs consistent with a CE, which included any of the following: vomiting, diarrhea, increased borborygmi, abdominal pain, increased or decreased appetite, and weight loss. All dogs were considered eligible if they had histopathological evidence of inflammation within the small or large intestine and there were no clinically relevant abnormalities detected on hematology, biochemistry, or abdominal ultrasonography, which were not attributable to CE. In addition, a stored frozen serum sample from each dog, collected at the time of diagnosis was required for retrospective analysis of 25(OH)D concentrations. Hematology variables (total white blood cell count, mature neutrophils, band neutrophils lymphocytes, monocytes, eosinophils, basophils, total red blood cell counts, packed cell volume, hemoglobin, mean cell volume, mean cell hemoglobin concentration, and platelet number) were measured on ADVIA(r) 2120i System with Autoslide.^a Biochemistry variables (albumin, alanine transaminase [ALT], alkaline phosphatase [ALP], bile acids, bilirubin, total calcium, creatinine, globulin, phosphate, potassium, sodium, chloride, urea, and glucose) were measured on an ILab650 biochemistry analyzer.^b Clinical records were also reviewed for the results of fecal parasitology. Canine inflammatory bowel disease activity index (CIBDAI) scores were calculated as follows: appetite, activity levels, vomiting, fecal consistency, fecal frequency, and weight loss were each scored from 0 to 3. A score of 0 indicated no changes were present, a score of one indicated mild changes, 2 moderate changes, and 3 severe changes. In addition, a score of one point was added if the feces contained blood or mucus. The area of intestinal tract which was biopsied (duodenum or duodenum and colon) was determined based on the presenting clinical signs and was at the discretion of the primary clinician managing the case. A diagnosis of CE was made if there was histological evidence of intestinal inflammation and no clear underlying cause.

Vitamin D Analysis

Serum samples retained for 25(OH)D measurement were frozen after being used for routine biochemical analysis. They were stored at -70°C before being sent to the laboratory for analysis on dry ice. Serum 25(OH)D has been shown to be stable under these conditions.¹⁸ Serum concentrations of 25(OH)D were measured as previously described in detail.^{19,20} Samples were extracted using acetonitrile and applied to C18 Silica Sep-paks. Separation of metabolites was by straight phase high performance liquid chromatography (HPLC)^c using a Hewlett-Packard Zorbax-Sil Column^d eluted with hexane:propan-2-ol:methanol (92 : 4 : 4). Serum 25(OH)D₂ and 25(OH)D₃ were measured separately by application to a second Zorbax-Sil Column eluted with hexane:propan-2-ol (98 : 2) and quantified by ultraviolet absorbance at 265 nm and corrected for recovery (sensitivity 5 nmol/l, intra- and interassay coefficients of variation 3.0 and 4.2%, respectively).²¹ Total 25(OH)D was defined as the sum of 25(OH)D₂ and 25(OH)D₃. This laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the international Vitamin D Quality Assurance Scheme (DEQAS).

Histopathology

Where available, the slides of the original duodenal biopsies were reviewed by a single veterinary pathologist. A qualitative scoring system (World Small Animal Veterinary Association [WSAVA] Standards for the Diagnosis of Gastrointestinal Inflammation in Endoscopic Biopsy Samples)²² was used to assess the degree of inflammation. The template for this system assesses the following histological changes (villous stunting, epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis) and inflammatory infiltrates (intraepithelial lymphocytes, lamina propria lymphocytes and plasma cells, lamina propria eosinophils, and lamina propria neutrophils). The changes for each of the variables listed were graded as normal (0), mild (1), moderate (2), or severe (3). The sums of all these variables were added together to determine an intestine inflammatory score which ranged from 0 (normal) to 30 (very severe).

Outcome

For each dog enrolled, follow-up data was obtained by reviewing clinical records and by telephone contact with referring veterinary surgeons and owners. Outcome was recorded as survivors if the dogs were alive at follow up or had died because of a non-CE-related illnesses or nonsurvivors if dogs had died or were euthanized because of the complications associated with CE.

Statistical Analysis

Univariable measures were compared between dogs which survived and nonsurviving dogs using a Mann-Whitney *U*-test. A Fisher's exact test was used to compare the sex of surviving and nonsurviving dogs. A binary logistic regression model was used to estimate the association between outcome (survivors versus nonsurvivors) and serum 25(OH)D concentrations conditional on a range of other candidate predictors. Stepwise selection of variables was used to minimize Akaike Information Criteria (AIC), which is a parameter-penalized measure of model fit. Duodenal histology scores were classified into 3 approximate tertiles (low <7, medium 7–8, high >8) for the regression model. The statistical analysis was performed using R statistical system (R Development Core Team 2012).

Ethical Review

Informed consent for the use of residual clinical blood samples for research purposes was obtained at admission for each dog enrolled. Ethical approval for the study was obtained from the University of Edinburgh's Veterinary Ethical Review Committee.

Results

Signalment

Forty-one dogs were included in the study. There were 15 nonsurvivors and 26 survivors. In the nonsurvivors group, 2 dogs were intact males, 7 were neutered males, 1 was an intact female, and 5 were neutered females. In the survivors group, 7 dogs were intact males, 11 were neutered males, and 8 were neutered females. Breeds in the nonsurvivors groups included Border Collie (n = 1), Boxer (n = 3), Cavalier King Charles Spaniel (n = 1), Cross Breed (n = 3), German Short Haired Pointer (n = 1), Hungarian Vizsla (n = 1), Italian Greyhound (n = 1), Pyrenees Mountain Dog (n = 1), Springer Spaniel (n = 1), Staffordshire Bull Terrier (n = 1), and West Highland White Terrier (n = 1). In the survivors group, breeds included Border Terrier (n = 1), Boxer (n = 7), Cocker Spaniel (n = 1), Cavalier King Charles Spaniel (n = 1), Chinese Crested (n = 1), Cross Breed (n = 1), Irish Setter (n = 2), Labrador Retriever (n = 2), Lurcher (n = 2), Rottweiler (n = 1), Shar-pei (n = 1), Shetland Sheep Dog (n = 1), Springer Spaniel (n = 1), Staffordshire Bull Terrier (n = 1), Toy Poodle (n = 2), and Yorkshire Terrier (n = 1).

Clinical Findings

The median duration of clinical signs at diagnosis was 3 months in the nonsurvivors (range 1–10 months) and 3.5 months (range 0.75–24 months) in the survivors group. Hematology, biochemistry, and abdominal ultrasonography findings did not reveal any clinically relevant abnormalities in any of the 41 dogs which could not be attributed to their CE. Fecal parasitology was performed in 36 dogs and did not reveal evidence of parasitic infection in any of the samples. Twelve dogs underwent gastroduodenoscopy and 29 dogs had both gastroduodenoscopy and colonoscopy.

Histopathological examination of duodenal biopsies in the nonsurvivors revealed lymphoplasmacytic enteritis (5) and mixed lymphoplasmacytic and eosinophilic enteritis (10). In the survivor group histopathological diagnosis based on duodenal biopsies included lymphoplasmacytic enteritis (8) and mixed lymphoplasmacytic and eosinophilic enteritis (18). Follow up for the survivor group ranged from 18 to 75 months (median 27 months). In the nonsurvivors group the follow up ranged from 4 days to 24 months (median 2 months).

Outcome

Fifteen dogs died or were euthanized as a result of CE (Tables 1 and 2). The age of the dogs at presentation which subsequently died or were euthanized

because of their CE ranged from 9 to 114 months (median 96 months). Dogs which subsequently died had been treated with dietary changes and antibiotics (n = 2), dietary changes, prednisolone and antibiotics (n = 7), and dietary changes, antibiotics, prednisolone and other immunosuppressive medications (n = 6). Twenty-six dogs did not die as a result of gastrointestinal disease (Tables 1 and 2). Sixteen were alive at follow up and 10 had died because of nongastrointestinal diseases. The age of the dogs in the survivors group ranged from 6 to 96 months (median 60.5 months). Dogs in this group were treated with dietary changes and antibiotics (n = 10), dietary changes and prednisolone (n = 1), dietary changes, prednisolone and antibiotics (n = 3) and dietary changes, prednisolone, antibiotics and other immunosuppressive drugs (n = 1), diet alone (n = 10), and gastroprotectants (n = 1).

Univariable analysis revealed that serum 25(OH)D, albumin and total calcium concentrations were significantly lower in nonsurvivors compared to survivors (Table 1, Fig 1). Age and CIBDAI scores were significantly higher in nonsurvivors compared to survivors (Table 1). There was no significant difference in the number of male and female dogs between the 2 groups (Table 1).

A binary logistic regression model was performed to estimate the association between outcome and serum 25(OH)D concentrations conditional on a range of other candidate predictors. Canine inflammatory bowel disease activity index was not included in this model as it has previously been shown that this measure strongly

Table 1. Univariable analysis of clinical and biochemical variables in surviving and nonsurviving dogs. The data show the median value for each variable and the interquartile range.

	Survivors (n = 26)	Nonsurvivors (n = 15)	P Value
Age (months)	60.5 (36–73)	96.0 (60–108)	.004
Calcium mg/dL	9.68 (9.04–10.12)	8.24 (6.76–9.04)	.002
Calcium mmol/L	2.42 (2.26–2.53)	2.06 (1.69–2.26)	
Albumin mg/dL	3.34 (2.73–3.52)	2.45 (1.3–2.69)	.0007
Albumin g/L	33.35 (27.3–35.2)	24.50 (13–26.9)	
CIBDAI	6 (5–9)	10 (7–12)	.0022
25(OH)D ng/mL	24.90 (15.63–39.45)	4.3 (1.6–17.0)	.0003
Male/female	18/8	9/6	.73

Table 2. Impact on model akaike information criteria (AIC) after addition of dropped predictors. An increase in AIC represents a poorer parameter-penalized model fit.

Added Predictor	AIC
Final model which includes 25(OH)D and age	36.97
Albumin	38.97
Calcium	38.81
Sex	38.60
Histology tertile	40.31

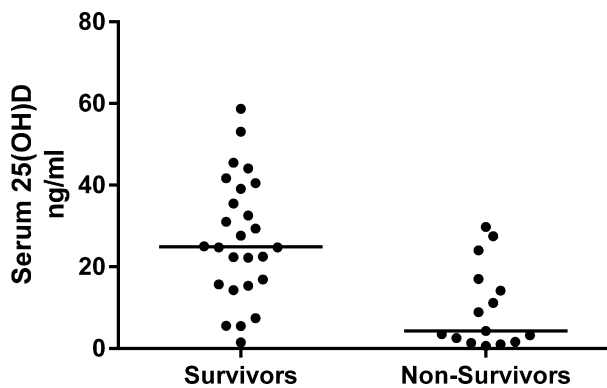


Fig 1. Serum 25 hydroxyvitamin D concentrations in surviving and nonsurviving dogs with a chronic enteropathy.

correlates with serum 25(OH)D concentrations.⁵ In this analysis duodenal histology severity scores were also included which were available for 34 of the 41 dogs. There was no significant difference in the histopathology scores between dogs which died compared to the ones which survived ($P = .06$). There were 10 dogs with a score of <7, 10 dogs with a score of 7 or 8, and 14 dogs with a score of 9 or greater. The initial model included age, sex, histopathology score as a tertile, serum calcium, albumin, and 25(OH)D concentrations. After stepwise AIC selection, the optimal predictive final model used serum 25(OH)D concentrations and age demonstrating that vitamin D status was an independent predictor of mortality in dogs with CE. The odds ratio for death was 1.08 (95% confidence interval 1.02–1.18) for vitamin D status and for age was 0.97 (95% confidence 0.93–1.00). Table 2 shows the consequences on model fit following the reintroduction of discarded predictors, demonstrating that the model based on 25(OH)D concentrations and age was optimal.

Discussion

The main finding of this study is that serum 25(OH)D concentrations are significantly lower at the time of diagnosis in dogs which died or were euthanized as a result of a CE. This is an important finding as it is presently difficult to predict outcomes in dogs with a CE. Older age, hypoalbuminemia, and higher CIBDAI scores are predictive of a poorer outcome in dogs with a CE, findings which are further supported by our research.^{1,23,24} This study, which demonstrates that 25(OH)D concentration at point of diagnosis is a prognostic marker in dogs with a CE, will provide additional prognostic information to owners and veterinarians managing dogs with a CE. Further work is needed to determine the optimal cut-off value for vitamin D as a prognostic marker.

The mechanism(s) underlying a hypovitaminosis D state in canine CE is unknown. Reduced dietary intake of vitamin D in dogs with a CE maybe an important cause of hypovitaminosis D especially as dogs do not cutaneously produce vitamin D.²⁵ As low vitamin D status in dogs with a CE has been associated with a

decreased appetite⁵, this may, in part, explain the reduced serum 25(OH)D concentrations that are frequently observed in dogs with a CE. However, dogs with a CE were found to have a lower 25(OH)D concentrations than hospitalized ill dogs with nongastrointestinal illnesses, many of which also have reduced appetites.⁵ Consequently, the influence of appetite on the vitamin D status in dogs with a CE remains unclear.

Circulating 25(OH)D is bound to vitamin D binding protein and albumin.²⁶ Enteric loss of albumin is regarded to be a significant problem in many dogs with a CE, notably in cases of protein losing enteropathies.²⁷ Consequently, loss of protein-bound vitamin D into the gastrointestinal tract could account for the low vitamin D status in some dogs with a CE. This may explain our earlier finding of a correlation between serum albumin concentration and vitamin D status in dogs with a CE.⁵ Similarly, albumin concentrations has also been shown to be a predictor of serum 25(OH)D concentration in humans with IBD.²⁸ Malabsorption might also contribute to low vitamin D status in human patients with Crohn's disease and this could potentially influence serum 25(OH)D concentrations in dogs with CE. However, while vitamin D absorption appears to be reduced in patients with Crohn's disease compared to healthy controls, there seems to be substantial variation in absorption of vitamin D in these patients.²⁹ Further studies are needed in both people and dogs with CE to clarify the potential role of vitamin D malabsorption in driving a hypovitaminosis D state.

Although hypovitaminosis D in CE has traditionally been considered to be a result of intestinal disease, there is growing evidence that hypovitaminosis D may contribute to the initiation of intestinal inflammation. Supporting evidence for a link between hypovitaminosis D and CE comes from rodent models which have demonstrated that vitamin D receptor knock out ($VDR^{-/-}$) mice are more susceptible to experimental forms of inflammatory bowel disease. For example, experimentally induced colitis in $VDR^{-/-}$ mice was significantly more severe compared to wild-type mice.^{30,31} Furthermore it has also been demonstrated that supplementing wild-type mice with vitamin D can decrease the severity of gastrointestinal inflammation with chemical induced colitis.³⁰ In addition, it has been shown that feeding mice a vitamin D-restricted diet can predispose to IBD.³² A recent meta-analysis of animal and human trials concluded that while many studies reported an improvement with supplementing vitamin D, there is insufficient evidence to currently recommend vitamin D treatment for human cases of IBD.³³ Therefore, further research is needed into the potential role of vitamin D in the management of these diseases.

The vitamin D receptor is expressed on many immune cell types and it is clear that vitamin D can modulate both the innate and acquired immune responses via effects on monocytes, macrophages, dendritic cells, and lymphocytes.^{34,35} Vitamin D has also been shown to profoundly modulate proinflammatory responses.^{36,37} Therefore, lack of vitamin D might drive abnormal inflammatory and immune processes.

Loss of tolerance to normally harmless bacterial and dietary antigens is hypothesized to be important for the development of canine CE.³ Vitamin D may also be important in regulating the immune response to commensal gut flora and maintaining normal bacterial populations. For example, dysbiosis was also reported in VDR^{-/-} mice and Cyp27B1^{-/-} mice³⁸ and dysbiosis may contribute to CE in dogs.³⁹

There is growing evidence linking vitamin D deficiency with disrupted intestinal mucosal barrier function. It has been proposed that altered epithelial barrier function, resulting in increased epithelial permeability, leads to increased exposure of the mucosal immune system to luminal antigens and that this may contribute to the initiation and perpetuation of chronic inflammation.⁴⁰ This hypothesis is supported by the observation that intestinal permeability is increased in people with naturally occurring inflammatory bowel disease and their unaffected relatives.⁴¹ Similar findings have been reported in dogs with naturally occurring CE where increased paracellular permeability has been demonstrated by lactulose to rhamnose absorption tests.^{42,43} In vitro active vitamin D metabolite, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], markedly enhances tight junctions by increasing tight junction protein expression.⁴⁴ The same study also demonstrated that VDR^{-/-} mice were more susceptible to mucosal injury than wild-type mice. These results suggest that vitamin D might be important in mucosal integrity and gastrointestinal barrier function which could contribute to CE.

Limitations of this study include the lack of standardization in treatment regime, which is a result of the retrospective nature of the study design.

In summary, this study demonstrates the serum vitamin D concentrations are predictive of clinical outcome in dogs with CE. Although, causality cannot be inferred from these results, the finding that low serum 25(OH)D concentrations are negatively correlated with outcome highlights the need to further examine the relationship between vitamin D homeostasis and disease development and outcome in dogs with CE.

Footnotes

^a Siemens Medical Solutions Diagnostics Ltd, Los Angeles, CA

^b Diamond Diagnostics, Los Angeles, CA

^c Waters Associates, Milford, MA

^d Hichrom, Reading, UK

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Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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