Journal of Veterinary Internal Medicine

ACVIM
American College of
Veterinary Internal Medicine

STANDARD ARTICLE

Serum concentrations of lipid-soluble vitamins in dogs with exocrine pancreatic insufficiency treated with pancreatic enzymes

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Background: In humans, exocrine pancreatic insufficiency (EPI) is associated with deficiencies in lipid-soluble vitamins. Little is reported regarding lipid-soluble vitamin status in dogs with EPI.

Hypothesis/Objectives: Compare serum concentrations of retinol, 25-hydrocholecalciferol (25OHD), and α-tocopherol among dogs with EPI, those with subclinical EPI (sEPI), and healthy dogs. Detect associations between serum concentrations of lipid-soluble vitamins and residual clinical signs in treated dogs with EPI and sEPI.

Animals: Twenty dogs with EPI and five dogs with sEPI receiving pancreatic enzyme replacement therapy. Ten healthy dogs sampled before and after 10 days of pancreatic enzyme supplementation.

Methods: Case-control study. Serum retinol and α-tocopherol concentrations were measured by high-performance liquid chromatography. Serum 25OHD concentrations were measured by radioimmunoassay.

Results: Serum retinol concentration was significantly lower in dogs with EPI (median, 490 ng/mL; range, 322-990 ng/mL) and serum α-tocopherol concentration was significantly lower in dogs with EPI (median, 11.51 μg/L; range, 4.8-27.1 μg/L) and sEPI (median, 12.66 μg/L; range, 10.21-21.03 μg/L) compared with healthy dogs (median, 1203 ng/mL; range, 637-1768 ng/mL and median, 43.54 μg/L; range, 34.26-53.97 μg/L, respectively). Dogs with weight loss had significantly lower 25OHD (mean, 243.50 nmol/L; standard deviation [SD], 3.54 nmol/L) than dogs with stable weight (314.0 nmol/L; SD, 138.38 nmol/L).

Conclusions and Clinical Importance: Altered homeostasis of lipid-soluble vitamins is present in dogs with EPI and sEPI, despite enzyme replacement therapy. Additional studies are needed to determine the clinical relevance of these findings and the therapeutic potential of lipid-soluble vitamin supplementation in dogs with EPI and sEPI.

KEYWORDS

malabsorption, retinol, tocopherol, vitamin A, vitamin D, vitamin E

1 | INTRODUCTION

Exocrine pancreatic insufficiency (EPI) is a malabsorptive syndrome caused by deficient secretion of pancreatic enzymes.[1] Fat malabsorption caused by deficient pancreatic lipase secretion is a prominent feature in dogs with EPI.[2,3] Lipid-soluble vitamins are essential to mammalian health, and deficiencies are associated with a variety of disease states. The effects of retinol (vitamin A) are mediated by retinoic acid (RA), a

ligand for nuclear receptor transcription factors that regulate diverse cellular functions including cell differentiation, lipid metabolism, immune function, and maintenance of epithelial barriers.[4–8] Alpha-tocopherol (vitamin E) is an antioxidant molecule that influences lipid membrane oxidation, inflammation, and immune function.[9,10] Vitamin D deficiency, determined by assay of 25-hydrocholecalciferol (25OHD), has been associated with altered calcium homeostasis, oncogenesis, cardiovascular disease, and inflammation.[11]

Previous studies have identified deficiencies in lipid-soluble vitamins in association with clinical abnormalities including vision loss, decreased bone density, and coagulopathies in humans with EPI.[12–15] A single published report, designed to assess the impact of dietary medium-chain triglycerides on the nutritional state of dogs with EPI, has described lipid-soluble vitamin status in dogs with EPI.[16] Significantly lower serum concentrations of retinol and 25OHD were identified in dogs with EPI compared with healthy dogs. No published reports had evaluated vitamin E status in dogs with EPI, but preliminary evidence suggests that vitamin E deficiency also is associated with EPI in dogs.[17]

Correcting micronutrient deficiencies (eg, cobalamin) is a critical aspect of managing patients with EPI. Documentation of micronutrient deficiencies could inform novel therapeutic approaches to the treatment of EPI and provide new insights on its pathophysiology. Our primary objective was to assess serum concentrations of retinol, 25OHD, and α-tocopherol in dogs with EPI and subclinical EPI (sEPI) receiving pancreatic enzyme replacement therapy (PERT), compared with healthy dogs fed pancreatic enzymes for 10 days. Subclinical EPI is a poorly-understood syndrome characterized by persistently subnormal (i.e. 2.6-5.6 μg/L) canine trypsin-line immunoreactivity (cTLI), variably identified in association with clinical signs of chronic enteropathy, EPI, or both.[18–20] Dogs with cTLI concentrations in this range do not only meet the diagnostic criteria for EPI but also persistently subnormal cTLI concentrations are indicative of a substantial reduction in functional exocrine pancreatic mass in these dogs. We also intended to describe associations between serum concentrations of lipid-soluble vitamins and the presence of residual clinical signs in dogs with EPI and sEPI receiving PERT. A secondary goal was to assess the impact of pancreatic enzyme administration on serum concentrations of lipid-soluble vitamins in healthy dogs. We hypothesized that serum concentrations of lipid-soluble vitamins would be significantly lower in dogs with EPI and sEPI compared to the healthy controls, and that the presence of residual clinical signs in these dogs would be associated with deficiencies in lipid-soluble vitamins.

2 | MATERIALS AND METHODS

2.1 | Animals, sample collection, and screening assays

All serum samples used in this study were obtained from residual material collected for a related study of dog EPI. All sampling methods and experimental procedures were approved by the University of Illinois Institutional Animal Care and Used Committee (protocol ID 14284), and expressed written consent was obtained from the owner of each study participant.

Dogs with a historical diagnosis of EPI or subclinical sEPI were recruited from an internet-based patient registry (www.epi4dogs.com; Epi4Dogs Inc., Farmville, VA) between October 2015 and May 2017. All dogs living in the United States with a serum cTLI concentration and owner contact information recorded in the database were identified. Their owners were contacted by phone or email and asked to complete a questionnaire to collect data regarding clinical signs (vomiting, diarrhea, weight loss, and appetite), diet, and therapies used in the 3 months before sample collection. Dogs were eligible for inclusion if they had a confirmed historical cTLI concentration ≤ 5.6 μg/L, their owners had completed the questionnaire, and their primary care veterinarian had agreed to participate. To account for the impact of pancreatic enzyme supplementation on lipid-soluble vitamin absorption, administration of PERT was an additional inclusion criterion. Dogs were excluded if a review of the medical record identified any evidence of concurrent systemic or gastrointestinal disease. Blood samples were collected into empty sterile tubes by venipuncture at primary care clinics after food had been withheld for 12 hours. Blood samples were centrifuged to obtain serum, which was divided into individual aliquots and immediately frozen on-site. All samples were mailed overnight on dry ice to the investigators where they were stored at −80°C. Aliquots of serum were submitted for assay of cTLI using a commercially available immunoassay (Texas A&M Gastrointestinal Laboratory, College Station, TX). Dogs with cTLI concentrations ≤2.5 μg/L were enrolled in the EPI group. Dogs with historical cTLI concentrations between 2.6 and 5.6 μg/L and confirmed by the investigators by reassessment of cTLI were enrolled in the sEPI group.

Ten healthy adult dogs were recruited from clients of the University of Illinois Veterinary Teaching Hospital. To screen for confounding diseases a CBC, serum biochemistry profile, urinalysis, total T4, cTLI, cPLI, cobalamin, and folate were assessed in each dog. Blood was collected into sterile empty and EDTA-containing tubes and urine samples were collected into empty sterile tubes by voiding or cystocentesis. Samples for CBC, serum biochemistry, urinalysis, and total T4 assays were submitted to an accredited veterinary diagnostic laboratory (University of Illinois Veterinary Diagnostic Laboratory; Urbana, IL) and cTLI, cPLI, folate, and cobalamin were measured by a commercial laboratory (Texas A&M Gastrointestinal Laboratory, College Station, TX). Dogs were excluded from this group if they had a serum cTLI concentrations <5.7 μg/L, clinical signs of vomiting, diarrhea, weight loss, anorexia, or polyphagia within 3 months of sample collection, laboratory abnormalities consistent with any systemic or gastrointestinal disease, or some combination of these. Pancreatic enzyme extract powder (Enzyme Diane 6X Pancreatin Powder; www.enzymediane.com) was administered PO (1/2-1 tsp per cup of food) for 10 days. On the final day of pancreatic enzyme supplementation, serum samples were collected as previously described.

2.2 | Vitamin analysis

All residual serum samples used for lipid-soluble vitamin analysis were frozen in individual aliquots immediately after collection, stored at −80°C, and thawed before analysis at the reference laboratory.

Previous studies have found that retinol, α-tocopherol, and 25OHD are stable for months to years at temperatures lower than −70°C.[21-23] Serum retinol, α-tocopherol, and 25OHD concentrations were assayed by a commercial veterinary laboratory (Michigan State University Veterinary Diagnostic Laboratory, Lansing, MI). Retinol and α-tocopherol concentrations were measured by high-performance liquid chromatography using a Waters Acquity system (Waters Corporation, Milford, MA) by detection of ultraviolet absorption at 292 and 325 nm, respectively. Serum 25OHD concentrations were measured by a commercially available radioimmunoassay (Immunodiagnostics Systems Limited, Bolden, UK).

2.3 | Statistics

All statistical analyses were performed using commercially available software (IBM SPSS Version 24.0.0.0, SPSS Inc., Armonk, New York). The distribution of the data was assessed by the Shapiro-Wilk test. Parametric variables were compared by 1-way analysis of variance (ANOVA) with post hoc pairwise comparisons corrected using the Hochberg method. Nonparametric variables were compared using the Kruskal-Wallis test with post hoc pairwise comparisons corrected by the Bonferroni method. The effect of enzyme administration in healthy dogs was assessed by paired t tests for parametric variables and by the Wilcoxon signed rank test for nonparametric variables. Differences in clinical signs across groups were assessed by Fisher's exact test. All results are reported as the mean ± standard deviation (SD) for parametric variables and the median and range (min-max) for nonparametric variables. A significance level of $P < .05$ was used for all statistical comparisons.

3 | RESULTS

3.1 | Animal and group characteristics

During the study period, 114 dogs met the initial inclusion criteria and their owners were contacted by email. Eighty-two dogs were excluded because of a lack of response to the historical questionnaire, an inability to obtain medical records, or both. Serum samples were collected from 32 dogs with historical cTLI concentrations ≤5.6 μg/L. Six dogs were excluded after their follow-up cTLI concentration was found to be >5.6 μg/L. One dog was excluded after it was found not to have been receiving PERT at the time of sampling. The EPI group consisted of 20 dogs with a mean (± SD) age of 4.3 (±2.04) years including 12 spayed females, 7 neutered males, and 1 intact male of the following breeds: German shepherd (GSD; n = 9), mixed breed (MBD; n = 4), Australian shepherd (n = 2), Akita (n = 1), border collie (n = 1), Labrador retriever (n = 1), and West Highland white terrier (n = 1). The sEPI group consisted of 5 dogs with a mean (± SD) age of 7.1 (± 2.53) years including 3 spayed females, 1 castrated male, and 1 intact male of the following breeds: GSD (n = 2), MBD (n = 1), Boxer (n = 1), and Standard Poodle (n = 1). The control group of healthy dogs consisted of 10 dogs with a mean (± SD) age of 5 (± 1.76) years including 7 castrated males and 3 spayed females of the following breeds: MBD (n = 4), GSD (n = 4), pit bull (n = 1), and Labrador retriever (n = 1). The distribution of breeds

was not significantly different across groups ($P = .678$). The sEPI group was significantly older than the EPI group ($P = .006$), but no other significant differences in age were identified among the other groups.

All dogs were fed diets that claimed to meet or exceed Association of American Feed Control Officials (AAFCO) adult minimum maintenance requirements for dietary vitamin A, D, and E (Association of American Feed Control Officials 2017 Official Publication; www.aafco.org). One dog in the EPI group was fed home-prepared, nutritionally balanced diet (chicken, sweet potato, white rice, spinach, carrots, and apples with a multivitamin powder) formulated by a veterinary nutritionist. Five of 20 dogs with EPI, 0/5 dogs with sEPI, and 2/10 healthy dogs were receiving dietary supplements containing vitamins A, D, E, or some combination of these at the time of sample collection. No significant difference was found in the proportions of dogs receiving vitamin supplements among the any of the groups ($P = .693$). Several dogs in the EPI group (5/20) received supplemental home-cooked foods or treats with their commercial diet including raw pancreas (1/5), chicken and sweet potato (1/5), ground pork (1/5), chicken paté (1/5), and a mixture of chicken, salmon, and cottage cheese (1/5).

3.2 | Impact of pancreatic enzyme supplementation on vitamin concentrations in healthy dogs

Ten healthy dogs received pancreatic enzyme supplements with each meal for 10 days with no reported adverse events before resampling. Retinol was measured in 3/10 dogs before and 9/10 dogs after enzyme administration. The median (range) retinol concentrations were 1116.0 ng/mL (709-1523 ng/mL) for the pre-enzyme (PreEnz) group and 1203.0 ng/mL (637-1768 ng/mL) for the postenzyme (PostEnz) group. Serum 25OHD was measured in 7/10 dogs before and 9/10 dogs after enzyme administration with mean (± SD) concentrations of 281.0 nmol/L (± 59.4 nmol/L) and 223.44 nmol/L (± 73.95 nmol/L), respectively. Serum tocopherol concentrations were measured in 3/10 dogs before, and 9/10 after enzyme administration with median (range) concentrations of 38.89 μg/mL (31.56-46.21 μg/mL) and 43.54 μg/mL (34.26-53.97 μg/mL), respectively. No effect on serum concentrations of retinol ($P = .285$), 25OHD ($P = .187$), or α-tocopherol ($P = .785$), was found in association with pancreatic enzyme supplementation in the healthy dogs. All of the following statistical comparisons utilize the PostEnz dogs as the healthy control group.

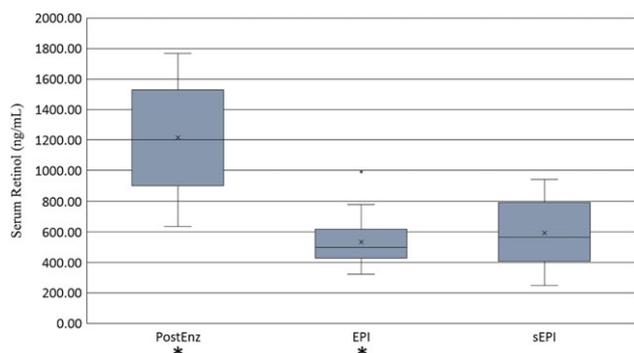### 3.3 | Differences in serum concentrations of lipid-soluble vitamins for experimental variables

Serum concentrations of retinol, 25OHD, and α-tocopherol were measured in all dogs in the EPI and sEPI groups (Table 1). The median (range) serum retinol concentrations were 490 ng/mL (322-990 ng/mL) and 566 ng/mL (248-940 ng/mL) in the EPI and sEPI groups, respectively (Figure 1). Serum retinol concentration was significantly lower in the EPI group compared with healthy dogs ($P < .001$). No significant differences in serum retinol concentrations were found between the sEPI group and the EPI ($P = 1.0$) or healthy control groups ($P = 0.126$). Mean (± SD) serum 25OHD concentrations were 336.74 nmol/L (±140.06 nmol/L) in the EPI group and 208.0 nmol/L (±67.7 nmol/L) in the sEPI group

TABLE 1 Summary statistics for serum lipid-soluble vitamins

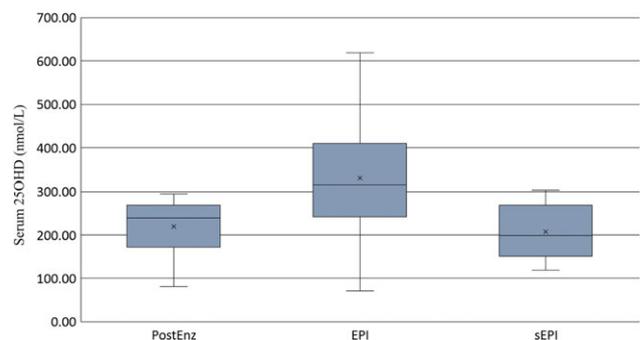| Group	Retinol (ng/mL) median (range)	25OHD (nmol/L) mean (±SD)	α-tocopherol (μg/L) median (range)
PreEnz (n = 10)	1116 (709-1523)	281.0 (59.4)	38.89 (31.56-46.21)
PostEnz (n = 10)	1203 (637-1768)	223.44 (73.95)	43.54 (34.26-53.97)
EPI (n = 20)	490 (322-990)	336.74 (140.06)	11.51 (4.8-27.1)
sEPI (n = 5)	566 (248-940)	208.0 (67.7)	12.66 (10.21-21.03)
EPI/sEPI by diarrhea			
Yes (n = 16)	470 (322-650)	304.81 (126.73)	8.11 (4.80-27.08)
No (n = 9)	566 (248-990)	308.44 (159.96)	12.91 (8.92-22.57)
EPI/sEPI by vomiting			
Yes (n = 7)	490 (322-650)	326.29 (107.51)	15.34 (4.80-25.59)
No (n = 18)	554 (248-990)	298.28 (148.00)	12.09 (6.61-27.08)
EPI/sEPI by weight			
Stable (n = 14)	554 (248-742)	368.64 (140.97)	13.41 (7.38-22.57)
Decreased (n = 6)	527 (322-650)	217.67 (66.98)	10.58 (7.99-27.08)
Increased (n = 5)	458 (416-940)	237.20 (99.349)	10.40 (4.80-25.59)
EPI/sEPI by appetite			
Stable (n = 21)	530 (248-990)	314.00 (138.38)	12.66 (4.80-25.59)
Decreased (n = 2)	480 (470-490)	243.50 (3.54)	17.6 (8.11-27.08)
Increased (n = 2)	607 (564-650)	159.00 (56.57)	15.62 (10.21-21.03)
RI	400-1200 ng/mL	109-423 nmol/L	4-12 μg/L

Serum concentrations of retinol, 25OHD, and α-tocopherol were measured in dogs with EPI, sEPI, and healthy dogs before (PreEnz) and after (PostEnz) pancreatic enzyme supplementation. Additionally, serum concentrations of lipid-soluble vitamins were assessed when dogs with EPI and sEPI were combined into a single group and stratified based on the presence or absence of residual clinical signs. Parametric variables are presented by the mean ± SD and nonparametric variables are presented by the median and range (min-max). SD, standard deviation; RI, reference interval.

(Figure 2). A statistically significant difference in serum 25OHD concentrations was identified by ANOVA among the 3 groups (P = .023), but no statistically significant differences were identified in the post hoc pairwise comparisons. The median (range) serum α-tocopherol was 11.51 μg/mL (22.28-27.1 μg/mL) in the EPI group and 12.66 μg/mL (10.21-21.03 μg/mL) in the sEPI group (Figure 3). Serum α-tocopherol concentration was significantly lower in dogs with EPI (P < .001) and sEPI (P = .021) compared with healthy dogs. No significant differences were found in serum α-tocopherol concentration between the EPI and sEPI groups (P = 1.0).

Based on responses to the questionnaire, the prevalence of clinical signs of gastrointestinal disease was compared between the EPI and sEPI groups. In the EPI group, residual clinical signs observed within 3 months of sample collection included hyporexia (2/20), weight loss (3/20), vomiting (6/20), and diarrhea (8/20). In the sEPI group, residual clinical signs included weight loss (3/5), vomiting (1/5), and diarrhea (1/5). No significant differences were identified in the proportions of dogs with hyporexia (P = .633), weight loss (P = .070), vomiting (P = .564), or diarrhea (P = .391) between the EPI and sEPI groups. When dogs with EPI and sEPI were combined into a single group and stratified based on the presence or absence of residual clinical signs, dogs with persistent weight loss had significantly lower serum 25OHD concentrations than did dogs with a stable weight (P = .047; Table 1, Figure 4). No significant differences were found in
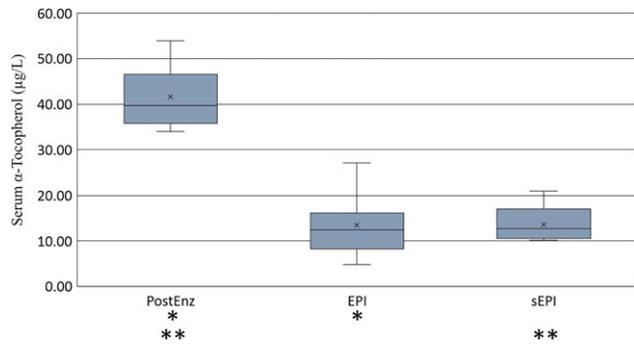

FIGURE 1 Comparison of serum retinol concentrations. Serum concentrations of retinol were compared between groups. Retinol was significantly lower in dogs with EPI compared with sEPI dogs and healthy controls. The line within the box represent the median value, the "x" within the box represents the mean, the box depicts the interquartile range, the whiskers show the maximum and minimum values less than or equal to 1.5 times the interquartile range. The open dot represents an outlier defined by a value greater than 1.5 times the interquartile range. An asterisk (*) denotes a statistically significant difference (*P < .001)

FIGURE 2 Comparison of serum 25OHD concentrations. Serum concentrations of 25OHD were compared between groups. There was no significant difference in 25OHD concentrations between groups. The line within the box represent the median value, the "x" within the box represents the mean, the box depicts the interquartile range, the whiskers show the maximum and minimum values

FIGURE 3 Comparison of serum α-tocopherol concentrations. Serum concentrations of α-tocopherol were compared between groups. α-tocopherol was significantly lower in dogs with EPI and sEPI compared with healthy controls. The line within the box represent the median value, the "x" within the box represents the mean, the box depicts the interquartile range, the whiskers show the maximum and minimum. Asterisks (*) denote statistically significant differences (*$P < .001$; **$P = .02$)

the distributions of serum tocopherol, retinol, or 25OHD concentrations across any other category of residual clinical signs.

4 | DISCUSSION

The purpose of our study was to compare serum concentrations of lipid-soluble vitamins retinol, 25OHD, and α-tocopherol among dogs with EPI, sEPI, and healthy dogs. The results indicate that, despite PERT, dogs with EPI have lower serum concentrations of retinol and α-tocopherol and dogs with sEPI have lower serum concentrations of vitamin E, compared with healthy dogs. These results are consistent with studies documenting altered homeostasis of lipid-soluble vitamins in humans with EPI. Those studies found that patients with EPI had deficiencies in ≥1 of vitamins A, D, E, and K, and steatorrhea despite pancreatic enzyme supplementation.[14,24] Vitamin deficiencies were associated with clinical abnormalities including increased dark-adapted visual threshold, decreased cortical bone density, and prolonged prothrombin times. Dietary supplementation with lipid-soluble
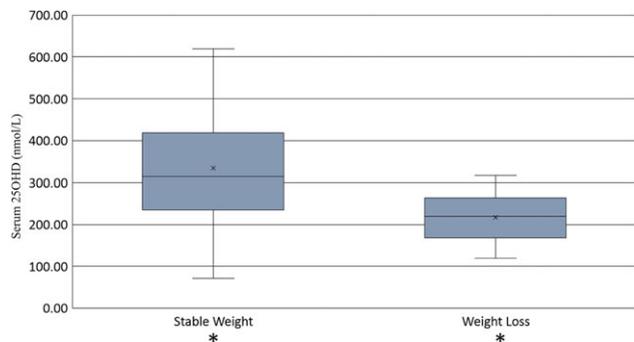
FIGURE 4 Serum concentrations of 25OHD in dogs with persistent weight loss. When dogs with EPI and sEPI were combined into a single group, those with persistent weight loss were found to have significantly lower serum 25OHD compared to dogs with stable weight. the line within the box represent the median value, the "x" within the box represents the mean, the box depicts the interquartile range, the whiskers show the maximum and minimum values. An asterisk (*) denotes a statistically significant difference (*$P < .043$)

vitamins resulted in correction of the deficiencies and normalization of the dark-adapted threshold and prothrombin times in several of the affected patients. These findings also support those from a study documenting lower serum concentrations of retinol in dogs with EPI treated with PERT.[16] Unlike the previous study, ours did not identify lower serum concentrations of 25OHD in dogs with EPI or sEPI, except in dogs with unresolved weight loss. The reason for this discrepancy is unknown, but it could reflect differences in the duration of PERT between the 2 populations of EPI or reflect the impact of a change in the testing method for 25OHD by the reference laboratory as of February 2017.

A comparison of our results to the laboratory-established reference intervals (RI) for retinol, 25OHD, and α-tocopherol indicates that a substantial number of dogs had serum vitamin concentrations within or above the RI. Within the PostEnz group, 5/9 healthy dogs had serum retinol concentrations above the RI whereas all had serum α-tocopherol concentrations above the RI. Despite highly significant differences in retinol and α-tocopherol concentrations among the EPI, sEPI, and control groups, it was uncommon for dogs with EPI and sEPI to have concentrations below the RIs. Retinol concentrations were below the RI (400-1200 ng/mL) in 2/20 dogs with EPI and in 1/5 dogs with sEPI, respectively. Serum 25OHD concentrations were below the RI (109-423 nmol/L) in 1/20 of dogs with EPI. Serum α-tocopherol concentrations were not below the RI (4-12 µg/L) in any of the dogs and were above the RI in 9/20 of dogs with EPI and 3/5 of dogs with sEPI. The RIs were provided by the reference laboratory where the tests were performed. They were derived from a database of all samples submitted to the laboratory for vitamin analysis and represent the 5th (lower reference limit) to 95th (upper reference limit) percentiles of samples from that population (personal communication). Compared with RIs generated from a population of healthy dogs, this method of RI construction is prone to bias introduced by the sampled population, which at a referral laboratory would include a larger number of diseased or malnourished animals than exist in a typical population of healthy animals. Therefore, the RIs provided by this laboratory may not apply to the population of dogs sampled for our study.

Absorption of dietary fat and fat-soluble vitamins is dependent on sufficient intake, bile acid secretion, micelle formation, and optimal duodenal pH in the presence of pancreatic lipase.[25,26] The nutrient profiles were not available for the diets of most dogs in our study. However, all dogs in the study were fed diets formulated to meet or exceed AAFCO standards for adult maintenance for vitamins A (5000 IU/kg DMB), D (500 IU/kg DMB vitamin D3), and E (50 IU/kg DMB). In fact, individual dogs within each group were found to have been fed diets that exceeded the minimum AAFCO vitamin A, D, and E standards for adult maintenance by as much as 5-, 4-, and 9-fold, respectively. In addition, a few dogs were receiving supplements containing vitamins A, D, and E. Because all dogs in the study were receiving balanced diets, and the proportions of dogs receiving vitamin supplements were not significantly different among groups, diet and vitamin supplementation are unlikely causes for the differences in serum vitamin concentrations among groups. It is our opinion that the differences in serum vitamin concentrations among groups are a result of persistent dietary fat malabsorption, changes in vitamin metabolism associated with EPI in dogs, or both.

Dietary vitamin A is derived from preformed retinol, retinyl esters, or provitamin carotenoids (eg, β-carotene).[6] Retinyl esters must undergo hydrolysis to retinol by pancreatic or intestinal brush border enzymes in order to be absorbed.[8] Within enterocytes, retinol is re-esterified to retinyl esters, which are transported to the liver and deposited in hepatic stellate cells.[6,8] When hepatic vitamin A is mobilized, the retinyl esters are hydrolyzed to retinol which is transported in plasma and delivered to tissues by retinol binding protein (RBP).[6,8] Unlike other mammals, substantial amounts of retinyl esters circulate with low-density lipoprotein (LDL) in dogs.[27–29] The relevance of this circulating pool of retinyl esters, and its impact on tissue metabolism of vitamin A is not well understood. However, in previous studies of dogs, circulating concentrations of retinyl esters were influenced substantially by dietary intake.[27,28] Based on these observations, it seems that circulating retinol is a more accurate indicator of long-term vitamin A homeostasis than LDL-associated retinyl ester concentrations, because it is influenced primarily by hepatic storage and mobilization, rather than by dietary intake. The physiologic effects of vitamin A are mediated by RA, which is generated intracellularly from retinol in a tissue-specific manner.[5,30] Retinoic acids are ligands for a family of nuclear receptor transcription factors, primarily the retinoic acid receptor (RAR) and the retinoid-X receptor (RXR), that regulate a diverse array of vital genes governing metabolism, the immune system, epithelial barriers, and cell proliferation and differentiation.[31]

Our data suggest that serum retinol concentrations are significantly lower in dogs with EPI, compared with healthy controls. Given the unique aspects of vitamin A metabolism in the dog, differences in dietary intake are unlikely to have resulted in the lower serum concentrations in dogs with EPI. We propose the following hypotheses as potential explanations for this finding: (1) decreased hepatic reserves of vitamin A associated with long-term dietary fat malabsorption, (2) decreased vitamin A absorption caused by decreased intestinal conversion of retinyl esters to retinol because of decreased intraluminal pancreatic and mucosal brush border enzymes, and (3) decreased mobilization of hepatic stores of retinol. As in humans, dogs with EPI treated with porcine enzymes are known to have dietary fat malabsorption that persists despite PERT.[2,32] As such, long-term lipid and lipid-soluble vitamin malabsorption could affect circulating pools of retinol because of depletion of hepatic reserves. Aside from pancreatic enzyme deficiencies, dogs and mice with EPI also have abnormal activities of multiple jejunal brush border enzymes.[17,33] Therefore, a direct effect of pancreatic or brush border enzyme deficiencies, or both on vitamin A absorption in dogs with EPI cannot be excluded. Finally, the regulation of retinol mobilization from hepatic reserves and relationship between circulating retinol and tissue-level RA metabolism are poorly understood. Some combination of these factors may contribute to altered vitamin A homeostasis in dogs with EPI. Additional studies are warranted to determine the relationships among hepatic mobilization of stored vitamin A, factors regulating mobilization and tissue utilization, and impact of dietary fat malabsorption on vitamin A homeostasis in dogs.

Vitamin D describes a group of steroid compounds that are known traditionally for their roles in calcium homeostasis. Unlike humans, dogs do not synthesize vitamin D in the skin and are reliant on dietary vitamin D to meet their physiologic requirement.[34] Dietary vitamin D is available as plant-derived ergocalciferol (D2) or cholecalciferol (D3), which is derived from animal tissues.[6] Aside from its well-established function to regulate calcium availability and bone growth, an increasing number of studies have posited roles for vitamin D beyond calcium regulation. In dogs, inflammatory bowel disease (IBD), chronic cardiac valve disease, and cancer all have been associated with serum 25OHD concentrations lower than those of healthy dogs.[11] Vitamin D concentrations were not significantly different among dogs with EPI, sEPI, and healthy controls. An ANOVA comparing all groups identified a significant difference in 25OHD concentration, but post hoc pairwise comparisons failed to identify a significant difference between groups. It is possible, given the low numbers of dogs in our study, that an increase in sample size would result in a statistically significant difference between dogs with sEPI and healthy controls. Dogs with EPI and sEPI and persistent weight loss had significantly lower serum concentrations of 25OHD. The reason for this finding is unknown, but it may reflect a poor nutritional state in dogs with unresolved weight loss. Alternatively, vitamin D deficiency may play a role in the response to PERT in some dogs. Additional studies of vitamin D metabolism in dogs with EPI are warranted.

Vitamin E is a term describing a group of structurally similar antioxidant compounds, among which α-tocopherol has the greatest biologic activity.[6] Tocopherols are absorbed by passive diffusion by enterocytes, a process dependent on solubilization in micelles.[6,35] Like retinol, α-tocopherol is stored in the canine liver and released with very low-density lipoproteins.[35] The most important physiologic effect of α-tocopherol is its role in stabilizing lipid membranes by preventing peroxidation of vital phospholipids.[36] Serum α-tocopherol concentrations were significantly lower in dogs with EPI and sEPI compared with healthy dogs. As with retinol, dietary fat malabsorption is likely an important contributor to the lower serum tocopherol concentrations in dogs with EPI. It is also possible that increased utilization of α-tocopherol because of oxidative stress could contribute to altered vitamin E metabolism in dogs with EPI. Long-term malabsorptive diseases such as EPI are associated with brown bowel syndrome (BBS), a condition associated with vitamin E deficiency.[37,38] In BBS, vitamin E deficiency is thought to induce oxidative damage to mitochondria in enteric smooth muscle, resulting in atony and lipofuscinosis which may exacerbate the malabsorptive disease process.[38]

According to a previous retrospective study, 25-50% of dogs with EPI have persistent clinical signs of pancreatic enzyme deficiency, including voluminous stool and flatulence, despite PERT.[39] In this study population, 40% of dogs with EPI had persistent diarrhea, despite PERT. There are several potential explanations for persistent diarrhea in treated dogs with EPI, and deficiencies in certain lipid-soluble vitamins could contribute to poor stool quality in affected dogs by influencing bile acid (BA) metabolism and mucosal immune responses. In humans, bile acid dysmetabolism is a relatively common complication-causing diarrhea in patients with EPI.[40–43] Bile acid diarrhea (BAD) is characterized by increased primary BA delivery to the colon. It traditionally has been attributed to BA malabsorption, but more recent studies in humans with idiopathic BAD have determined that dysregulation of BA signaling pathways leads to an increase in BA synthesis.[44] Bile acid synthesis is controlled by overlapping feedback

mechanisms in enterocytes and hepatocytes, ensuring that the rate of BA synthesis is inversely proportional to BA absorption, preventing BA synthesis beyond physiologic requirements.[45,46] Interestingly, vitamins A and D contribute to the regulation of BA synthesis by interactions between their nuclear receptors and specific regulatory gene elements.[47–49] Their effect is to increase expression of fibroblast growth factor-19, a potent antagonist of cholesterol 7-alpha-hydroxylase, the enzyme responsible for BA synthesis. A recent investigation identified dysregulation of BA metabolism in dogs with EPI compared with healthy controls.[50] Vitamins A and D also play roles in the regulation of mucosal immune responses through their influences on innate and adaptive immune responses.[51] Retinoic acid produced by dendritic cells enhances intestinal homing of IL-10 secreting regulatory T-cells and promotes increased production of IgA in activated B-cells.[7,52]

Vitamin D stimulates innate immune and adaptive responses by enhanced expression of pattern recognition receptors, cytokines, and antimicrobial peptides in addition to T-cell activation.[53,54] Thus, it is plausible that altered homeostasis of lipid-soluble vitamins participates in the pathophysiology of persistent diarrhea in treated dogs with EPI, a possibility that could be exploited as a target for novel therapies.

Our study had several limitations that should considered when interpreting the results. First, we had low numbers of dogs in each group, particularly in the sEPI group. Similarly, only three samples were available for analysis of retinol and α-tocopherol in the healthy dogs before enzyme supplementation. Because this study utilized excess sample material from a related investigation of dog EPI, a priori power analysis was not performed. The low numbers of samples available for analysis of retinol and α-tocopherol in the PreEnz group likely led to a reduction of statistical power such that an effect of pancreatic enzyme administration on serum concentrations of lipid-soluble vitamins in healthy dogs could not be properly evaluated. Consequently, these findings do not exclude the possibility that pancreatic enzyme administration could alter serum concentrations of retinol, 25OHD, and α-tocopherol. Regardless of the uncertainty related to the impact of pancreatic enzyme supplementation in the healthy dogs, the Post-Enz group was considered to be the most relevant reference group for our study because all dogs in the EPI and sEPI groups were receiving pancreatic enzyme supplements at the time of sample collection.

Other limitations of our study are related to differences in the diets and duration of PERT among individuals. Dogs in our study were not fed a standardized diet, and some were receiving supplements containing vitamins A, D, and E. Because the proportions of dogs receiving supplements were not different between groups, it is unlikely that supplement administration confounded these results. One dog that was fed a home cooked, balanced diet was included in the EPI group, because the individual results from this dog were not outliers within the EPI group and statistical comparisons excluding this individual were not different from the results reported here. The duration of PERT was not controlled in our study and dogs in the EPI and sEPI groups are likely to have been treated for variable lengths of time. However, this information was not recorded by the investigators, and the duration of PERT in the EPI and sEPI groups could not be assessed in this population. In addition, the healthy dogs were treated with enzymes for a relatively short duration of 10 days. The

duration of enzyme administration in the healthy dogs was limited by the availability of a donated pancreatic extract powder provided to the investigators. The relatively higher cost and invasiveness of long-term pancreatic enzyme administration in the healthy dogs further limited the enzyme trial to 10 days. As most of the dogs in the EPI and sEPI groups had been diagnosed months to years before enrollment, their average duration of enzyme treatment likely was longer than that of the healthy dogs. There are no published reports of the impact of duration of enzyme treatment on dietary fat absorption or lipid-soluble vitamin status in dogs or humans, and we are unable to speculate as to its potential effect on our results. Finally, dogs in our study could have had gastrointestinal disturbances from concurrent related or unrelated disease (eg, IBD, enteric dysbiosis) that could have confounded our results by affecting serum vitamin concentrations.

In addition, the sEPI group is a poorly defined clinical population. Previous studies of dogs with sEPI have found that its clinical course is highly variable.[18–20] Whereas some dogs progress to clinically overt EPI, others have equivocal cTLI concentrations that resolve spontaneously or persist for years without progressing to EPI. Given the small numbers of dogs in the sEPI group, and the variability of its clinical course, no firm conclusions can be drawn from these findings in terms of the clinical impact of lipid-soluble vitamin homeostasis in dogs with sEPI. However, these findings indicate that they are similar to dogs with EPI in that they have significantly lower serum α-tocopherol concentrations than healthy dogs, indicating similar alterations in lipid-soluble vitamin absorption, metabolism, or both.

In conclusion, we documented lower serum concentrations of retinol and α-tocopherol in dogs with EPI and lower serum α-tocopherol concentrations in dogs with sEPI despite PERT, relative to healthy dogs also receiving pancreatic enzymes. In addition, dogs with persistent weight loss had lower serum 25OHD concentrations than dogs with a stable weight. These findings could be because of long-term dietary fat malabsorption that is refractory to PERT. It is also plausible that altered systemic metabolism could contribute to the lower concentrations of lipid-soluble vitamins in this population of dogs with EPI. The factors governing tissue-level metabolism of lipid-soluble vitamins are not well understood and more research will be required to determine the clinical relevance of these findings. Additional studies to investigate the role of vitamins A and D in the pathophysiology of EPI and other conditions associated with dietary fat malabsorption are warranted. The therapeutic potential of supplemental dietary vitamins A, D, and E in dogs with deficiencies of these vitamins should be assessed in randomized, placebo-controlled clinical trials.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST DECLARATION

The authors receive research funding from Nestle-Purina, a corporation that manufactures pet feed and probiotic supplements. Additionally, the authors have received financial gifts for research and travel to a continuing education event from Epi4Dogs Inc., a nonprofit charity that supports EPI advocacy and research. The pancreatic enzyme supplement used in this study was donated by enzymediane.com, an e-commerce resource for pancreatic enzyme supplements. One of the authors (DAW) is an advisory board member for Nestle Purina Pet Care and a consultant with the Texas A&M GI Laboratory and IDEXX Inc. This same author holds patents and receives royalties for the canine and feline pancreatic lipase assays. None of the above entities were involved in the design of this study or in the interpretation of these findings.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

This study utilized only residual serum samples from an unrelated study of canine EPI. Samples collection protocols for this study were reviewed and approved by the University of Illinois IACUC (protocol ID number 14284).

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