Plasma glucagon-like peptide-2 concentrations are lower in dogs with chronic enteropathies than in healthy dogs

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OBJECTIVE
To compare plasma glucagon-like peptide-2 (GLP-2) concentrations in dogs with treatment-naïve chronic enteropathies to healthy dogs and describe changes over time in dogs with chronic enteropathies (CE).

ANIMALS
18 client-owned dogs with treatment-naïve CE and 17 client-owned healthy control dogs.

METHODS
This was a prospective study. Fasting, 1-hour, and 3-hour postprandial plasma GLP-2 concentrations were measured using a commercial immunoassay in healthy dogs and dogs with uncontrolled, untreated CE. Repeated fasting and postprandial plasma concentrations were measured in dogs with CE after initiating directed treatment for gastrointestinal disease.

RESULTS
There was no significant difference between fasting and postprandial GLP-2 concentrations in either group. Dogs with treatment-naïve CE had lower fasting (mean, 424 ± SD 176 pg/mL) plasma GLP-2 concentrations than healthy dogs (1184 ± 435 pg/mL; P < .0001). Fasted plasma GLP-2 concentrations (624 ± 314 pg/mL) remained lower in dogs with CE than in healthy dogs at recheck.

CLINICAL RELEVANCE
Dogs with CE have disrupted GLP-2 secretion. Future studies are required to evaluate subsets of CE and changes in response to therapy.

Keywords: enteroendocrine, enteropathies, gastrointestinal, glucagon-like peptide-2, inflammatory bowel disease

Glucagon-like peptide-2 (GLP-2) is a 33-amino-acid peptide hormone secreted by enteroendocrine L cells, with a predominant role in maintaining the gastrointestinal (GI) barrier and normal GI mucosal structure.1–2 GLP-2 stimulates GI epithelial cell proliferation and inhibits apoptosis.1–2 These roles have been demonstrated in rodent inflammatory bowel disease (IBD)-models in which treatment with a GLP-2 analog led to improvement in histopathologic lesions, decreased gene expression associated with GI inflammation,5 and increased mucosal thickness and villus height.1,4–6

It is generally accepted that GLP-2 is co-secreted from L cells in equimolar amounts to glucagon-like peptide-1 (GLP-1).7 Hence, it is assumed that most stimuli for GLP-1 secretion will stimulate GLP-2 secretion and mechanisms of GLP-2 regulation are extrapolated from well-established mechanisms of GLP-1 secretion.8 GLP-2 is secreted from L cells in response to intraluminal nutrients,9 GI bacterial metabolic by-products (eg, short-chain fatty acids, bile acids),10–11 and enteric nervous system stimuli.12–13

Human studies have explored changes in GLP-2 secretion in various forms of IBD. While effects on secretory pattern vary based on disease form and severity,14–18 pediatric human patients with ileal Crohn’s disease (CD) had decreased postprandial GLP-2 concentrations, which were associated with GI malabsorption and hyperpermeability.17 Idiopathic chronic enteropathies (CE) in dogs are associated with many GI histopathologic changes that are similar to changes in human IBD. These abnormalities include villus stunting and inflammatory infiltrates into the lamina propria. Ultrastructural changes to
the GI brush border, which aids nutrient digestion and absorption, also occur. In addition to histopathologic changes, microbial-associated functional changes, such as altered fecal short-chain fatty acid composition and decreased secondary bile acids, are also observed. In humans, postprandial GLP-2 concentrations normalize with increased GI mucosal area and improved GI function, suggesting that concurrent L cell loss contributes to decreased circulating GLP-2. Therefore, it is possible that similar histopathologic abnormalities in dogs could lead to differences in circulating GLP-2 concentrations, as is seen in humans with CE.

Importantly, GLP-2 plays a critical role in GI mucosal healing; therefore, decreased GLP-2 could also contribute to the persistence of histopathologic changes in individuals with CE. In 1 human IBD study, GLP-2 did not increase postprandial secretion, even with clinically stable disease. Treatment of those individuals with a GLP-2 analog improved measures of individual nutrient and total energy absorption. As histopathologic abnormalities do not consistently resolve in dogs after CE treatment, determining whether GLP-2 could contribute to the pathophysiology of disease could provide new targets for the treatment of canine idiopathic CE. In addition to the microscopic benefits noted above, rodents with induced IBD that were treated with GLP-2 analogs had increased body weight, improved fecal consistency, and prolonged survival. Human patients have achieved up to 90% disease response rates and experienced improved disease severity scores. Even in humans who are not reliant on parenteral nutrition, healing of GI lesions and improved biochemical parameters (eg, plasma proteins) have been noted.

As GLP-2 secretory patterns have not been explored in the context of canine CE, this study aimed to compare pre- and postprandial plasma GLP-2 concentrations in dogs with treatment-naïve CE to healthy dogs. A secondary objective was to compare GLP-2 concentrations in dogs with CE before and after starting treatment for GI disease. The hypothesis was that dogs with CE would have lower fasting and postprandial GLP-2 concentrations than healthy dogs. It was also hypothesized that GLP-2 concentrations would increase with clinical response to therapy for GI disease.

Methods

Chronic enteropathy population

Client-owned, adult dogs presenting to Kansas State University Veterinary Health Center with a diagnosis of chronic primary GI disease were enrolled prospectively between March 1, 2021, and May 26, 2022. Inclusion criteria were a minimum of 1 month’s duration of clinical signs consistent with GI disease (eg, vomiting, diarrhea, dysrexia, and weight loss) and exclusion of non-GI causes of clinical signs. All dogs had a CBC, chemistry profile, and abdominal ultrasound performed by a board-certified veterinary radiologist within 2 weeks of enrollment. Any dog with a creatinine >1.4 mg/dL had a urine-specific gravity performed to exclude clinically relevant renal insufficiency; dogs with urine-specific gravity <1.030 were excluded. Additional diagnostics (eg, baseline cortisol, fecal flotation, and Giardia antigen testing) were performed at the discretion of the attending clinician on the dog’s case. Exclusion criteria were ultrasound abnormalities concerning for infectious or neoplastic disease (eg, intra-abdominal lymphadenomegaly, GI masses, or loss of GI wall layering), as well as fecal, cytologic, or histopathologic diagnosis of infectious or neoplastic disease. Dogs with concurrent comorbidities on physical examination or baseline lab work (eg, liver enzyme elevation ≥1.5X the reference interval, cardiac disease requiring medical therapy) were also excluded. Use of antimicrobials, steroids, or probiotics within the previous 30 days was an additional exclusion criterion. Gastrointestinal histopathology was not required for inclusion.

Healthy control population

Healthy, adult dogs belonging to staff and students at the Kansas State University Veterinary Health Center were prospectively enrolled as a control population. In addition to ≥1 year of age, dogs were required to have a body condition score of 4–6 out of 9, where 5 is considered ideal. Exclusion criteria were medications aside from routine preventatives within the previous 6 months or any abnormalities on physical examination or routine lab work (CBC, chemistry profile) at the time of enrollment. Any dogs with a history of GI signs within 6 months before enrollment or use of a prescription diet to control historical GI signs were also excluded.

Due to the potential to cause GI microbiota dysbiosis or enteropathogen etiology of clinical GI signs, ingestion of raw food diets or raw food treats within 6 months was an exclusion criterion in both groups. All study procedures were approved by the Kansas State University IACUC (Protocol 4479), and informed owner consent was obtained before enrollment.

Sample collection

In all dogs, food was withheld 10–15 hours before sampling, initiated by owners at home and culminating when dogs were fed in-hospital for the study. Periods of fasting were confirmed at the time of the appointment. A preprandial, baseline blood sample (3 mL) was obtained via peripheral venipuncture (lateral saphenous or jugular vein). Dogs were then fed a standard commercial diet (CN; Nestle Purina Petcare Comp) at 25% resting energy requirement and allowed 15 minutes to eat the offered meal. Subsequent blood draws were performed at 1 and 3 hours (3 mL each) postfood ingestion. For all samples, whole blood was collected into chilled EDTA tubes and placed on ice. Proteinase inhibitors at 3% volume each (Aprotinin 3–8 trypsin inhibitor units/mg; 0.1 mM Diprotin A [ILE-PRO-ILE]) were immediately added to the sample, as previously described to prevent in vitro enzymatic GLP-2 degradation. Immediately after collection, samples were centrifuged.
were centrifuged (4°C; 1,794 X g; 20 minutes), plasma separated, and aliquots frozen (−80°C) and analyzed in bulk, with storage time within assay manufacturer reported stability. Before storage, repeat centrifugation was performed on any samples with gross hemolysis or lipemia. After recentrifugation, only samples without gross pigmentation were used for analysis.

After initial enrollment, treatment was individualized per dog at the discretion of the veterinarian managing the dog’s care. All procedures were repeated approximately 30 days after enrollment in the CE population.

**GLP-2 assay**

Plasma GLP-2 concentrations were quantified in-house using a commercially available canine-specific competitive ELISA (Canine GLP-2 ELISA Kit; Kendall Scientific). This kit has a reported detection range of 123.5–10,000 pg/mL and a sensitivity of 49.1 pg/mL. The manufacturer reports no cross-reaction with other proglucagon-derived peptides. ELISA procedures were performed according to manufacturer instructions. In brief, samples and reagents were allowed to thaw at room temperature for 1 hour; 50 μL of each sample or standard was added to each precoated well in duplicate. Optical density was read at 450 nm immediately after the addition of the stop solution using a microplate reader (BioTek Epoch). A minimum of 8 blank wells were allotted per plate to calculate background absorbance. Intra-assay variability was calculated using the duplicate results from all samples on a single plate; results from individual plates were averaged (245 sample pairs). An additional 22 sample pairs were duplicated between plates for the calculation of inter-assay variability.

**Disease severity scores**

Disease severity was scored at enrollment and the 30-day recheck in the CE population using previously established CE disease scoring systems, the canine inflammatory bowel disease activity index (CIBDAI) and canine chronic enteropathy activity index (CCECAI), based on a standardized questionnaire. This questionnaire was also completed for the healthy control dogs to screen for possible undisclosed GI or systemic disease.

**Statistical analysis**

Statistical analyses were performed using commercial software (Prism Version 9.1.4; GraphPad Software Inc). Data were assessed for normality using the Shapiro-Wilks test. Data are presented as median and IQR for normally distributed data, respectively. For all analyses, \( P < .05 \) was considered significant.

The number of dogs with CE was chosen based on the difference between postprandial GLP-2 concentrations in humans with active IBD (24.1 pmol/L) vs remission (38.9 pmol/L). A priori sample size calculation was performed based on a difference in means, standard deviation from human data = 10.9, with a desired \( \alpha = 0.05 \), and power = 0.8. Based on this calculation, 6 dogs per group would be needed. It was aimed to include 20 dogs in each group to account for an expected 30% remission rate in dogs with CE (ie, expecting that only 1/3 of enrolled dogs would have disease remission at recheck).

Population characteristics (eg, weight, body condition score, and age) were compared between CE and healthy dogs using an unpaired t-test (normally distributed data) or Mann-Whitney test (non-normally distributed data). A Wilcoxon test was used to compare disease severity scores between day 0 and recheck in dogs with CE.

To evaluate changes in GLP-2 concentrations after feeding in CE dogs at baseline, at day 30, and in healthy dogs, mixed-effects analyses using a compound symmetry covariance matrix and fit using restricted maximum likelihood were performed. In the absence of missing values, this is equivalent to a 1-way ANOVA with repeated measures. For statistically significant models, post hoc pairwise testing was performed using Tukey’s multiple comparisons test. A mixed ANOVA accounting for repeated measures, within-subject effects, and between-subject effects was used to evaluate the effect of study day (ie, baseline vs day 30 in CE dogs) or study group (ie, CE vs healthy dog) on GLP-2 concentrations. For statistically significant models, post hoc pairwise testing was performed between study days or study groups at each feeding time-point using Sidak’s multiple comparisons test. As there was no difference between fasting and postprandial GLP-2 concentrations, Spearman’s correlation was used to evaluate the relationship between disease severity scores and fasting plasma GLP-2 concentration. Spearman values were defined as previously described, with 0–0.19 = very weak, 0.20–0.39 = weak, 0.40–0.59 = moderate, 0.60–0.79 = strong, and 0.80–1.0 = very strong.

**Results**

**Dog demographics**

There were 18 CE and 17 healthy control dogs enrolled. Dog signalment, CE dog definite diagnosis, and CE dog disease severity scores were recorded. CE dogs consisted of 8 castrated males, 8 spayed females, 1 intact male, and 1 intact female. Healthy dogs included 5 castrated males, 10 spayed females, 1 intact male, and 1 intact female. Breeds included in the CE group were Brittany Spaniel (n = 2), golden-doodle (n = 2), mixed breed (n = 2), Yorkshire terrier (n = 2), and 1 each Australian shepherd, boxer, Cairn terrier, French bulldog, golden retriever, Jack Russel terrier, Maltese, miniature dachshund, Newfoundland, and Siberian husky. The healthy dog group was comprised of 8 mixed breed dogs, Labrador retriever (n = 2), and 1 each Border collie, Bassett hound, Dutch shepherd, German shepherd, Gordon setter, Irish setter, and Siberian husky. There was no significant difference in weight (\( P = .99 \)) or body condition score (\( P = .40 \)) between CE dogs at study enrollment and healthy dogs. Healthy dogs were younger (mean, 3.9 ± 2.3 years) than CE dogs (6.0 ± 3.6 years).
Clinical signs in CE dogs at the time of diagnosis included vomiting (n = 14), diarrhea (n = 12), weight loss (n = 12), and dysrexia (n = 11). The median duration of clinical signs was 3.75 months (range = 1–17 months). And 16 out of 18 CE dogs had GI histopathology performed at enrollment, all of which were consistent with idiopathic inflammatory CE. Most dogs (n = 9) had either lymphoplasmacytic enteritis or gastroenteritis. Eosinophilic enteritis (n = 3), histiocytic or granulomatous enteritis (3), and neutrophilic enteritis (1) were also observed. Three dogs had lymphangiectasia associated with their disease. Two dogs had lymphoplasmacytic colitis, both of which also had small intestinal inflammatory infiltrates. Recheck evaluation of CE dogs occurred at a median of 31.0 days (range = 25–59 days), with 14 dogs completing the study. Three dogs died before study re-evaluation (n = 2 dogs euthanized due to reasons other than GI disease; n = 1 dog died at home of unknown cause); 1 additional dog was lost to follow-up. Treatments in CE dogs between enrollment and follow-up included a novel protein or hydrolyzed diet trial (n = 9), glucocorticoids (n = 8), antithrombotics (n = 4), cyanocobalamin (n = 3), antimicrobials (n = 3), calcium carbonate (n = 2), antacids (n = 1), chemotherapeutics (chlorambucil; n = 1), folic acid (n = 1), appetite stimulants (n = 1), and bismuth subsalicylate (n = 1). Some dogs received more than 1 treatment.

Plasma GLP-2 concentrations

All CE and healthy dogs consumed the entire offered meal within 15 minutes. Mean intra-assay and inter-assay variability were 14.9 ± 11.3% and 19.2 ± 13.1%, respectively.

GLP-2 concentrations were higher in healthy dogs compared to CE dogs at all time points on day 0 (P < .0001, F 41.2) and study recheck (P < .001, F 14.0). At enrollment, mean plasma GLP-2 concentrations were 424 ± 176 pg/mL after fasting, 440 ± 169 pg/mL at 1-hour postprandial, and 473 ± 155 pg/mL at 3-hours postprandial (P = .16, F 2.15) in CE dogs. In healthy dogs, median fasting GLP-2 concentrations were 1028 pg/mL (range = 602–1966 pg/mL), compared to 1,052 pg/mL (range = 699–3645 pg/mL) at 1-hour, and 1,044 pg/mL (range = 642–2665 pg/mL) at 3-hours postprandial. There was no difference between fasting and postprandial concentrations in either group (CE dogs, P = .16, F 2.15; healthy dogs, P = .64, F 0.33; Figure 1).

At the study follow-up, the mean fasting GLP-2 in CE dogs was 624 ± 314 pg/mL. There was no significant difference in GLP-2 concentrations at 1-hour (677 ± 253 pg/mL) or 3-hours (667 ± 230 pg/mL) postmeal ingestion compared to fasting concentrations (P = .33, F 1.15). At study recheck, 1-hour postprandial GLP-2 concentrations were higher than at day 0 (P = .02, F 6.3). Three-hour postprandial GLP-2 concentrations trended higher at study follow-up compared to day 0, but this was not statistically significant in post hoc analysis (P = .07). Changes in individual fasted and postprandial CE dog plasma GLP-2 concentrations between study enrollment and recheck are demonstrated (Figure 2).

Disease severity scores

Median CCECAI scores in CE dogs at enrollment and at study recheck were 9 (range = 2–18) and 2.5 (range = 0–12), respectively. Median CIBDAI scores in CE dogs at enrollment and at study recheck were 8 (range = 2–11) and 2.5 (range = 0–11), respectively. Both CCECAI and CIBDAI scores were significantly lower (improved) on day 30 compared to day 0.
GLP-2, as well as a blunted postprandial response.\(^\text{17}\) which demonstrated decreased basal circulating plasma GLP-2 concentrations in this population of CE ing ulcerative colitis and CD.\(^\text{14-18,36}\) The decreased responses in humans with chronic GI disease, includ- increase after treatment of GI disease. It also aimed to evaluate changes in GLP-2 concentrations in dogs after the initiation of GI disease treatment,\(^\text{17}\) the exact time- points for this study were based on times to maxi- mum postprandial GLP-2 secretion in humans with GI disease is unknown. A 30-day follow-up was chosen based on previously reported response times for dogs with inflammatory CE.\(^\text{25,26,34,38}\) However, as human studies often span at least several months to evaluate clinical response\(^\text{19-40}\) and canine literature demonstrates significant continued improvement up to 2 months,\(^\text{25}\) a longer time to reevaluation may be needed. Although clinical disease severity scores sig- nificantly improved between study enrollment and recheck, some dogs still had “moderate” to “severe” disease and different results could have been real- ized with longer treatment duration. However, dis- ease severity scores did not correlate with GLP-2 concentrations in this population, and previous lit- erature has demonstrated that the severity of histopathologic lesions does not consistently correlate with disease severity.\(^\text{19}\) Therefore, the use of disease severity scoring to reflect clinical disease response may not be a beneficial marker of histopathologic response; repeat histopathology is not standard-of-care in veterinary CE patients and was considered unnecessarily invasive. Given that GI histopathologic abnormalities, microbiota dysbiosis, and metabo- lome indices do not consistently normalize in dogs with CE,\(^\text{21,22,24,25}\) persistent disease may also account for the lack of complete normalization. To the authors’ knowledge, this was the first com-parison of fasting and postprandial GLP-2 concentra- tions in either sick or healthy dogs. Based on GLP-2 secretion in humans and other animal species,\(^\text{9,41,42}\) as well as GLP-1 secretion in dogs,\(^\text{43-46}\) the lack of difference between fasting and postprandial GLP-2 concentrations in either population was unexpected. Many variables including diet type, volume ingested, and sample timing could have impacted these find- ings. The diet utilized in this study was chosen due to its relatively high fat content (7.36 g/100 kcal), a macronutrient that has been demonstrated to stimulate GLP-2 secretion in humans.\(^\text{8}\) In dogs, a high-fat diet led to greater postprandial increases in GLP-1 compared to a high carbohydrate diet within 30 minutes of meal ingestion.\(^\text{45}\) Although, within mixed meals, other factors may play a role, includ- ing fermentability of included fiber sources and type of protein source.\(^\text{45,46}\) In addition to macronutrient content, caloric intake and meal size may impact postprandial GLP-2 secretion.\(^\text{9}\) In humans, meals less than 400 kcal, regardless of nutrient content, do not reliably stimulate GLP-2 secretion;\(^\text{9}\) although, postprandial increases have been noted after meals as low as 220 kcal.\(^\text{41}\) Dogs in this study received a standardized diet based on the percentage of rest- ing energy requirement. This study design was based on other animal models demonstrating glu- cagon-like peptide secretion proportional to body weight calculated energy requirements.\(^\text{47-49}\) Due to this approach, the caloric intake varied, and greater caloric intake, either absolute or on a resting energy requirement basis, may be required to stimulate GLP-2 secretion in dogs. Additionally, the time at which maximal postprandial GLP-2 secretion occurs in dogs is unknown. Therefore, postprandial time- points for this study were based on times to maxi- mum postprandial GLP-1 concentrations in dogs, which have demonstrated peaks within 30 minutes

\[ P = .003 \text{ CCECAI}; P = .004 \text{ CIBDAI}. \]
to 3 hours of meal ingestion, and concentrations remaining above baseline for up to 8 hours. In other species, postprandial GLP-2 peak concentrations occur within 30 minutes to 2.5 hours, with variations based on the predominant nutrient type. While feeding a mixed-content meal may require a longer time for L cell stimulation due to the need for digestion into individual components (eg, fatty acids, amino acids), an early postprandial increase has been demonstrated in multiple species after complex nutrient meals. It is also possible that the dog demonstrates a different glucagon-like peptide secretion pattern than other species. In dogs fed a mixed nutrient content meal twice daily, GLP-1 levels remained above defined basal levels. However, a more typical postprandial increase was noted in dogs fed once daily. Although the dogs in this study had food withheld before sampling, all had received food the previous evening, which would more closely mimic twice daily feeding. If GLP-2 secretion mirrors GLP-1 secretion in dogs, it might be difficult to detect a distinct postprandial increase. Lastly, the ELISA kit used was designed to measure active (1-33) GLP-2. It is possible that the ELISA is not sensitive enough to detect the 2 amino acid differences between active and inactive (3-33) GLP-2, effectively measuring total (ie, combined [1-33] and [3-33]) GLP-2. While active GLP-2 concentrations increase after meal ingestion in humans, the total concentrations remain unchanged; therefore, detection of both forms may prevent detection of a pre- and postprandial difference in active GLP-2.

This study had several limitations, including the lack of requirement for histopathologic diagnosis. However, 16 of 18 dogs had GI histopathology performed, confirming a diagnosis of idiopathic inflammatory disease; neoplasia or infiltrative infectious disease was considered unlikely in the other 2 dogs based on history and abdominal ultrasound findings. The control population was also considered healthy based on history, physical exam, disease severity scores of “zero,” and biochemical analysis. While lack of further evaluation, such as abdominal imaging or GI histopathology, prevents complete exclusion of subclinical GI disease, this was considered a low likelihood based on the absence of any GI signs, including subtle weight loss, within 6 months. In addition, this study was not designed to differentiate GLP-2 responses between various subsets of idiopathic CE (eg, IBD without protein-losing enteropathy vs lymphangiectasia) or response to specific treatments (eg, food-responsive enteropathy vs steroid-responsive enteropathy). There were too few dogs in each category, and many received multiple treatments, precluding this analysis. However, this would be an area of focus in future treatment-controlled prospective studies. There was also some variability in follow-up time. While reevaluation was planned at 30 days postenrollment, follow-up occurred up to 59 days after starting CE treatment, due to owners’ ability to return for rechecks. It is possible that a longer time to follow-up could allow greater disease response and subsequently greater normalization of GLP-2. However, even allowing for longer treatment duration in some dogs, overall GLP-2 concentrations remained decreased compared to healthy dogs, suggesting incomplete normalization of enteroendocrine responses. This might suggest that a longer follow-up time than 2 months would be beneficial.

In conclusion, there is a disrupted enteroendocrine (ie, GLP-2) response in dogs with CE compared to healthy dogs. Further studies are needed to evaluate whether normalization of GLP-2 secretion could serve as a marker for disease response, as well as the utility of GLP-2 therapy as a treatment in dogs with chronic GI disease.

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Disclosures

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