# Evaluation of plasma glucagon-like peptide-2 in dogs with chronic enteropathies

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## Abstract

The enteroendocrine hormone glucagon-like peptide-2 (GLP-2) promotes gastrointestinal mucosal growth and healing in response to injury or disease. In humans with inflammatory and malabsorptive enteropathies, mucosal damage impacts GLP-2 secretion, with effects dependent on disease severity. In dogs with idiopathic chronic enteropathies, similar histopathological and biochemical changes to those observed in humans are noted. Glucagon-like peptide-2 has yet to be evaluated in dogs with chronic enteropathies.

A prospective study was performed to compare fasting and post-prandial plasma GLP-2 concentrations in dogs with uncontrolled chronic enteropathies to healthy dogs, with the hypothesis that both fasted and fed concentrations would be lower in dogs with gastrointestinal disease. Short-term GLP-2 responses were compared in dogs with chronic enteropathies one month after starting treatment for their disease, with the hypothesis that GLP-2 concentrations would increase concurrent with clinical disease response.

Eighteen client-owned dogs with chronic enteropathies were enrolled prior to targeted therapy for gastrointestinal disease. Seventeen healthy client-owned dogs were enrolled as a control group. In all dogs, fasted blood samples were obtained, followed by samples 1 and 3 hours after feeding a standardized meal. Blood was collected into chilled EDTA tubes, and two proteinase inhibitors were added to the sample at the time of collection. Samples were immediately centrifuged, plasma separated, and frozen (-80°C). Same-day fecal samples were collected and scored using a previously established fecal scoring system. Standardized, previously established scoring systems for canine chronic enteropathies were used to score disease severity. All

procedures were repeated 30 days following enrollment in the chronic enteropathy population. Plasma GLP-2 concentrations were measured using a canine-specific ELISA. A mixed-effects analysis accounting for repeated measures was used to compare post-prandial GLP-2 changes in all dogs. A mixed analysis of variance, accounting for repeated measures, within-subject effects, and between-subject effects, was used to analyze effects of study day or study group on GLP-2 concentrations.

Fasted and post-prandial plasma GLP-2 concentrations were lower in dogs with uncontrolled gastrointestinal disease compared to healthy dogs at the time of diagnosis ($P < 0.0001$) and at study follow-up ($P < 0.001$). Post-prandial GLP-2 concentrations were higher at the 1-hour post-prandial time-point at study recheck compared to enrollment in CE dogs ($P = 0.02$).

The results of this study suggest altered enteroendocrine responses, specifically decreased GLP-2 secretion, in dogs with chronic enteropathies compared to healthy dogs. Treatment for gastrointestinal disease may help to normalize GLP-2 response

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# List of Abbreviations

| | |
|---|---|
| EEC | Enteroendocrine cells |
| GI | Gastrointestinal |
| SI | Small intestine |
| PYY | Peptide YY |
| CCK | Cholecystokinin |
| GIP | Glucose-dependent insulinotropic peptide |
| GLP | Glucagon-like peptide |
| IV | Intravenous |
| SS | Somatostatin |
| CNS | Central nervous system |
| IP-2 | Intervening peptide 2 |
| PC | Proconvertase |
| DPP-IV | Dipeptidyl peptidase IV |
| GLP-2R | Glucagon-like peptide-2 receptor |
| GRP | Gastrin releasing peptide |
| FFAR | Free fatty acid receptor |
| GPCR | G protein-coupled receptor |
| SGLT | Sodium-glucose co-transporter |
| STR | Sweet taste receptor |
| GLUT 2 | Glucose transporter 2 |
| CaSR | Calcium sensing receptors |
| TGR5 | Takeda G protein-coupled receptor 5 |
| cAMP | Cyclic adenosine monophosphate |
| T1R2 | Taste receptor type 2 |
| T1R3 | Taste receptor type 3 |
| GLUT 5 | Glucose transporter 5 |

| | |
|---|---|
| Pept1 | Peptide transporter 1 |
| ASBT | Apical sodium bile acid transporter |
| LPS | Lipopolysaccharide |
| SQ | Subcutaneous |
| EGF | Epidermal growth factor |
| IGF | Insulin-like growth factor |
| ErbB | Intestinal epithelial receptor |
| TPN | Total parenteral nutrition |
| VIP | Vasoactive intestinal peptide |
| CRI | Continuous rate infusion |
| NO | Nitric oxide |
| SBS | Short bowel syndrome |
| IBD | Inflammatory bowel disease |
| DSS | Dextran sodium sulfate |
| UC | Ulcerative colitis |
| IL | Interleukin |
| TNF-α | Tumor necrosis factor-alpha |
| IFN-γ | Interferon gamma |
| GH | Growth hormone |
| KGF | Keratinocyte growth factor |
| BCS | Body condition score |
| CE | Chronic enteropathy |
| CBC | Complete blood count |
| RER | Resting energy requirement |
| CIBDAI | Canine inflammatory bowel disease activity index |
| CCECAI | Canine chronic enteropathy activity index |

# Chapter 1: Overview of the enteroendocrine system and glucagon-like peptide-2 regulation

*1.1 The enteroendocrine system*

Enteroendocrine cells (EEC) are specialized epithelial cells distributed throughout the gastrointestinal (GI) tract.[1] Enteroendocrine cells comprise the largest endocrine organ in the body. There are at least 15 different cell types subdivided based on polypeptide and amine production.[2] Enteroendocrine cells account for 1% of the total GI epithelial cell population.[3-5] **Table 1.1** demonstrates the predominant EEC and traditionally associated hormone products. Broad functions of products secreted by EEC include regulation of food intake, gallbladder contraction, exocrine pancreatic enzyme secretion, GI motility, GI immune responses, GI nutrient absorption, and maintenance of the normal GI mucosal structure and barrier function.[1,6-8]

| **EEC cell** | **Hormones** | **GI segment location in humans** |
|---|---|---|
| A cells | Ghrelin | Stomach |
| D cells | Somatostatin | Stomach and intestine |
| I cells | Cholecystokinin | Proximal small intestine |
| K cells | Glucose-dependent insulinotropic peptide | Proximal small intestine |
| L cells | Glucagon-like peptide-1 | Terminal ileum, colon and rectum |
| | Glucagon-like peptide-2 | Terminal ileum, colon and rectum |
| | Peptide YY | Terminal ileum, colon and rectum |

**Table 1.1. Enteroendocrine cells and localization**

**Summary of the different EEC, hormones produced, and anatomic localization.[9-10]** ***EEC, enteroendocrine cells; GI, gastrointestinal***

*1.2 Enteroendocrine cell structure and localization*

Enteroendocrine cell type and cell morphology vary along the different anatomic regions of the GI tract (e.g., small versus large intestine), as well as functional regions (e.g., intestinal villus tip versus crypt).[5,11] These variations likely reflect mechanisms of hormone secretion from

a specific EEC type, in addition to the roles of hormones produced by that cell.[12-14] For example, EEC responding directly to post-prandial vagal input or secreting hormones with primary roles in small intestine (SI) feedback on gastric emptying are located more proximally within the GI tract. Further, EEC that respond to intraluminal nutrients exhibit morphologies that promote direct contact with luminal contents, which are different from EEC that respond predominantly to paracrine stimuli. These anatomic and structural variations are further described below.

There are two general EEC morphologies, termed "open-type" and "closed-type" cells.[5] Open-type EEC exhibit a high degree of polarization, with a flask-like apical process covered with microvilli that contact the GI lumen.[5,15] The open-type cell morphology is exhibited by EEC that have primary roles in nutrient response (e.g., L cells).[5] Chemoreceptors within the microvilli facilitate EEC responses to intraluminal nutrients.[16] Intraluminal nutrients activate receptors on open-type EEC that result in calcium-dependent intracellular signaling and release of hormones from secretory granules on the basolateral cell surface into systemic circulation.[5,17] Closed-type EEC exhibit a manifold shape with cytoplasmic processes that extend from the basal aspect of the cell and are closely associated with capillary beds and nerves.[5,17-18] Closed-type EEC, such as enterochromaffin cells, do not have luminal contact and are less reliant on intraluminal nutrients for regulation of hormone secretion, relying on neuronal and paracrine or endocrine signals.[5,16] By-products of GI microbiota metabolism also stimulate hormone secretion from closed type EEC.[18] Enteroendocrine cells of a single type may also exhibit distinct morphologies in the small versus large intestine or along different regions of an intestinal villus.[9-10] For example, peptide YY (PYY)-secreting L cells within ileal villi have limited apical contact with intraluminal content. However, they express basilar pseudopod-like processes, which extend between the GI mucosa and lamina propria. As these processes synapse onto neighboring cells, it is theorized

that there is direct paracrine secretion of PYY by those cells. Colonic PYY-secreting L cells exhibit a spindle-like morphology, with basal processes extending between GI epithelial cells. PYY-secreting cells in this location contact both the GI lumen and lamina propria; secretory granules localize at the basal aspect of the cells. Concurrent contact with the lumen and deeper GI layers suggests combined roles of colonic L cells to both sense intraluminal nutrients and mediate paracrine secretion.[9] Cholecystokinin (CCK)-secreting I cells may also exhibit either a flask-like or spindle-shaped morphology, and some, though not all, CCK-secreting cells have basilar pseudopod-like processes.[10] While CCK-secreting I cells are detectable along the intestinal villus and within crypt, they are more prominent within crypt regions. The presence or absence of basilar processes varies based on localization, with a higher percentage of villus I cells exhibiting basilar processes than those in crypts. Basilar processes are also longer in villus-located versus crypt-located I cells. Variation in process length likely reflects their secretory role and need for villus-located cells to exert paracrine effects across a longer distance. Like PYY-secreting cells, some CCK-secreting cells also appear to have dual functions, with apical microvilli contacting the GI lumen.[10]

In addition to open versus closed-type morphology, other modifications to EEC structure mediate direct communication with the central nervous system.[19] The secretory granules of CCK and PYY-secreting cells described above also contain neurofilaments, proteins that comprise neuronal axons.[20-21] Further, enteric glial cell processes have direct contact with these EEC pseudopods to provide support to neuronal cells. Synapses between EEC and vagal neurons confer excitatory signals from the GI tract to the brain via glutamine.[20-22]

Nutrient stimulation on EEC leads to secretion of hormones, which may lead to paracrine secretion of other enteroendocrine hormones.[23-24] K cells in the proximal SI secrete glucose-

dependent insulinotropic peptide (GIP), which leads to vagal-mediated glucagon-like peptide (GLP) secretion from more distally located L cells.[23] This paracrine-mediated EEC stimulation was demonstrated by combined GIP and GLP-1 secretory patterns in rats. When fat (corn oil) was placed directly into the ileal lumen, increased circulating GLP-1 was observed. Fat placed directly into the duodenum led to increased circulating GIP, followed by increased circulating GLP-1; GLP-1 concentrations were not different than those noted following ileal fat placement.[24] Similar increases in circulating GLP-1 concentrations were noted following intravenous (IV) GIP administration. In an *in vitro* model, L cells co-incubated with GIP secreted GLP-1, but GLP-1 secretion was not observed following co-culture with glucose alone.[25] L cells also express somatostatin (SS) receptors, which inhibit GLP-1 secretion when activated by SS binding, even in the absence of D cells in close proximity to L cells.[26]

Based on multiple immunohistochemistry studies, localization of EEC to specific GI segments is relatively conserved among humans, rodents, cats, and dogs; although, some differences between species have been described.[17,26-27] D cells are predominantly located within the gastric fundus, as well as the proximal SI and secrete SS.[17,26] X/A-like cells are predominantly located within the stomach in humans and mice and are responsible for the secretion of ghrelin.[27] K cells are predominantly located in the duodenum and proximal jejunum in most species.[28,29] In cats, they are distributed diffusely throughout the SI, as well as the colon.[29] L cells are predominantly localized to the distal SI (ileum) and colon in humans, rodents, and cats.[29,30] Dogs have a higher concentration of L cells within the jejunum, compared to pigs, rats, humans, and cats; although, the majority of L cells are still located within the colon.[28-30] In dogs, L cells co-localize with K cells in the jejunum.[28]

While it is generally accepted that EEC type determines the hormone secreted, there is some evidence that EEC anatomic or microanatomic location (e.g., duodenum versus ileum; villi versus crypts) also impacts the secretory product.[29,31] Chicken L cells stained positive via immunohistochemistry for both GLP-1 and GLP-2 throughout the entire ileum when located in the lower and middle parts of the intestinal villi.[15] When localized to the mid-villus, immunohistochemistry demonstrated only GLP-1 positivity.[15] Immunohistochemistry staining for GLP-1 and the human GLP-2 sequence demonstrated that feline L cells in the proximal SI (e.g., duodenum and jejunum) were positive for GLP-1 but not GLP-2; those in the distal ileum and colon were positive for both GLP-1 and GLP-2.[29] In rats, GLP-1 was secreted from L cells in both the duodenum and ileum, while PYY was only secreted from ileal L cells.[31] It has been suggested that differential staining for hormone products in a single cell type represents two populations of a given EEC cell.[29,31] This could be mediated through enzymatic precursor processing that results in a different complement of hormone products (see proglucagon processing below).

*1.3 Gcg gene and proglucagon-derived peptides*

The *Gcg* gene encodes preproglucagon mRNA in EEC, pancreatic alpha cells, and the central nervous system (CNS), specifically the brainstem and hypothalamus.[32-35] Preproglucagon is post-translationally processed to glucagon and the 160-amino acid peptide major proglucagon fragment.[36] The major proglucagon fragment is subsequently processed to the proglucagon-derived peptides GLP-1, GLP-2, intervening peptide 2 (IP-2), oxyntomodulin, and glicentin in a tissue-dependent manner (**Figure 1.1**).[36-37] Post-translational processing is mediated by the proconvertase (PC) family of enzymes, of which PC2 and PC1/3 are highly specific to nervous and endocrine tissues.[37-38] PC2 cleaves proglucagon to glucagon in pancreatic alpha cells.[39] In L

cells, proglucagon is cleaved by PC1/3 into GLP-1, GLP-2, IP-2, oxyntomodulin and glicentin.[37] Proconvertase enzyme expression likely contributes to the variation in hormone products secreted in different tissues and by individual cell EEC types.[37,39] In humans, there is relatively increased expression of PC1/3 in SI EEC compared to colonic EEC; there is similar expression of PC1/3 and PC2 in SI EEC.[40] Canine L cells express both PC1/3 and PC2, but unlike in pancreatic alpha cells, glucagon is not secreted by L cells.[38] The reason for detection of PC2 positive cells in human and canine intestinal EEC is unknown; although, some proglucagon cleavage sites may not be specific for PC1/3 versus PC2.

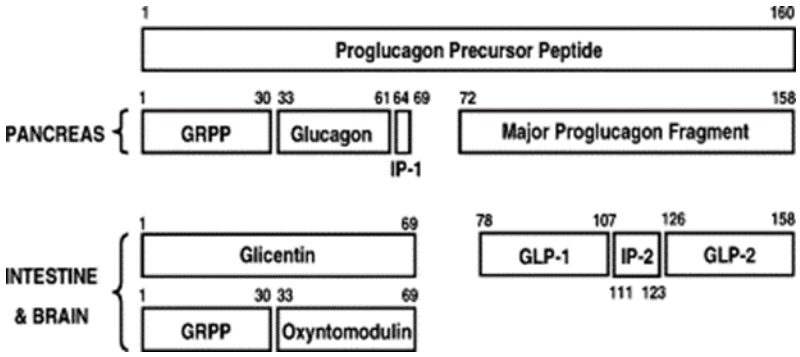


**Figure 1.1. Proglucagon peptide and post-translational products**

**Tissue-dependent processing of the proglucagon precursor peptide and subsequent post-translational processing of the major proglucagon fragment into GLP-1, GLP-2, IP-2, oxyntomodulin, and glicentin.[41]** ***GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; IP-2, intervening peptide 2.***

*1.4 Glucagon-like peptide-2*

GLP-2 is a 33-amino acid peptide hormone that is post-translationally cleaved by PC1/3 from the C-terminal portion of proglucagon.[37-38] The N-terminus of GLP-2 is essential for signal transduction, and the C-terminus is important for receptor binding.[42-43] Eighteen different GLP-2

sequences have been confirmed in vertebrates.[44] Among mammals with a known peptide sequence, there is high overall sequence conservation (88-97% homology to humans) and 100% conservation of the N-terminal sequence (**Figure 1.2**).[41] High sequence conservation among mammalian species allows for relative extrapolation of regulation and action among various species.[45-46]


**Figure 1.2. GLP-2 amino acid sequence homology between different species**

**GLP-2 sequence conservation among species. The box denotes the N-terminal sequence, demonstrating 100% conservation among mammals with a known sequence. Underlined peptides denote variation from the human sequence. Percentages represent percent homology to the human sequence.[41]**

Glucagon-like peptide-2 is secreted from L cells in an active form [GLP-2 (1-33)].[42-43] GLP-2 (1-33) is degraded in circulation to inactive GLP-2 (3-33) via the enzyme dipeptidyl peptidase IV (DPP-IV) through cleavage at the N-terminal histidine and alanine. Affinity of GLP-2 (3-33) for the GLP-2 receptor (GLP-2R) is approximately 10-fold lower than GLP-2 (1-33).[42-43,47] At low, physiologic doses, exogenously administered GLP-2 (3-33) acts as a competitive GLP-2 receptor antagonist; however, when administered at supraphysiologic doses, it exhibits partial agonist activity.[47] The role of DPP-IV was demonstrated in a study comparing

incubation of GLP-2 in plasma from DPP-IV deficient rats and wild-type control rats.[43] Following incubation, high performance liquid chromatography revealed both GLP-2 (1-33) and GLP-2 (3-33) in serum from wild-type rats. High performance liquid chromatography on DPP-IV deficient rat serum, demonstrated minimal GLP-2 (3-33) following incubation.[43]

Dipeptidyl peptidase-IV activity has been documented in multiple locations, including free in circulation, capillary endothelial cells, adipocytes, apical portion of enterocytes, proximal renal tubule brush border cells, and on the surface of hepatocytes.[48-50] There is high DPP-IV sequence conservation among species, with 83% homology and identical active site in humans and rats. There is also approximately 92% DPP-IV sequence homology in bovines and rats.[51] However, differences in DPP-IV activity result in variable circulating times of active GLP-2 among species.[49,52-54] Degradation of GLP-2 (1-33) by DPP-IV occurs rapidly, leading to a half-life of 7 minutes in circulation in humans.[47] Dipeptidyl peptidase- IV activity varies between rats and mice, and rats appear to degrade GLP-2 faster than mice.[43]

Routes of GLP-2 elimination have been investigated through tissue-accumulation studies of radioactive-labeled GLP-2 (1-33).[55] Several studies have demonstrated renal elimination.[55-56] While renal excretion is the primary route of elimination, the presence of radioactive-labeled GLP-2 within hepatocytes and bile ducts suggested possible hepatic metabolism and clearance; although, the exact mechanisms and extent of hepatic metabolism have not been demonstrated.[55]

It is generally accepted that GLP-2 is co-secreted from L cells in equimolar amounts to GLP-1.[57] 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 known mechanisms of GLP-1 regulation. Therefore, demonstrated mechanisms and patterns of GLP-1 and GLP-2 secretion are considered interchangeable in this discussion, except where otherwise noted. In

humans, GLP-1 and GLP-2 have biphasic patterns of secretion, with an early peak secondary to indirect neuronal and endocrine stimulation of L cells shortly after meal ingestion.[25,58] Due to direct connectivity between EEC and vagal synapses, GI signals are directly transmitted to the CNS via afferent nerves.[58-59] The second peak occurs due to direct intraluminal nutrient exposure to L cells in the distal SI and colon.[60] There is also some evidence that suggests direct intraluminal nutrient stimulation of the small L cell population in the proximal SI contributes to the first peak.[61] Maximum GLP-2 secretion occurs with the first peak approximately 15-30 minutes following food ingestion in humans and rats and approximately 1 hour in piglets regardless of feeding strategies (e.g., bolus versus continuous infusion).[62-65] The initial peak is characterized by GLP-2 (1-33) concentrations 2-5 times above fasting concentrations in humans and 5-9 times above fasting concentrations in pigs.[60,63] In dogs, circulating GLP-1 appears to peak approximately 15-30 minutes post-meal ingestion;[66] though, GLP-2 secretory patterns have not been specifically evaluated. In humans, the second peak occurs 1-2 hours following meal ingestion.[60] In a human study monitoring circulating GLP-1 concentrations over a 24 hour period, increased post-prandial concentrations only returned to basal levels following an approximate 10.5 hour fast.[65] In addition, peak timing and maximal concentration of GLP-2 secretion are impacted by the meal nutrient profile and caloric intake, which is further described below.

Vagal nerve signaling is the primary neural regulator of GLP secretion.[67-68] This is supported by surgical/functional (capsaicin) vagotomies in rats, which reduced GLP-1 and GLP-2 secretion, respectively.[59,69] Vagal stimulation of L cell secretion is paired with paracrine and endocrine effects.[12,70-71] Corn oil (i.e., fat) infusion directly into the proximal GI lumen of mice still resulted in subsequent GIP, followed by GLP-1 secretion even in mice with distal SI ligation

preventing ileal and colonic L cell nutrient exposure.[24] Vagal nerve involvement in this neuroendocrine loop was confirmed by abolished GLP-1 response following vagal nerve transection.[23] GIP-stimulated GLP-1 secretion likely occurs through either vagal or myenteric gastrin-releasing peptide (GRP) release.[72] This pathway was supported by a study in which IV administration of a GRP antagonist (BW10) to rats under anesthesia abolished proglucagon derived peptide secretion in response to intraduodenal fat. [24,72-73] In dogs, the co-localization of GIP-secreting K cells with L cells in the jejunum may suggest a paracrine mechanism for L cell stimulation, thought this has not been directly demonstrated.[25,28] Muscarinic receptors have been identified on human L cells, which may provide an additional neuronal pathway for GLP-1 and GLP-2 secretion.[67] When bethanechol, a muscarinic receptor agonist, was placed in human-derived L cell cultures, GLP-1 secretion was increased 200% over control cell cultures. Further, when atropine, a muscarinic antagonist, was administered IV to healthy humans there was decreased total GLP-1 secretion, observed as decreased post-prandial peak concentrations; basal GLP-1 secretion was not affected.[74-75]

L cell surface receptors that respond directly to intraluminal contents and mediate secondary GLP peaks include free fatty acid receptors (FFARs), G protein-coupled receptors (GPCRs), sodium-glucose co-transporter (SGLT), sweet taste receptor (STR), calcium sensing receptors (CaSR), and Takeda G protein-coupled receptor 5 (TGR5).[65,76-79] Fat stimulation of L cell secretion is associated with the presence of intraluminal fatty acids and triglycerides.[80] Fatty acid breakdown creates a 2-monoacyl-glycerol moiety, which activates GPCRs.[81] Free fatty acid receptor family 1 and 4 [FFAR1 (GPCR40), FFAR4 (GPCR120)] are key lipid sensing GPCRs on EEC.[82-83] Following binding by medium- to long-chain fatty acids, increases in intracellular calcium result in enteroendocrine hormone secretion.[83-84] Other GPCRs located on K and L cells,

such as GPCR 119, can increase post-prandial CCK, GIP, GLP-1, and PYY secretion via activation of cyclic adenosine monophosphate (cAMP).[85] Fatty acid type also influences the extent of EEC response.[80-81,86-87] In a rodent study, GLP-1 secretion increased from L cells exposed to monounsaturated fatty acid (oleic acid) compared to a saturated fatty acid (palmitic acid).[86] Following IV administration of different fatty acids to healthy humans, GLP-2 concentrations were increased when longer chain fatty acids [dodecanoid acid (C12) versus decanoic acid (C10)] were administered.[87] In healthy humans fed olive oil (unsaturated fatty acid) or butter (saturated fatty acid), unsaturated fatty acids resulted in higher circulating concentrations of GLP-1 and GIP compared to saturated fatty acids.[80]

Carbohydrate stimulation of L cell secretion occurs via activation of sodium-glucose co-transporter-1 (SGLT1) and glucose transporter 2 (GLUT 2).[67,88-90] SGLT1 is expressed on the apical L cell surface in mice, and GLUT 2 is predominantly expressed on the basolateral aspect of the cell surface.[88,90] The role of SGLT1 in L cell responses has been demonstrated in an SGLT1 knockout mouse models showing diminished or abolished GLP-1 secretion in response to intraluminal glucose.[77,88,91-92] L cells located in the duodenum and ileum were stimulated to secrete GLP-1 by intraluminal glucose concentrations >200 mmol/L, with maximum GLP-1 secretion at an intraluminal glucose concentration of 300 mmol/L.[91] Colonic L cells were not stimulated to secrete GLP-1 by intraluminal glucose. SGLT1 activation without concurrent glucose exposure also increased GLP-1 secretion but was less potent than oral glucose.[77,91] Dietary carbohydrate content may regulate SGLT1, as SGLT1 expression and activity increased when a high glucose meal was administered orally to mice,[93] supporting its role in enteroendocrine glycemic response. In piglets, higher carbohydrate contents (i.e., 52.6 or 60.3%) increased SGLT1 expression by approximately two-fold compared to 7 or 35.9% carbohydrate

diets.[94] In addition to direct induction of SGLT1 expression by glucose exposure, indirect mechanisms mediated via cAMP and protein kinase A also increase SGLT1 expression.[95] This was demonstrated whereby infusion of a membrane-impermeable D-glucose analog, di(glucos-6-yl)poly(ethylene glycol) 600, into a sheep intestine led to an increase in SGLT1 expression and increase in intracellular cAMP without impacting sodium/glucose transport into intraluminal brush border cells.[96] Upregulation of SGLT1 by a non-substrate molecule suggests that another intraluminal sugar-sensing mechanism must be present to mediate this response. The role of STRs [taste receptor type 2 (T1R2) / taste receptor type 3 (T1R3)], which are present on rat and piglet EEC, was confirmed via similar upregulation of SGLT1 following intraduodenal infusion of the SGLT1 substrate D-glucose and non-substrate molecules D-fructose or saccharin into the SI of anesthetized rats.[97-98] The extent of SGLT1 upregulation by non-SGLT1 substrates mirrored T1R2/T1R3 expression (i.e., there was minimal increase in SGLT1 expression in SI segments with low T1R2/T1R3 expression).[97-98] Piglet EEC with apical border STRs also contain intracellular alpha-gustducin, which is necessary for receptor function in human and mouse L cells. [78,97] Support for this mechanism of EEC regulation was demonstrated by failed GLP-1 secretion following oral glucose administration in mice lacking the alpha-gustducin gene.[78] Additionally, sucralose, another STR substrate, leads to GLP-1 secretion from mouse and human L cells; secretion is inhibited when T1R3 or alpha-gustducin are blocked.[78,99] Importantly, these are rodent and human models, and it is unknown whether these mechanisms can be extrapolated across species, particularly cats, which lack a functional oral T1R2.[100-102] Additional evidence suggests that glucose does not independently stimulate secretion from canine L cells even with functional STR and SGLT1.[25,102] Addition of glucose alone (range up to 25mM) to L cell culture did not increase GLP-1 secretion above basal concentrations (growth

media, DMEM/bovine serum albumin, 5.5 mm glucose).[25] Addition of GIP to the same L cell cultures, increased GLP-1 secretion in a concentration-dependent manner.[25] Glucose transporter 5 (GLUT 5), a facilitative glucose transporter with a primary role in GI fructose absorption, is also expressed on colonic L cells and may play a role in luminal sugar sensing; though, there is minimal direct evidence that GLUT 5 regulates GLP release.[90]

Proteins, predominantly di- or tripeptides and individual amino acids, stimulate GLP-1 secretion via activation of the transmembrane peptide transporter 1 (Pept1) and CaSR on L cells.[103-104] Following exposure of mouse L cell cultures to glycine-sarcosine residues, as well as other small peptides, Pept1 activation increased intracellular calcium, which subsequently increased GLP-1 secretion; peptide stimulated GLP-1 secretion was decreased approximately 50% in L cell cultures from Pept1 knockout mice.[103] These mechanisms of GLP-1 secretion have not been evaluated *in vivo.* Administration of Calhex, a CaSR antagonist, reduced GLP-1 secretion from mouse L cells in the presence of peptides.[1,89]

Ingested fiber likely stimulates GLP secretion through metabolic degradation product (e.g., short chain fatty acid) interaction with FFAR, which is present on colonic L cells.[105-107] Mice administered intra-colonic propionate had increased circulating GLP-1 and PYY concentrations compared to intra-colonic saline infusion, an effect that was abolished in FFAR2 knockout mice.[105] In rats fed a fiber-free elemental diet, 2.5% pectin supplementation for 15 days caused a two-fold increase in circulating GLP-2 concentrations compared to rats fed the unsupplemented diet.[106] Small intestinal GLP-2R mRNA expression was not upregulated with pectin supplementation despite the increased circulating GLP-2 concentrations.[106]

In mouse L cell and STC-1 cultures, addition of bile acids stimulates GLP-1 and GLP-2 secretion.[108-110] This effect has been mirrored following direct luminal exposure of L cells to bile

in isolated colon models.[110-111] Conjugated primary and secondary bile acids are absorbed by L cells via the apical sodium bile acid transporter (ASBT) and the G-protein-coupled bile acid receptor TGR5 to stimulate secretion of GLP-1 and GLP-2.[109,112-113] Oral administration of a TGR5 agonist (RO5527239) in mice increased tissue concentrations of GLP-1 and GLP-2 in the colon compared to placebo (vehicle) administration.[109] Supplementation of chenodeoxycholic acid, a bile acid, via a gastroduodenal feeding tube in pigs increased circulating GLP-2 by 80% but did not alter GLP-1 secretion.[114] Finally, evidence for direct L cell stimulation by intraluminal bacteria has been demonstrated via lipopolysaccharide (LPS) activation of toll like receptor 4 in mice following IV or intraperitoneal LPS injections.[115]

While mechanisms for individual nutrient-mediated secretion are described above, multiple studies have compared effects of mixed-nutrient meals, effects between different nutrients (e.g., fat versus carbohydrates), and effects of caloric intake, regardless of nutrient composition.[24,60,65,116-117] In healthy dogs fed a mixed-nutrient diet of approximately equal percent gross energy content from each macronutrient, active GLP-1 concentrations were maintained at or greater than fasting concentrations in dogs fed twice daily; however, in dogs fed once daily, GLP-1 concentrations peaked 4 hours after feeding and dropped below fasting 8 hours after feeding.[116] Over the 24-hour study period, cumulative GLP-1 concentrations were greater in dogs fed twice daily versus once daily.[116] In dogs fed a meal standardized based on percent of daily metabolizable energy, carbohydrate-based (maltodextrin) and fat-based (lard) meals resulted in peak post-prandial GLP-1 concentrations 60 minutes following meal ingestion which fat-based meals maintained through 360 minutes.[116] A second post-prandial GLP-1 peak was noted approximately 360 minutes following maltodextrin ingestion.[116] Fasting GLP-1 concentrations in healthy dogs were higher when fed protein-based meals (chicken breast)

compared to carbohydrate-based or fat-based meals, but no post-prandial peak was noted.[116] Evaluation of healthy dogs consuming high-carbohydrate or high-fat diets demonstrated that basal levels of GLP-1 were increased in dogs consuming a high-fat diet; however, both diets resulted in a post-prandial GLP-1 peak at 180 minutes.[117] Both diets also resulted in increased GLP-1 within the first 30 minutes following meal ingestion but only a statistically significant increase with the high-fat diet.[117] In dogs, higher post-prandial GLP-1 concentrations were noted following ingestion of a diet high in a mixture of fermentable fiber sources compared to a low-fermentable fiber diet (wood cellulose).[66] Contradictory to this finding, dogs fed a low-fermentable fiber diet compared to a high-fermentable fiber diet in a different study had no difference in post-prandial GLP-1 concentrations, but voluntary food intake was decreased and SCFA production was increased in dogs consuming the high-fermentable fiber diet.[118] A human study compared the effects of mixed composition, solid diets, fed as meals throughout the day, to a liquid morning meal of individual nutrient compositions on circulating GLP-2 concentrations.[60] The mixed diet caused an approximate 200-300% increase in circulating GLP-2 above premeal concentrations when fed as a >500 kcal meal (i.e., not <400 kcal snacks).[60] The previously described biphasic pattern of GLP-2 secretion was observed following the liquid (400 kcal) fat or carbohydrate meals; a greater increase was observed following the carbohydrate versus fat meal.[60] Post-meal GLP-2 concentrations were no different than premeal concentrations following the protein meal.[60] Interestingly, given the role of GIP in relation to GLP-2 secretion, circulating GIP was also not different following ingestion of a protein-based meal in a different study.[65] GLP-1 levels returned to fasting levels at nighttime. In humans fed calorically standardized meals with different carbohydrate sources, a glucose test meal led to higher 90-minute post-

prandial GLP-1 concentrations compared to rice or barley meals; neither rice nor barley meals significantly increased circulating GLP-1 compared to pre-prandial concentrations.[65]

The major roles of GLP-2 include maintenance of the normal GI mucosal morphology and barrier, as well as GI mucosal healing following disease or injury, with minor influences on GI motility and GI blood flow.[45,119-125] Multiple rodent models have correlated the trophic effects of GLP-2 with both circulating and tissue hormone concentrations.[119,126] Across several mouse studies, effects of subcutaneously (SQ) administered exogenous GLP-2 included increased SI mucosal surface area, increased crypt cell proliferation, increased villus height, and inhibition of enterocyte apoptosis.[1,45,119,127] Effects of GLP-2 on intestinal growth, including increased villus height and crypt depth, were also noted in ruminating calves receiving twice-daily SQ GLP-2 compared to placebo (0.5% bovine serum albumin in saline).[121] Intestinotrophic effects occur through GLP-2R binding on L cells in close proximity to GI epithelial and crypt stem cells, with subsequent signaling via epidermal growth factor (EGF) and insulin-like growth factor (IGF) leading to crypt cell proliferation.[128] Epidermal growth factor and GLP-2 operate in a synergistic manner. Subcutaneous human GLP-2 and long-acting $hGly^2$GLP-2 analog administration induced expression of intestinal epithelial growth factor receptor (ErbB) ligands, including heparin-binding EGF, in mice small and large intestines.[129] Upregulation of ErbB ligands was abolished with SQ pre-treatment of mice with the ErbB inhibitor CI-1033.[129] Intestinotrophic effects of GLP-2 were also abolished with Cl-1033 administration.[129] Insulin-like growth factor-1 is produced by subepithelial myofibroblasts following stimulation by GLP-2.[130] In IGF-1 receptor-knockout mice, intestinotrophic effects of combined $hGly^2$GLP-2 and EGF treatment were reduced but not abolished, suggesting IGF-1 is needed for maximal response.[128] Pretreatment of mouse intestinal subepithelial myofibroblasts in cell culture with

phosphatidylinositol 3 kinase/Akt inhibitors (LY294002, wortmannin) prevented upregulation of IGF mRNA by GLP-2.[130] This suggests that a phosphatidylinositol 3 kinase/Akt pathway facilitates the effects of GLP-2 that are mediated through IGF. Vasoactive intestinal peptide (VIP) also regulates small intestinal growth in conjunction with GLP-2, as demonstrated in an VIP-knockout mice model.[1] Inflammatory polyps formed in VIP-knockout mice that also had reduced proglucagon,[1] suggesting that the lack of GLP-2 contributes to the aberrant polyp growth. Following administration of SQ GLP-2 to VIP-knockout mice, crypt cell proliferation and increased SI weight similar to that in wild-type control mice were noted, suggesting an interaction between GLP-2 and VIP for normalization of intestinal growth.[1] In addition to paracrine and endocrine mediators, intraluminal nutrients are key to maintaining normal GLP-2 secretion and subsequently GI mucosal structure.[131] Total parenteral nutrition (TPN) causes mucosal atrophy that is reversible with exogenous GLP-2 treatment.[131]

Anti-inflammatory effects of GLP-2 are facilitated through neuropeptide activity, specifically VIP, and anti-inflammatory effects are not observed in the absence of either GLP-2 or VIP.[1,132] Subcutaneous GLP-2 administration in rats increased VIP-expressing submucosal and mucosal neurons.[132-133] While in an experimentally induced rat enteritis and colitis model, VIP antagonism blocked the anti-inflammatory effects of GLP-2.[132] However, GLP-2 can still promote GI mucosal growth even with VIP antagonism,[132] indicating independent pathways for anti-inflammatory and intestinotrophic effects.

The role of GLP- 2 in maintenance of the GI barrier has been demonstrated through several molecular and ion transport studies.[122-123] Improved barrier function is documented prior to increased mucosal thickness,[122] suggesting separate mechanisms for GI permeability and intestinotrophic effects. Glucagon-like peptide 2 inhibits endocytic uptake of macromolecules,

thereby regulating molecules that can cross the GI mucosal barrier.[122] Compared to control mice, SQ administration of either native or degradation resistant GLP-2 resulted in decreased sodium CR-EDTA and horseradish peroxidase translocation, demonstrating decreased paracellular and transcellular permeability.[122] Upregulated zonulin-1 and occludin expression is also presumed to contribute to improved barrier function.[123] This has been indirectly demonstrated in mice administered oral prebiotics, which subsequently led to increased GLP-2 in conjunction with increased zonulin and occludin expression.[123] Additionally, these mice had increased jejunal and colonic proglucagon mRNA, which correlated with tight junction mRNA.[123] Twice daily SQ GLP-2 antagonist administration over 4 weeks decreased the observed prebiotic effects, decreasing proglucagon, zonulin, and occludin mRNA.[123] Direct evidence of decreased GI permeability in these mice was suggested through correlation of plasma LPS concentrations and 4000 Da fluorescein isothiocyanate-dextran with plasma GLP-2 concentrations.[123]

GLP-2 impact on GI nutrient absorption has been demonstrated predominantly through healthy models.[134] Both IV and intraperitoneal administration of GLP-2 (1-33) increased GI absorption of leucine, triolein, and galactose in mice and triolein in hamsters, respectively.[134-135] In addition, mice treated SQ with native or degradation resistant GLP-2 had increased disaccharidase expression at the GI brush border, as well as increased glucose transport via GLUT-2 and SGLT-1 through SI basolateral membrane vesicles and brush border membrane vesicles.[135] In healthy humans, an IV infusion of GLP-2 (1-33) increased post-prandial uptake of triglycerides and free fatty acids compared to a placebo (1% human serum albumin in 0.9% NaCl).[136]

Effects of GLP-2 on GI motility and tone have been demonstrated in both animals and humans.[124-125,137] Rat GLP-2 added to an organ bath containing an isolated mouse stomach led to

decreased fundic tone.[124] Combined IV continuous rate infusion (CRI) of GLP-1 and GLP-2 in healthy rats significantly lengthened the migrating motor complex cycle length, resulting in decreased GI motility.[138] However, this effect was not observed when a CRI of only GLP-2 was administered.[138] In healthy anesthetized pigs, a recombinant GLP-2 CRI reduced gastric antral motility in a dose-dependent manner.[125] In healthy mice treated intraperitoneally with a degradation-resistant GLP-2 analog (hGly$^2$GLP-2), gastric emptying rate decreased by 13% compared to placebo (PBS).[137] However, in healthy humans, a synthetic human GLP-2 IV infusion did not affect gastric emptying compared to placebos.[136,139]

GLP-2 increases mesenteric blood flow predominantly through VIP-induced nitric oxide (NO) synthase expression on submucosal enteric neurons.[133,140] An IV infusion of GLP-2 increased portal blood flow within 10 minutes, with peak blood flow velocity observed within 45 minutes.[140] In neonatal pigs given IV native GLP-2 while receiving TPN, GLP-2 resulted in a NO-dependent increase in portal-drained visceral blood flow, demonstrating NO-mediated effects independent of luminal nutrition.[140] A GLP-2 CRI in ruminating calves increased mesenteric blood flow by 175% at initial use (day 0), followed by a 137% reduction after chronic use (day 10) compared to placebo (bovine serum albumin).[121] Subcutaneous and IV administration of native GLP-2 in humans increased mesenteric blood flow comparable to post-prandial induced increases in mesenteric blood flow.[141]

*1.5 GLP-2 receptor*

The GLP-2R is a seven transmembrane, G-protein-coupled receptor related to a subclass of glucagon/GIP receptors.[142] The GLP-2R is expressed in the hypothalamus, gallbladder, and lungs, in addition to EEC, enteric neurons, and subepithelial myofibroblasts.[121,126,143] In rats, GLP-2R mRNA expression is highest in the jejunum, followed by the duodenum, ileum, colon,

and stomach, reflecting where GLP-2 has its strongest effects.[143] In humans, the GLP-2R was identified in the epithelium of the stomach, large intestine, and small intestine. In pigs, GLP-2R mRNA expression was upregulated in the neonatal period compared to fetal pigs.[144] This results in increased circulating GLP-2 in neonatal pigs and may imply a role of GLP-2 in GI maturation.[144]

## Chapter 2: Glucagon-like peptide-2 in gastrointestinal disease

Multiple studies have demonstrated altered circulating GLP-2 concentrations in different GI diseases, with disruptions in secretion patterns dependent on the underlying disease and disease severity.[6,145-146] Decreased GLP-2 secretion may lead to impaired GI mucosal growth and barrier function, along with decreased mesenteric blood flow and reduced nutrient absorption.[147-148] Changes observed in specific diseases are detailed below.

*2.1 Short bowel syndrome/intestinal failure*

In diseases associated with intestinal failure, disrupted GLP-2 secretory responses depend on the anatomic region of the GI tract impacted.[145-148] Differences in GLP-2 concentrations are reflected as altered GI motility, as well as absorptive capacity. In humans with massive ileal resection, GLP-2 concentrations varied depending on whether there was colonic preservation.[147-148] Glucagon-like peptide-2 was increased in short bowel syndrome (SBS) humans with colonic preservation compared to those individuals with a resected colon.[147-148] In addition, post-prandial increases in GLP-2 were not observed when the colon was not preserved, while a minor post-prandial GLP-2 increase was seen with colonic preservation.[147-148] GLP-2 concentrations were inversely correlated with residual SI mass in pediatric patients with intestinal failure but were not different between patients with ileocolic resection versus ileal resection alone.[145] In adult humans with SBS secondary to both primary GI (e.g., Crohn's disease) and non-GI (e.g., resection due to mesenteric infarction) disease but with 60-100% colonic retention (e.g., jejunoileo-colonic anastomosis), both fasting and meal-stimulated GLP-2 concentrations were increased compared to healthy individuals.[148] Those individuals with colonic preservation showed enhanced intestinal adaptation, including mucosal growth, nutrient absorption, and mucosal integrity compared to individuals with colonic resection.[147-148] This was also seen in human pediatric patients with SI

failure, where colonic preservation led to increased post-prandial circulating GLP-2 compared to SI endostomy.[145] Gastric emptying time was shorter in humans with SBS and an end jejunostomy (i.e., lacking jejunal-colonic continuity) but remained similar to healthy individuals in SBS patients with colonic continuity.[149] It is presumed that remaining colonic L cells produce sufficient GLP-2 to maintain the normal colonic break feedback to control gastric emptying.

In regard to GI absorptive function, circulating GLP-2 concentrations correlate with disease severity, location of GI resection, and parenteral nutrition requirements.[145,147-148] In a rat SBS model, ileal-jejunal transposition increased circulating GLP-2 concentrations two-fold and subsequently increased mucosal growth of the transposed ileal segment and the remaining SI compared to sham rats.[150-151] This finding suggested that stimulation of L cells within the transposed segment and subsequent GLP-2 secretion might promote intestinotrophic effects without restoration of normal SI mass.[151] Further, GI tissue GLP-2 concentrations remained unchanged, indicating that there was enhanced GLP-2 secretion from an unchanged L cell mass.[151]

*2.2 Inflammatory intestinal disease*

There have been multiple rodent and human studies evaluating enteroendocrine responses in patients with inflammatory intestinal disease.[152-154] In a mouse T-cell induced inflammatory bowel disease (IBD) model, colonic tissue GLP-2 concentrations were decreased compared to healthy mice and severe combined immunodeficiency mice.[153] Decreased colonic GLP-2 concentrations corresponded to anatomic regions with the most severe histopathologic lesions.[153] Colonic tissue GLP-2 concentrations were reduced in dextran sodium sulfate (DSS) colitis mice compared to healthy controls, which may reflect depletion of cellular storage or cellular loss from mucosal damage.[154] However, human patients with ulcerative colitis (UC) and Crohn's

disease had tissue GLP-2 levels which were unchanged in controlled or active disease state.[152] In juvenile human patients (mean age, 15 years) with active Crohn's disease, fasting GLP-2 (1-33) concentrations were comparable to healthy individuals, but reduced post-prandial concentrations were observed.[146] With disease remission, post-prandial GLP-2 concentrations and GI hyperpermeability (evaluated with lactulose/mannitol absorption) normalized. In adult humans with either UC or Crohn's disease, fasting total (1-33 & 3-33) circulating GLP-2 concentrations were unchanged compared to healthy individuals, but the ratio of GLP-2 (1-33) to GLP-2 (3-33) was increased 60%.[6] This, in combination with a 3.5-fold decrease in circulating DPP-IV activity in individuals with IBD compared to healthy individuals, suggested a compensatory GLP-2 response to GI disease.[6] In contrast, a different study of humans with UC or Crohn's disease found no difference in circulating (fasted or post-prandial) or tissue GLP-2 (1-33) concentrations compared to healthy individuals; this lack of difference was noted in individuals with active disease and disease remission.[152] However, another study in human UC patients demonstrated decreased serum GLP-2 concentrations compared to healthy individuals; it was not specified whether fasted or post-prandial GLP-2 measurements were performed.[155] Decreased GI mucosal microbiota diversity and abundances were observed in UC patients versus healthy controls; lower serum GLP-2 concentrations correlated with lower microbiota diversity and abundance indices.[155]

# Chapter 3: Treatment with exogenous glucagon-like peptide-2, glucagon-like peptide-2 analogs, glucagon-like peptide-2 receptor agonists, & proteinase inhibitors

*3.1 Short-acting (native structure) GLP-2 therapy*

In multiple species, exogenous short-acting GLP-2 administration can provide temporary, but similar, benefits to endogenous GLP-2, including intestinal proliferation, reduced inflammation, and improved nutrient uptake.[131,156-157] Short bowel syndrome rats co-treated with TPN and a GLP-2 CRI experienced greater crypt cell proliferation and reduced enterocyte apoptosis compared to a control group receiving only TPN.[157] In a rat SBS model, daily human sequence-based GLP-2 administration IV increased jejunal mucosal cellularity, villus height, and crypt depth compared to rats receiving only TPN.[158] GLP-2 supplementation also increased jejunal mucosal sucrase and alkaline phosphatase activity compared to rats receiving only TPN.[158] Greater increases in jejunal villus height and cellularity, crypt cellularity, and mucosal sucrase activity were observed with longer duration of combined partial parenteral nutrition, enteral nutrition, and GLP-2 treatment. In rats with SI resection, rats receiving a CRI of human sequence-based GLP-2 for 7 days had increased adaptive intestinal growth (i.e., increased SI and colonic mucosal mass and protein content) compared to rats receiving a placebo (0.9% phosphate buffered saline).[159] Notably, GLP-2 administration did not decrease plasma active GLP-2 concentrations or ileal proglucagon mRNA expression compared to placebo-treated rats;[159] these findings suggested that exogenous GLP-2 augmented intestinal growth without blunting the natural enteroendocrine response. However, sustained exogenous GLP-2 administration is required to maintain enhanced GI proliferation.[160] Mucosal growth, defined by increased SI mucosal mass and DNA content, was reversed when GLP-2 supplementation was discontinued

in an ileojejunal resection SBS rat model.[160] These parameters were no different in rats following GLP-2 discontinuation than SBS rats receiving only TPN that never received GLP-2.[160] In neonatal pigs treated with human sequence-based GLP-2 IV, there was increased epithelial cell proliferation, increased SI and colonic mass, increased crypt cell depth, and reversal of TPN-associated gut atrophy compared to neonatal pigs treated with IV 0.1% porcine serum albumin in 0.9% buffered saline IV.[144] In humans with colonic resection resulting in SBS and reduced L cell mass, SQ GLP-2 administration enhanced energy absorption, measured by the difference between dietary intake and stoma output, increased lean body mass, and promoted modest weight gain; microscopic disease (e.g., increased crypt depth and villus height) also improved with treatment compared to pre-treatment histopathology.[156] In addition, increased urine creatinine and resolution of edema supported a true increase in lean body mass versus weight gain due to fluid retention.[156]

In 2,4,6-trinitrobenzene sulfonic acid and DSS induced mouse colitis models, there was increased crypt cell proliferation and decreased enterocyte apoptosis following SQ human sequence-based GLP-2 treatment compared to saline placebo.[132] There was also downregulation of mucosal inflammatory cytokines, such as interleukin (IL)-1β, interferon-gamma (IFN-γ), and tumor necrosis factor- alpha (TNF-α), improved histopathologic inflammatory scores, and increased myeloperoxidase activity in mice receiving GLP-2.[132] In IL-10 knockout mouse colitis models, twice daily SQ human recombinant GLP-2 (1-33) injections induced crypt cell proliferation similar to the degree of proliferation seen in wild-type mice administered a saline placebo, suggesting an alternative GLP-2 anti-inflammatory pathway to IL-10 mediated effects.[161] Further, as IL-1β, IFN-γ, and TNF-α decreased, colonic inflammation and proportion of activated CD4 lymphocytes also decreased.[161] In a rat HLA-B27 IBD model, an IV GLP-2

CRI reduced intestinal inflammatory infiltrate by 40% and intestinal gene expression of TNF- α and IFN- γ by 100% compared to untreated HLA-B27 mice.[162] In a mouse DSS-colitis model, SQ h[Gly$^2$]GLP-2 administration promoted GI growth, despite decreased colonic GLP-2 tissue concentrations and unchanged proglucagon mRNA compared to healthy mice.[154]

*3.2 Treatment with GLP-2 analogs*

Due to the short circulating half-life of active GLP-2, various analogs have been designed to increase duration of action in the body and thereby, prolong therapeutic potential.[18] Modifications to the endogenous GLP-2 structure are made to decrease degradation by DPP-IV. Teduglutide is produced by replacing alanine with glycine in position 2, resulting in a half-life of 3-4 hours in humans.[43,163] Other GLP-2 analogs, such as glepaglutide and apraglutide, have half-lives of 50 hours and 72 hours, respectively, following SQ administration in humans.[164-167] Lipophilic amino acids are substituted at positions 11 and 16 to create apraglutide.[164] Glepaglutide has 9 amino acid substitutions to native GLP-2 and a C-terminal tail with 6 lysines, which allows stability in water.[168] Intestinotrophic effects seen with GLP-2 analogs are dose-dependent and independent of changes in food intake.[169]

A GLP-2 analog (h[Gly$^2$]GLP-2) given SQ twice daily to DSS colitis mice downregulated circulating and mucosal inflammatory cytokines (e.g., TNF-α, IFN-γ, IL-1, IL-2) and decreased histopathologic evidence of mucosal damage compared to a placebo-treated (phosphate buffered saline) disease control group.[154] There was also an increase in SI and colonic crypt depth and increased SI villus height in the GLP-2 treated group despite decreased colonic GLP-2 tissue concentration.[154] In an aminosalicylate mouse UC model, treatment with h[Gly$^2$]GLP-2 SQ in combination with conventional therapy, such as methylcellulose and sulfasalazine, showed improved survival (88%) compared to conventional treatment alone (75%)

or GLP-2 analog therapy alone (71%).[170] In that study, corticosteroids alone had a survival benefit of 50% but negated the beneficial effects of GLP-2, leading to a 38% survival rate in groups treated with both corticosteroids and h[Gly$^2$]GLP-2.[170]

In a rat SBS model (mid-jejunoileal resection), twice daily SQ teduglutide administration for 3 weeks increased villus height and crypt depth within the residual SI compared to placebo (vehicle) treatment.[171] In SBS rats (mid-jejunoileal resection) treated SQ with a degradation-resistant GLP-2 analog similar to teduglutide, recombinant growth hormone (GH), recombinant keratinocyte growth factor (KGF), or saline, GLP-2 analog treatment resulted in the greatest increase in jejunal villus height and total mucosal height.[172] However, KGF and GH resulted in greater colonic crypt depth than GLP-2 analog supplementation. In a piglet SBS model (total ileal resection), SQ administration of apraglutide on days 0 and 4 following intestinal resection increased SI weight and villus height compared to saline treated controls, indicating that apraglutide may provide a benefit for intestinal adaptation.[173] In neonatal SBS piglets (jejunocolic anastomosis) treated with teduglutide (SQ twice daily), apraglutide (SQ twice weekly), or placebo (saline), treatment with apraglutide induced villus hyperplasia, which was not sustained following discontinuation of therapy.[174] Intestinal growth (i.e., small intestinal length) was noted with teduglutide and apraglutide, which was maintained following discontinuation of therapy in the case of teduglutide and further increased with apraglutide.[174] Teduglutide was first approved for human use in patients with intestinal failures secondary to SBS, with an overall 93% rate in achieving 20-100% reduction in parenteral fluid and nutritional support.[169,175] Specific effects of teduglutide treatment included increased SI villus height and crypt depth, as well as enhanced fluid and macronutrient absorption in SBS patients.[169] These findings were supported by an increase in intestinal wet weight absorption, reduction in diarrhea,

and increased urine production.[169] In adults with varying degrees of intestinal resection or inflammatory etiologies, there was a reduction in parenteral fluid requirements and improved fluid balance (e.g., decreased oral intake, increased urine output) over a 24-week period particularly in patients with higher initial parenteral support requirements following administration of teduglutide SQ.[176] In a 24-week study, 54% of SBS human patients supplemented with teduglutide SQ gained an additional parenteral nutrition free day per week compared to 23% in the placebo-treated group; all patients were continued on pre-existing diet or medical therapy for SBS.[177] In pediatric human patients with intestinal failure secondary to SBS, parenteral nutrition requirements were decreased by 20% within 24 weeks of SQ teduglutide therapy daily.[178] Twelve of seventeen patients were weaned off parenteral nutrition, including three within 3 months of initiating teduglutide therapy, an additional four patients within 6 months, and an additional five patients within 12 months.[178] All 12 had failed previous attempts to reduce parenteral nutrition requirements.[178] Due to reductions in parenteral nutrition requirements, quality of life in individuals with SBS significantly improves.[179] In humans with malabsorptive diseases, such as Crohn's disease, but without intestinal failure, individuals exhibited improvement in body condition score (BCS), improvement in biochemical parameters such as albumin, and improvement in microscopic lesions, such as abscesses after initiation of teduglutide SQ.[180] Further, teduglutide is effective in inducing remission in humans with Crohn's disease.[181] Within 8 weeks of initiating teduglutide treatment (0.2 mg/kg/day SQ) in addition to ongoing standard therapy, 40% of patients achieved remission versus 33% of patients receiving a placebo in addition to standard therapy. Continued administration of teduglutide is recommended since discontinuation resulted in a return to baseline of citrulline, a marker of SI enterocyte mass, four weeks following treatment cessation in pediatric patients with SBS.[182]

Apraglutide is a long-acting GLP-2 analog, which is currently undergoing safety and efficacy trials in human SBS patients.[183] Three weeks of apraglutide treatment at 3 mg/kg SQ three times weekly in healthy mice increased colonic length, SI crypt depth, and villus height.[184] While crypt depth and villus height did not increase further after 3 weeks, colonic length continued to increase at 7- and 10-week evaluation points.[184] Eight adult humans with SBS were treated with either 5 mg or 10 mg SQ once weekly for 4 weeks prior to crossover; increased urine volume, suggesting increased intestinal fluid absorption, was observed at both doses.[183] Adverse effects included decreased thirst, stoma complications, and edema formation.[183]

Elsiglutide is another GLP-2 analog, which has been investigated for treatment of chemotherapy-induced diarrhea in rats.[185] Changes in distal ileum and proximal colon myeloperoxidase concentrations were not different between rats treated with daily oral lapatinib chemotherapy alone or those with concurrent SQ elsiglutide treatment.[185] However, reduced ileal histological damage (e.g., villous blunting, brush border disruption, crypt loss, dilation of lymphatics) was noted in elsiglutide-treated rats.[185] Incidence of diarrhea was also lower (31%) in rats treated with elsiglutide in combination with lapatinib than those treated with laptinib alone (88%).[185] Further support for GLP-2 mitigation of chemotherapy-induced GI damage includes prevention of 5-fluorouracil-induced SI mass loss and villus loss when a proprietary GLP-2 analog (Leu17 replaced by Lysine and Lys30 replaced by Arginine) was administered SQ once daily for 5 days prior to chemotherapy in rats.[186]

Glepaglutide is a GLP-2 analog, which has been investigated for the treatment of SBS.[165,167,182] Different SQ dosing regimens were investigated in human SBS patients, and improved intestinal absorption (i.e., reduced fecal output) was observed with 1 mg and 10 mg doses given SQ daily for 3 weeks.[167] The 10 mg dose increased time to 10% gastric emptying by

27 minutes for solid foods and time to 50% gastric emptying for liquids by 40 minutes compared to prior to treatment.[187]

*3.3 GLP-2 Receptor Agonists*

As stimulation of GLP-2R mediates the intestinotrophic effects of GLP-2, several rodent studies have evaluated effects of GLP-2R agonists.[188-190] Subcutaneous administration of h[$Gly^2$]GLP-2 (0.1 mg/kg, twice daily), exendin-4, a GLP-1 receptor agonist (0.1 ug/g twice daily), combination treatment, or placebo (vehicle) demonstrated that combination treatment increased villus height and SI length compared to placebo or either therapy alone in healthy mice.[190] Combination therapy did not result in decreased appetite or weight loss compared to placebo.[190] Dapiglutide, a dual GLP-1 and GLP-2 receptor agonist, was administered to mice following 40% ileocecal resection to evaluate the effects on intestinal growth and motility.[191] Following a 14-day duration of dapiglutide treatment (10 nmol/kg SQ every 24 hours), SI villus height and body weight were increased in mice receiving dapiglutide compared to vehicle treated mice; there was comparable voluntary oral caloric intake between groups.[191] In healthy rats, SQ dapiglutide also caused a dose-dependent prolongation in intestinal transit time compared to placebo (vehicle), but gastric emptying was not affected.[191]

*3.4 Proteinase inhibitors as treatment of intestinal disease*

DPP-IV inhibitors decrease degradation of GLP-2, along with other enteroendocrine hormones, in vivo and in vitro.[190,192] When administered in combination with an elemental diet, oral sitagliptin increased ileal mucosal total GLP-2 concentrations and improved ulcer healing in mice with indomethacin-induced ulcers; these effects were noted in comparison to placebo, as well as sitagliptin administration alone (i.e., lack of elemental diet).[190] Mucosal DPP-IV activity

was also downregulated in the jejunum and ileum following administration of sitagliptin.[190] Colonic mucosal DPP-IV activity was unchanged,[190] which was relatively expected given the higher colonic expression of DPP-VIII and -IX compared to DPP-IV. This has implications for therapeutic utilization of DPP-IV inhibitors based on anatomic localization of GI disease. Adult humans with SBS, who were administered sitagliptin orally twice daily for 8 weeks, had an increase in median AUC post-prandial (0-3 hours post-prandial) circulating endogenous GLP-2 compared to values at study baseline; however, median fecal wet weight and intestinal wet weight absorption were not significantly altered.[192] As GLP-2 receptors have been found on carcinoid cell lines, caution is required when considering supplementation or methods to prolong action of GLP-2 in individuals with neoplastic disease.[193] Enhanced tumor growth and cell migratory activity was noted secondary to DPP-IV inhibitor (P32/98) administration on human colonic carcinoma cell lines (HT29, SW480) in cell culture.[193]

# Chapter 4: Plasma glucagon-like peptide-2 concentrations in dogs with chronic enteropathies

## *4.1 Introduction*

Chronic idiopathic, inflammatory GI disease in dogs is associated with histopathologic changes similar to changes observed in human IBD.[194-195] Abnormalities include villus stunting and inflammatory infiltrates into the lamina propria.[194-196] Ultrastructural changes to the GI brush border, which aids nutrient digestion and absorption, also occur. GLP-2 is a 33-amino-acid peptide hormone secreted by EEC L cells, with a predominant role in maintaining the GI barrier and normal GI structure.[37-38,122] Human IBD is associated with altered fasting and post-prandial circulating GLP-2 concentrations.[6,146,152] While specific changes (e.g. decreased or increased circulating concentrations) are dependent on disease subtype, it is proposed that abnormal enteroendocrine responses are, in part, related to decreased functional L cell mass. Therefore, it is possible that the similar histopathologic abnormalities in dogs could lead to differences in circulating GLP-2 concentrations, as is seen in humans with chronic enteropathies (CE).

## *4.2 Objectives and hypotheses*

The primary objective of this study was to compare fasting and post-prandial plasma GLP-2 concentrations in dogs with uncontrolled CE to healthy dogs using a canine-specific ELISA. The secondary objective was to evaluate short-term changes in fasting and post-prandial plasma GLP-2 concentrations in response to therapy in dogs with CE, through serial GLP-2 measurements and correlation with disease severity scores.

The hypothesis was that both fasting and post-prandial plasma GLP-2 concentrations would be lower in dogs with CE compared to healthy dogs. Further, post-prandial GLP-2

concentrations would increase with response to standard-of-care treatment. All study procedures were approved by the Kansas State University institutional animal care and use committee (Protocol 4479).

*4.3 Material and methods*

*Study Population*: Client-owned, adult dogs with a history of clinical signs of chronic GI disease were enrolled prospectively following informed owner consent. Inclusion criteria were chronic, uncontrolled GI signs (e.g., vomiting, diarrhea, hyporexia, tenesmus, weight loss) of at least one month duration, complete blood count (CBC) and biochemistry profile excluding systemic disease, and abdominal ultrasound performed within two weeks of enrollment by a board-certified radiologist or radiology resident under supervision of a board-certified radiologist. Exclusion criteria were ultrasound abnormalities concerning for infectious or neoplastic disease (e.g. intra-abdominal lymphadenomegaly, GI masses, or loss of GI wall-layering), fecal, cytologic, or histopathologic diagnosis of infectious or neoplastic disease, or concurrent comorbidities on physical examination or baseline lab work (e.g., ≥ International Renal Interest Society stage 2 chronic kidney disease, cardiac disease requiring medical therapy). Use of antibiotics, steroids, or probiotics within the previous 30 days was an additional exclusion criterion. Gastrointestinal biopsies were not required for enrollment in the study.

*Healthy Control Population*: Healthy, adult client-owned dogs were prospectively enrolled following informed owner consent. Dogs were required to be ≥ 1 year of age and have a BCS of 4-6 out of 9, where 5 is considered ideal.[197] Exclusion criteria were medications aside from routine heartworm/flea/tick preventatives within the previous six months, raw food diets or

raw food treats within six months, or any abnormalities on physical examination or routine lab work (CBC, biochemistry profile) at the time of enrollment. Any dogs with a history of GI signs within six months prior to enrollment or use of a prescription diet to control historical GI signs were also excluded.

*Study Design and Sample Collection:* In all dogs, food was withheld 10-15 hours prior to sampling. A pre-prandial, baseline blood sample (3 ml) was obtained via peripheral venipuncture (lateral saphenous or jugular vein). Dogs were then fed a standard commercial diet (Purina CN; Nestle Purina Petcare Company, Saint Louis, Missouri) at 25% resting energy requirement (RER). RER was calculated based on the predictive RER equation 70 x (body weight in kg)$^{0.75}$. Dogs were allowed 15 minutes to eat the offered meal. Subsequent blood draws were performed at 1 hour (3 ml) and 3 hours (3 ml) following meal ingestion for GLP-2 analysis. For all samples, whole blood was collected into chilled EDTA tubes and placed on ice. Proteinase inhibitors at 3% volume each per sample volume [Aprotinin 3-8 trypsin inhibitory units/mg; Sigma-Aldrich, St. Louis, MO; 0.1 mM Diprotin A (ILE-PRO-ILE); Sigma-Aldrich, St. Louis, MO] were immediately mixed with the sample, as previously described to prevent in vitro enzymatic GLP-2 degradation.[6,194-195] Immediately following collection, samples were centrifuged (4ºC; 1,794 X g; 20 minutes), plasma separated using manual transfer pipettes, and plasma aliquots frozen (-80°C) and analyzed in bulk at study completion, with storage time within manufacturer reported stability.

Fecal samples, sampled at the time of natural voiding or collected via digital rectal examination, were obtained for fecal scoring.

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

*Plasma GLP-2 Concentrations*: Plasma GLP-2 concentrations were quantified using a commercially available canine-specific competitive ELISA (Canine GLP-2 ELISA Kit; Kendall Scientific, Lincolnshire, IL). The ELISA was performed according to manufacturer instructions. In brief, samples were allowed to thaw at room temperature for one hour. Fifty microliters of each sample or standard were added to each well. Plates were incubated 3 times at 37$^{o}$C, once following addition of hydrogen peroxide (reagent A; one hour), once following addition of 3,3',5,5'-tetramethylbenzidine (reagent B; 30 minutes), and once following addition of 3,3',5,5'-tetramethylbenzidine substrate (15 minutes). Optical density was read at 450 nm immediately following addition of the stop solution using an ELISA plate reader. All samples were run in duplicate, along with a minimum of 8 blank wells per plate to account for background absorbance. Intra-assay variability was calculated using the duplicate results from all samples on a single plate; results from individual plates were averaged. Additional samples were duplicated between plates for calculation of inter-assay variability. Manufacturer reported detection range is 123.5-10,000 pg/ml, with a sensitivity of 49.1 pg/ml and no cross reaction with other proglucagon-derived peptides.

*Disease Severity Scores*: Disease severity was scored at enrollment and the 30-day recheck in the CE population using previously established disease scoring systems for CE in dogs, the canine inflammatory bowel disease activity index (CIBDAI) and canine chronic

enteropathy activity index (CCECAI), based on a standardized questionnaire.[194,198] This questionnaire was completed for the healthy control dogs, as well, to screen for possible undisclosed GI or systemic disease.

*Fecal Scores*: Fecal scoring was performed using a previously established 1-5 scoring system in all enrolled dogs.[199] A fecal score of 1 represents stool which is dry and crumbles readily, and a score of 5 is a liquid stool with no solid material. Fecal scoring was performed by an individual observer, who was blinded to the dog's disease status (i.e., CE or healthy population, as well as clinical signs if CE population) and evaluation date (Day 0 or Day 30). For this study, a fecal score $\geq 3.5$ was considered diarrhea.

*4.4 Statistical Analysis:*

Statistical analyses were performed using commercial software (GraphPad Prism Version 9.1.4 Software Inc, San Diego, CA). Data were assessed for normality using the Shapiro-Wilk test. Data are presented as median (range) or mean +/- standard deviation for nonnormally distributed data and normally distributed data, respectively. For all analyses, $P < 0.05$ was considered significant.

Population characteristics (e.g., weight, BCS, age) and fecal scores were compared between CE and healthy dogs using an unpaired *t* test (normally distributed data) or Mann-Whitney test (nonnormally distributed data). A paired *t* test (normally distributed data) or Wilcoxon test (nonnormally distributed data) was used to compare population characteristics, fecal scores, and disease severity scores between day 0 and study recheck in CE dogs.

To evaluate changes in GLP-2 concentrations following feeding in CE dogs at baseline, at day 30, and in healthy dogs, a mixed-effects analysis using a compound symmetry covariance matrix and fit using restricted maximum likelihood was performed. In the absence of missing values, the mixed-effects analysis is equivalent to a one-way ANOVA with repeated measures. For statistically significant models, post-hoc pairwise testing was performed using Tukey's multiple comparisons test. Effect of study day (i.e., baseline versus day 30 in CE dogs) or study group (i.e., CE versus healthy dog) on GLP-2 concentration was assessed using a mixed analysis of variance accounting for repeated measures, within-subject effects, and between-subject effects. 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.

Relationships between fecal scores and disease severity scores with fasting plasma GLP-2 concentration were assessed using Spearman's correlation.

*4.5 Results*

*Study Population* 18 CE dogs were enrolled, and 14 dogs completed the study. 16 out of 18 CE dogs had GI histopathology performed at enrollment. Dog signalment, definite diagnosis if obtained, fecal scores, and disease severity scores are listed in **Table 4.1**. Clinical signs at the time of diagnosis included vomiting ($n$ = 14), diarrhea ($n$ = 12), weight loss ($n$ = 12), and dysrexia ($n$ = 11).

| Age (years) | Sex | Breed | Weight (kg) | BCS (1-9) | MCS (0-3) | Duration of clinical signs (months) | Fecal Score | CIBDAI | CCECAI | Predominant histopathology findings |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 | MC | Australian shepherd | 21.4 | 5 | 0 | 4 | 2.5 | 5 | 5 | Plasmacytic enteritis |
| 10 | MC | Golden retriever | 32.2 | 3 | 1 | 4 | 4 | 10 | 13 | Lymphoplasmacytic gastroenteritis |
| 12 | MC | Jack russell terrier | 7.8 | 2 | 3 | 3 | 5 | 13 | 18 | Macrophagic enteritis with lymphangiectasia |
| 3 | FI | Siberian husky | 16.2 | 2 | 2 | 18 | 2.5 | 8 | 9 | n/a |
| 1 | MC | Goldendoodle | 23.7 | 6 | 0 | 3.5 | 5 | 10 | 15 | Lymphoplasmacytic enteritis |
| 3 | MC | Newfoundland | 57.2 | 7 | 0 | 12 | 2.5 | 6 | 6 | Neutrophilic enteritis and lymphoplasmacytic colitis |
| 3 | MC | Hound mix | 25.8 | 4 | 0 | 1.5 | 2.5 | 6 | 6 | Lymphoplasmacytic gastritis and eosinophilic enteritis |
| 5 | FS | Hound mix | 21 | 6 | 0 | 8 | 3.5 | 9 | 14 | Lymphoplasmacytic gastritis and plasmacytic, pyogranulatomatous enteritis with lymphangiectasia |
| 10 | MI | Yorkshire terrier | 3.26 | 3 | 1 | 4 | 4.5 | 6 | 8 | Lymphoplasmacytic gastroenteritis |
| 4 | MC | Boxer | 38.7 | 7 | 0 | 3 | 1.5 | 5 | 5 | Lymphoplasmacytic gastroenteritis |
| 3 | FS | Goldendoodle | 24.9 | 4 | 0 | 1.5 | 5 | 11 | 13 | Lymphoplasmacytic gastritis, eosinophilic enteritis, and lymphoplasmacytic colitis |
| 13 | FS | Miniature dachshund | 5.58 | 5 | 0 | 1 | 3.5 | 8 | 12 | n/a |
| 6 | FS | Brittany spaniel | 20 | 6 | 0 | 12 | 2.5 | 2 | 2 | Lymphoplasmacytic gastroenteritis |
| 7 | FS | Cairn terrier | 8 | 6 | 0 | 1 | 3.5 | 8 | 10 | Lymphoplasmacytic gastritis and lymphoplasmacytic, eosinophilic enteritis with lymphangiectasia |
| 9 | MC | Yorkshire terrier | 4.54 | 4 | 0 | 6 | 3.5 | 6 | 8 | Lymphoplasmacytic gastroenteritis |
| 3 | FS | Brittany spaniel | 16.5 | 5 | 0 | 1 | 2 | 4 | 4 | Lymphoplasmacytic gastroenteritis |
| 4 | FS | French bulldog | 8.47 | 4 | 1 | 3 | 5 | 8 | 9 | Lymphohistiocytic enteritis |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | FS | Maltese | 2.3 | 2 | 3 | 5 | 5 | 11 | 16 | Lymphoplasmacytic enteritis |

**Table 4.1 CE dog demographic data**

**Summary of individual dog demographics and definite diagnoses for 18 client-owned dogs with chronic enteropathies at the time of enrollment in a case-control study.**

***BCS, Body condition score; CIBDAI, Canine inflammatory bowel disease activity index; CCECAI, Canine chronic enteropathy clinical activity index; MCS, Muscle condition score where 0 = Normal MCS, 1 = Mild muscle atrophy, 2 = Moderate muscle atrophy, 3 = Severe muscle atrophy; FI, Female intact; FS, Female spayed; MC, Male castrated, MI, Male intact; NA, Not available***

Recheck evaluation of CE dogs occurred at a median of 31.0 days (range, 25 - 59 days). Three dogs died prior to study re-evaluation ($n$ = 2 dogs euthanized due to reasons other than GI disease; $n$ = 1 dog died at home of unknown cause); one 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), glucocorticoid therapy ($n$ = 8), antithrombotics ($n$ = 4), cyanocobalamin ($n$ = 3), antimicrobials ($n$ = 3), calcium carbonate ($n$ = 2), antiacids ($n$ = 1), chemotherapeutics (chlorambucil; $n$ = 1), folic acid ($n$ = 1), appetite stimulants ($n$ = 1), and bismuth subsalicylate ($n$ = 1). Some dogs received more than one treatment.

Seventeen healthy dogs were enrolled. Dog signalment and fecal scores are listed in **Table 4.2**. There was no significant difference in weight ($P$ = 0.99) or BCS ($P$ = 0.40) between CE dogs at baseline and healthy dogs. Healthy dogs were younger (mean, 3.9 +/-2.3 years) than CE dogs (6.0 +/-3.6 years).

| Age (years) | Sex | Breed | Weight (kg) | BCS (1-9) | MCS (0-3) |
|---|---|---|---|---|---|
| 2 | FS | Shepherd mix | 20.6 | 4 | 0 |
| 6 | FS | Retriever mix | 24.2 | 6 | 0 |
| 4 | MC | Retriever mix | 14.4 | 5 | 0 |
| 1 | FS | Terrier mix | 9 | 5 | 0 |
| 1 | MC | Hound mix | 17.8 | 5 | 0 |
| 3 | MC | Terrier mix | 9.5 | 6 | 0 |
| 4 | MC | Gordon setter | 18.8 | 4 | 0 |
| 7 | FS | Terrier mix | 7.1 | 6 | 0 |
| 1 | FS | Border collie | 20.1 | 5 | 0 |
| 5 | FS | Terrier mix | 10.4 | 5 | 0 |
| 5 | MI | Bassett hound | 23.2 | 5 | 0 |
| 8 | FS | Labrador retriever | 28.2 | 5 | 0 |
| 6 | MC | Dutch shepherd | 23.9 | 5 | 0 |
| 2 | FI | German shepherd | 26.8 | 5 | 0 |
| 1 | FS | Irish setter | 16.4 | 4 | 0 |
| 5 | FS | Siberian husky | 28.1 | 6 | 0 |
| 5 | FS | Labrador retriever | 19.3 | 5 | 0 |

**Table 4.2 Healthy dog demographic data**

**Summary of individual dog demographics for 17 client-owned healthy dogs enrolled in a case-control study.** ***See Table 4.1 for the remainder of the key.***

*Plasma GLP-2 Concentrations*

All CE and healthy dogs consumed the entire offered meal within 15 minutes. Mean intra-assay variability was 14.9%. Inter-assay variability was 19.2%. The standard curves were sigmoidal. $R^2$ values are reported in **Table 4.3**.

| ELISA Plate number | $R^2$ |
|---|---|
| 1 | 0.9908 |
| 2 | 0.9952 |
| 3 | 0.9953 |
| 4 | 0.9909 |
| 5 | 0.9890 |
| 6 | 0.8799 |
| 7 | 0.9881 |
| 8 | 0.9939 |

**Table 4.3 Standard curve $R^2$ values**

In CE dogs on day 0, there was no significant difference between baseline (424 +/-176 pg/ml), one hour (440 +/-169 pg/ml), or three hour (473 +/-155 pg/ml) post-prandial GLP-2 concentrations ($P$ = 0.16; F 2.15). At study follow-up, 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) post-prandial compared to baseline. ($P$ = 0.33, F1.15; **Figure 4.1**) At study recheck, one-hour post-prandial GLP-2 concentrations were higher than at day 0. ($P$ = 0.02, F 6.3). Three-hour post-prandial GLP-2 trended toward higher concentrations at day 30 compared to day 0, but this was not statistically significant in post-hoc analysis. ($P$ = 0.07; **Figure 4.2**).

In healthy dogs, there was no difference between fasting (median, 1028 pg/ml [range, 602 - 1966 pg/ml]), one hour (median 1052 [range, 699 - 3645 pg/ml]), or three hour (median, 1044 [range, 642 - 2665 pg/ml]) post-prandial concentrations ($P = 0.84$). GLP-2 concentrations were higher in healthy dogs compared to CE dogs at all time-points on day 0 ($P < 0.0001$, F 41.2) and day 30 ($P < 0.0001$, F 14.0; **Figure 4.3**).

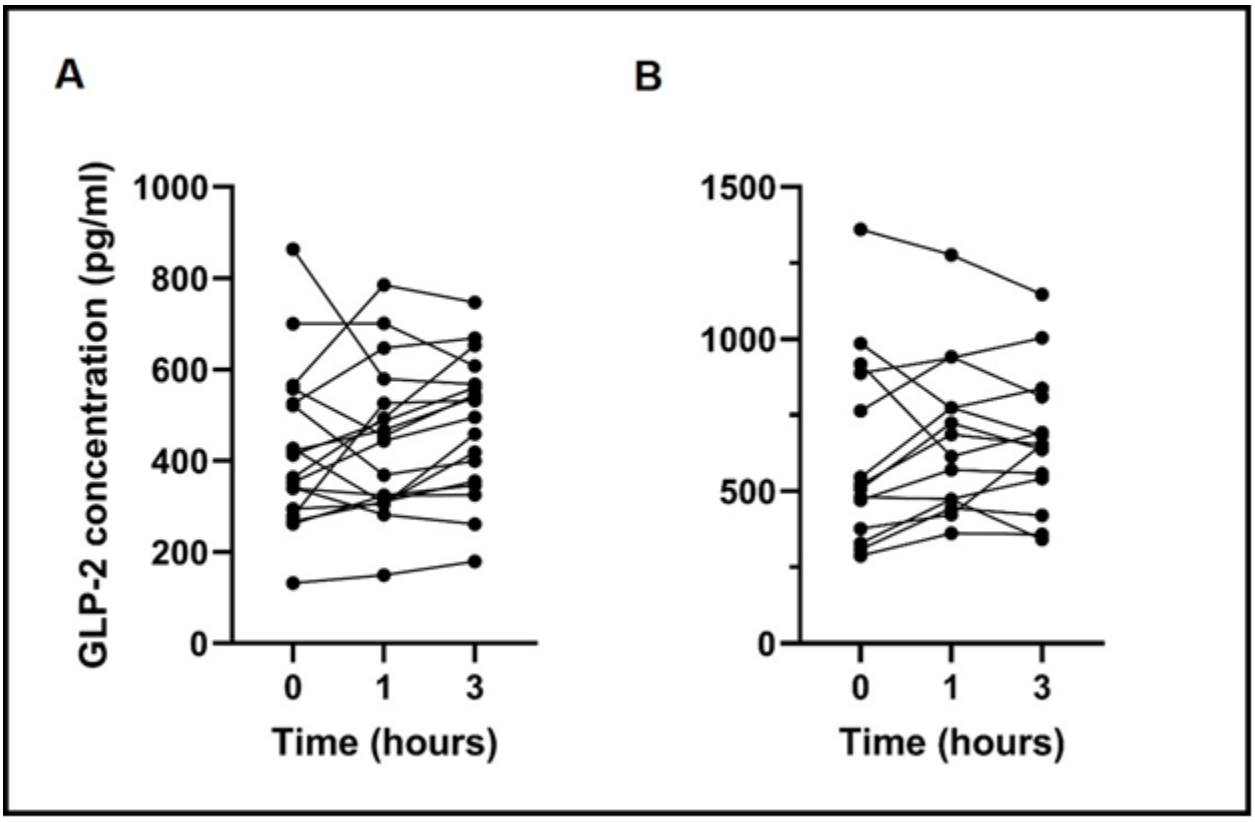


**Figure 4.1. Individual plasma GLP-2 concentrations in CE dogs**

**Individual pre- and post-prandial plasma GLP-2 concentrations in client-owned dogs with CE following ingestion of a standardized meal. Panel A depicts values from 18 dogs at study enrollment and prior to treatment of CE. Panel B depicts values from 14 of the same dogs after 30 days of CE treatment. No significant difference was noted between any time-points at either enrollment or 30 days. Each**

**line denotes an individual dog.** ***GLP-2, glucagon-like peptide-2; CE, chronic enteropathy.***

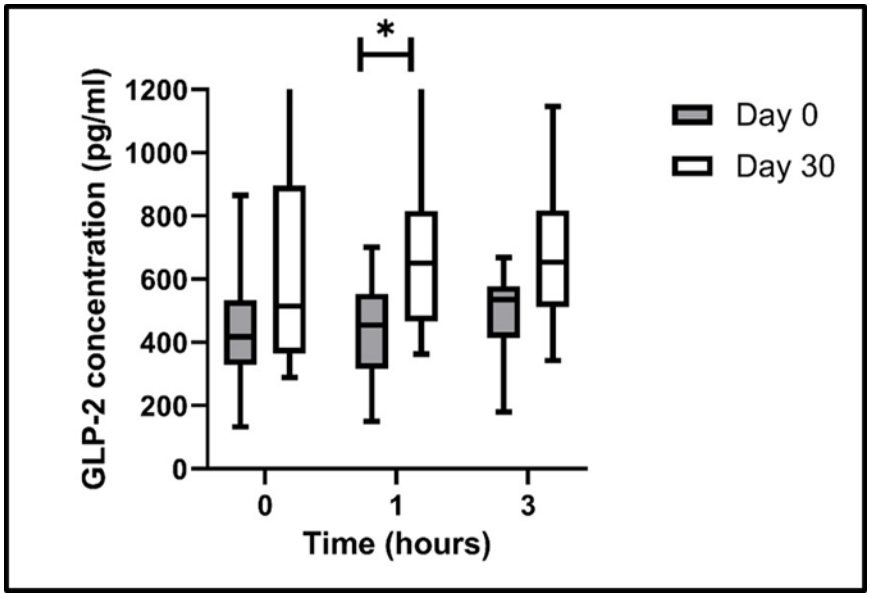


**Figure 4.2. Plasma GLP-2 concentrations in CE dogs**

**Fasted, 1-hour and 3-hour post-prandial plasma GLP-2 concentrations in client-owned dogs with CE at enrollment (n = 18) and after 30 days of gastrointestinal treatment (n = 14). Data presented as median, range. *P <0.05.** ***GLP-2, glucagon-like peptide-2; CE, chronic enteropathy.***

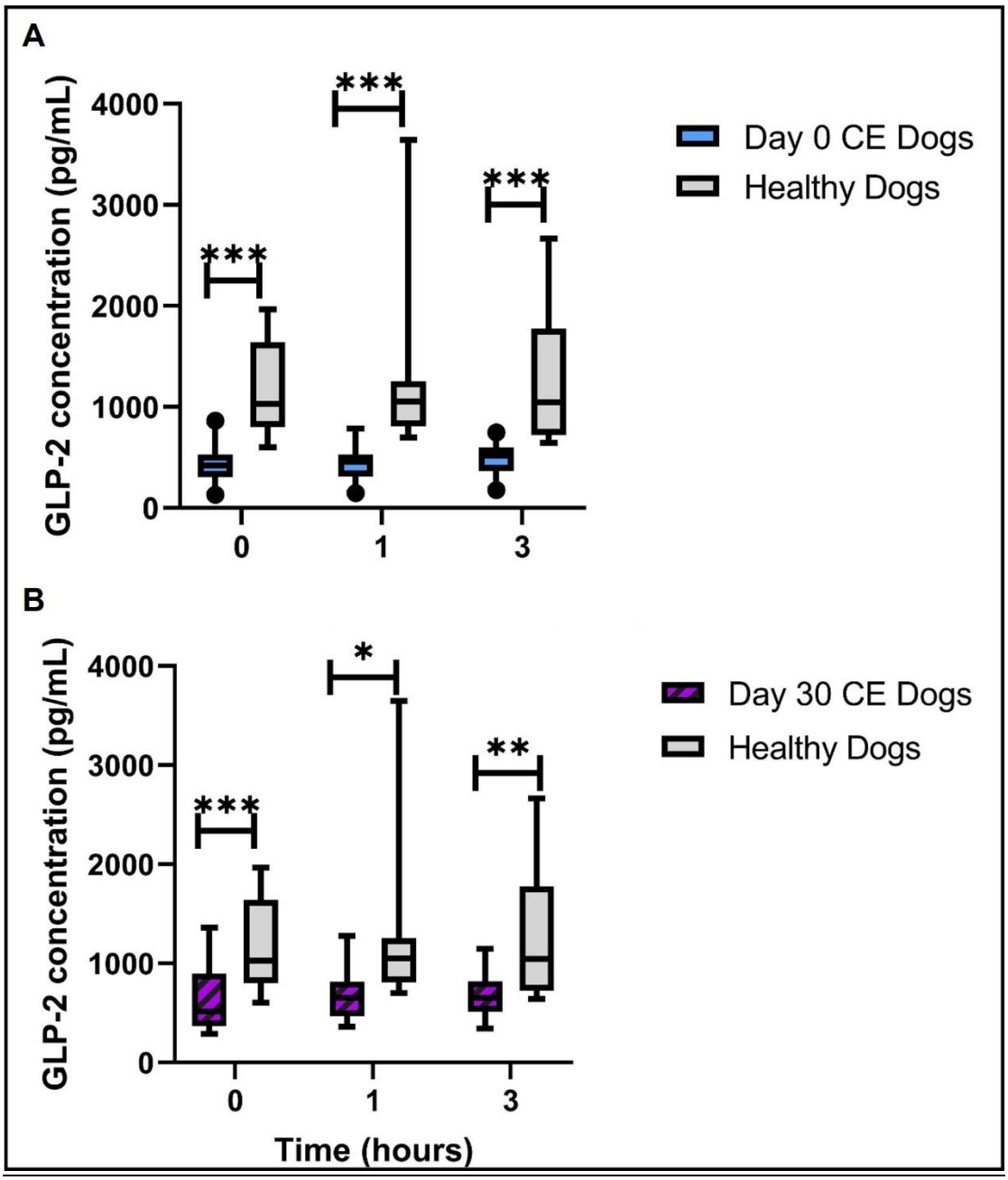


**Figure 4.3. Comparison of plasma GLP-2 concentrations in CE and healthy dogs**

**Fasted, 1-hour and 3-hour post-prandial plasma GLP-2 concentrations in client-owned dogs with CE compared to healthy dogs. Panel A depicts values from 18 dogs at study enrollment and prior to treatment of CE compared to 17 healthy dogs. Panel B depicts values from 14 of the same CE dogs after 30 days treatment compared to the same healthy**

**dogs. *P<0.05, ** P<0.01,*** P< 0.001.** ***GLP-2, glucagon-like peptide-2; CE, chronic enteropathy.***

*Disease Severity Scores*

Median CCECAI scores in CE dogs on day 0 and at study recheck were 9 (range, 2 - 18) and 2.5 (range, 0 - 12), respectively. Median CIBDAI scores in CE dogs on day 0 and at study recheck were 8 (range, 2 - 11) and 2.5 (range, 0 - 11), respectively. Both CCECAI and CIBDAI scores were significantly lower on day 30 than on day 0 ($P$ = 0.003 CCECAI; $P$ = 0.004 CIBDAI). There was no correlation between fasting GLP-2 and CIBDAI or CCECAI scores at either day 0 (CIBDAI $r_s$ = -0.21, $P$ = 0.40; CCECAI $r_s$ = -0.23, $P$ = 0.35) or day 30 (CIBDAI $r_s$ = -0.03, $P$ = 0.92; CCECAI $r_s$ = -0.06, $P$ = 0.85).

*Fecal Scores*

Mean fecal scores in CE dogs on day 0 and at study recheck were 3.6 out of 5 +/- 1.0 [range, 1.5 - 5] and 3.0 +/-0.68 [range, 2 - 4.5], respectively. Mean fecal score in healthy dogs was 2.38 +/-0.38 [range, 2-3]. Fecal scores were lower in healthy dogs than CE dogs on day 0 ($P$ < 0.0001). Fecal scores in CE dogs were lower on day 30 compared to day 0 ($P$ = 0.04). There was no correlation between fecal score and fasting GLP-2 concentration in CE dogs on either study day or healthy dogs.

## *4.6 Discussion*

To the author's knowledge, this is the first study to compare circulating GLP-2 concentrations in dogs with CE to healthy dogs. In this population, plasma GLP-2 concentrations were lower in dogs with uncontrolled CE compared to heathy dogs. In addition, post-prandial GLP-2 concentrations increased following the first month of treatment in dogs with CE.

In humans with IBD, there have been multiple studies demonstrating altered enteroendocrine responses.[6,146,148,152,156,200] In pediatric patients with Crohn's disease, decreased fasted and post-prandial GLP-2 concentrations have been observed,[146] However, there was no clear cut-off in circulating GLP-2 concentrations between the CE dogs and healthy dogs. This could indicate variation in disease severity or GI tract location within the CE population. Some human studies, including both UC and Crohn's disease, have also failed to demonstrate differences in post-prandial GLP-2 responses compared to healthy individuals.[152] Additional, study populations in the human literature include individuals with minimal GI inflammation or states of disease remission,[152]which could contribute to those findings, as enteroendocrine responses normalize with disease response.[200] Human infant circulating GLP-2 correlated with SI mass[200] and colonic preservation in adults,[148] suggesting that disease impacting GI tract regions with high L cell concentrations could lead to higher impact on GLP-2 secretion. We did not assess L cell mass in our CE dogs before or after therapy, but GI region affected could also result in GLP-2 concentration overlap in heathy and CE dogs.

Both fasted and post-meal GLP-2 concentrations remained lower in CE dogs than healthy dogs following 30 days of treatment for GI disease. Although, there was in increase in 1-hour post-prandial GLP-2 concentrations and trend toward an increase in 3-hour post-prandial concentrations in CE dogs at recheck compared to baseline. There are several possible factors that could contribute to lack of complete enteroendocrine response normalization. Normalization of GLP-2 secretion in humans with IBD occurs following remission, and it is possible that complete histopathologic remission was not achieved in our population or duration of remission too short to normalize EEC responses. After 6-8 weeks of treatment for IBD, post-prandial GLP-2 concentrations are similar to those in healthy humans,[146] but the exact timeline for this

improvement is unclear (i.e., unknown whether it occurs sooner than 8 weeks). Thirty-days was chosen as a follow-up point for our study population, as most dogs achieving remission from chronic inflammatory enteropathies will demonstrate improvement within one to two months, with one month being a standard time-point for reevaluation.[198,201,202-203] However, dogs can continue to experience substantial improvement in GI disease status even up to two months or longer following treatment initiation,[202] and human studies with enteroendocrine evaluation often last several months.[204-205] Therefore, it is possible that a longer time to study follow-up would have allowed for greater GLP-2 secretion normalization, particularly as some dogs still had "moderate" to "severe" disease based on severity scoring systems. However, disease severity scores do not correlate with histopathologic lesions in dogs,[194]and GLP-2 concentrations did not correlate with severity scores in this study population. In dogs with inflammatory enteropathies, incomplete resolution of histopathologic changes and GI microbiome dysbiosis is recognized even with clinical response is achieved;[196,201,202,206] therefore, microscopic pathology or continued microbiome or metabolome disturbances may also contribute to incomplete normalization of GLP-2 concentrations. Although repeat GI histopathology would be most ideal, this is not considered standard-of-care in dogs with idiopathic CE.

This was also the first study evaluating post-prandial GLP-2 responses in healthy or CE dogs. Based on GLP-2 secretory patterns in humans and other animal species,[42,60,63] a post-prandial peak was expected. However, this was not observed in either study population, and a number of variables (e.g., meal macronutrient composition, meal volume, sample timing) could have contributed to this finding. A relatively high-fat (7.36 g/100 kcal) diet was chosen, as fat reliably stimulates significant post-prandial GLP-2 secretion in humans.[60] Further, greater post-prandial increases in GLP-1 were noted in dogs receiving a high-fat versus high-carbohydrate

diet.[117] In addition to macronutrient content, caloric intake and meal size may impact post-prandial GLP-2 secretion.[60] Dogs in this study received a standard amount of this diet based on percentage of RER, as GLP secretion appears proportional to intake based on body-weight related energy requirements in other species.[46-48] However, in humans, total caloric amount seems important, as meals less than 400 kcal, do not consistently increase circulating GLP-2 concentrations.[60] Our study design based on RER meant that caloric intake varied based on dog size, and if a minimum calorie amount is required, some dogs may not have reached that threshold. Furthermore, post-prandial sampling times were based on post-prandial GLP-1 peak times in dogs, which are observed between 30 minutes to 3 hours following meal ingestion[116-118] and remain above baseline for up to 8 hours.[116] These times are also consistent if when post-prandial GLP-2 peaks occur in other species, ranging from within 30 minutes to 2.5 hours.[60] Need for nutrient digestion for L cell stimulation by basic components, such as SCFA,[207] the early peaks described above have been noted in mixed nutrient meals.[60] The feeding pattern used in this study may have also impacted our findings if GLP-2 secretion in dogs mirrors GLP-1 secretion. In dogs fed twice daily, a defined post-prandial GLP-1 increase is not observed; rather concentrations remain above basal levels.[6,116,147] Most of our CE population normally received twice daily feeding and received a meal the prior evening, so withholding the morning meal would still generally approximate a twice daily feeding design. Lastly, while the GLP-2 ELISA kit was designed to measure active (1-33) GLP-2, it might not be sensitive enough to differentiate between active and inactive (3-33) GLP-2, effectively measuring total (i.e., combined [1-33] and [3-33]) GLP-2. In some human studies, total GLP-2 concentrations remained unchanged in individuals with IBD even when active GLP-2 concentrations increased.[6] Therefore, measurement of total GLP-2 may prevent detection of a post-prandial peak.

Despite the novel findings of this study, there were some limitations. Histopathology was not required for enrollment, so all dogs did not have a definite diagnosis. However, idiopathic inflammatory CE was confirmed via histopathology in 16 of the 18 CE dogs and both duration of clinical signs and imaging findings made neoplasia or infiltrative infectious disease unlikely in this population. The healthy dogs were screened on the basis of history, physical exam, disease severity scores of "zero," and standard health screening lab work. While it was not possible to exclude subclinical GI disease without histopathology, this was considered unlikely based on the screening performed and lack of any abnormal GI signs withing the previous 6 months. In addition, variation in GLP-2 responses among various subsets of idiopathic CE (e.g., IBD without protein losing enteropathy versus lymphangiectasia) or response to specific treatments (e.g., food responsive enteropathy versus steroid responsive enteropathy) cannot be concluded by this study. However, this would be a goal for future treatment-controlled studies. There was also some variability in follow-up time, with reevaluation occurring up to 59 days in one dog. Longer time to follow-up could allow greater normalization of GLP-2 with more complete treatment response. However, overall GLP-2 concentrations remained lower in the CE population than healthy dogs, suggesting that longer follow-up time could be required.

In conclusion, there is disrupted GLP-2 secretion in dogs with CE versus healthy dogs. Future directions include evaluation of CE subsets and whether GLP-2 normalization could indicate disease response.

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