Effect of dietary nutrient profile on plasma glucagon-like peptide-2 in healthy cats

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Abstract

Glucagon-like peptide-2 (GLP-2) is a meal-induced enteroendocrine hormone responsible for intestinal mucosal growth and repair. GLP-2 secretion in humans and rodents is nutrient-dependent with differential plasma concentrations based on the major nutrient component of a meal. Pre- and post-prandial concentrations of plasma GLP-2 have not been measured in cats.

A feasibility study was performed which evaluated whether GLP-2 could be measured in the plasma of client-owned cats, with the secondary hypothesis that the addition of proteinase inhibitor to the plasma samples immediately after collection would increase measured concentrations of GLP-2. A subsequent nutritional study investigated plasma concentrations of GLP-2 in 9 healthy research cats after a fixed calorie meal high in carbohydrates, protein, or fat, with the hypothesis that maximal GLP-2 plasma concentration would occur 30 minutes after the meal, and a high-fat meal would lead to increased plasma GLP-2 concentrations compared to a high-protein or high-carbohydrate meal.

In the feasibility study, plasma samples were obtained from 6 fasted client-owned cats at baseline and then one hour after a standardized meal. In the nutritional study, 9 healthy research cats were fasted prior to being fed a calorically standardized meal of 3 different commercial diets relatively high in carbohydrates, protein, or fat. Blood samples were collected at baseline, 30, 60, 75, 90, and 120 minutes after finishing the meal. For both studies, two proteinase inhibitors were added to half of the blood sample immediately after collection. Plasma GLP-2 concentrations were measured using a commercial ELISA. Pre- and post-prandial GLP-2 concentrations and the concentrations of GLP-2 with and without proteinase inhibition were compared using a paired t test. The Friedman test and one-way ANOVA of repeated measures were used to evaluate maximal secretion of GLP-2 within each diet, for non-normally distributed data and normally distributed data, respectively. A mixed analysis of variance with repeated measures was used to evaluate effect of diet on GLP-2 concentrations over time.

GLP-2 was detected in all samples. There was no difference between measured GLP-2 concentrations with versus without proteinase inhibitor in either study. Mean GLP-2

concentration 30 minutes after the high-fat meal (1.64 ± 0.23 ng/mL) was significantly higher than at 90 minutes (1.39 ± 0.31 ng/mL; $P = 0.029$) or 120 minutes (1.44 ± 0.27 ng/mL; $P = 0.031$) but not significantly different than baseline (1.49 ± 0.28 ng/mL; $P = 0.085$). There was no difference in pre- or post-prandial GLP-2 concentrations among the high-carbohydrate or high-protein meals.

The results of this study suggest that a meal high in fat provides a stimulus for GLP-2 secretion in cats, while meals high in protein and carbohydrate do not. Proteinase inhibition does not affect GLP-2 measurement using this commercial ELISA.

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GI	Gastrointestinal
GIP	Glucose-dependent insulinotrophic peptide
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
CCK	Cholecystokinin
PYY	Peptide YY
5-HT	5-hydroxytryptamin
IBD	Inflammatory bowel disease
GPCRs	G-protein-coupled receptors
SGLT	Sodium-glucose transporter
cAMP	Cyclic adenosine monophosphate
PKA	Protein kinase A
CaSR	Calcium sensing receptor
FFAR	Free fatty acid receptor
SCFA	Short chain fatty acids
TLRs	Toll-like receptors
PC2	Prohormone convertase-2
PC1/3	Prohormone convertase 1/3
DPP-IV	Dipeptidyl peptidase IV
SBS	Short bowel syndrome
TPN	Total parenteral nutrition
DS	Dextran-sulfate
IL	Interleukin
TNF	Tumor necrosis factor
IFN	Interferon
TGF	Transforming growth factor
NOS	Nitric oxide synthase
Cr-EDTA	Chromium ethylene diamine tetra-acetic acid

HRP	Horseradish peroxidase
IgA	Immunoglobulin A
GLP-2R	Glucagon-like peptide-2 receptor
P13K-Akt	Phosphatidylinositol 3-kinase-protein kinase B
Mek1/2-Erk1/2	Mitogen-activated extracellular signal-regulated kinase
IGF-1	Insulin-like growth factor 1
KGF	Keratinocyte growth factor
EGF	Epidermal growth factor
NG	Nasogastric
BCS	Body condition score
RER	Resting energy requirement
Carb	Carbohydrate

Chapter 1 - Overview of the Enteroendocrine System

1.1 The GI Tract as an Endocrine Organ

The gastrointestinal (GI) tract is an extensive and diverse endocrine organ regulating digestion, absorption, blood nutrient levels, and appetite using hormonal secretions from specialized cells called enteroendocrine cells. It is the largest endocrine organ in the body; although individual enteroendocrine cells only comprise about 1% of all GI cells.[1-3] Enteroendocrine cells are epithelial cells originating from stem cells responsible for differentiating into the entire GI epithelium including Paneth cells, enterocytes, and goblet cells.[4] They are found embedded in the gut mucosa along the entire length of the GI tract.[5] Enteroendocrine cells are thought to be renewed every 3-5 days along with other enterocytes, a unique feature for an endocrine organ.[1]

There are up to fifteen types of enteroendocrine cells that produce over 30 unique peptide hormones and neurotransmitters. A letter system to name the cells was instituted based on the hormone produced or features seen during electron microscopy studies. For example, M cells were so named as they produced motilin and L cells were named for large secretory vesicles.[6] Further examples of enteroendocrine cells and their letter designations include K cells, which produce a hormone called glucose-dependent insulinotropic peptide (GIP), which plays an important role in glucose homeostasis and the incretin effect, X/A like cells which contribute to appetite regulation by secreting the hormone ghrelin, and D cells in the small intestine which produce somatostatin, a hormone regulating overall hormone secretion. L cells are found mainly in the distal small intestine and colon and produce at least two hormones, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2). The classic naming system is still widely used despite documented species' differences and location in the intestine. Even within species, there is growing evidence to suggest an overlap between each cell type and the peptide hormone they secrete, suggesting that cell localization within the GI tract determines hormone secreted more so than the type of cell.[1,6]

Enteric hormones play important roles in controlling a diverse range of physiologic functions such as appetite regulation, mucosal immunity, insulin secretion and inhibition, GI motility, and digestion.[3] The most widely studied enteroendocrine functions revolve around controlling digestion and subsequent nutrient metabolism. For example, the stomach empties at a

rate controlled closely by the hormones cholecystokinin (CCK), GIP, GLP-1, and peptide YY (PYY).[7] Gallbladder contraction and pancreatic enzyme secretion are stimulated by CCK and secretin.[8] GLP-1 and GIP are major players in the incretin response, the amplified release of insulin in response to ingested glucose.[9] Many of these enteric hormones also provide negative feedback to inhibit hunger after ingestion of a meal.[7] The enteroendocrine system mediates paracrine and endocrine effects in non-GI tissue. For example, GLP-1, PPY, and 5-hydroxytryptamin (5-HT) can signal nausea via receptors located in the central nervous system. There is also accumulating information showing a role for enteric hormones in regulating GI inflammation.[5] Dysregulation of enteric hormones have been implicated in the pathogenesis of various chronic and acute GI diseases, including immune-mediated processes such as inflammatory bowel disease (IBD).[9] GLP-2 is a peptide hormone that is intricately tied to mucosal barrier repair, decreasing intestinal inflammation, increasing mesenteric blood flow, and promoting villus growth. It is gaining recognition as a possible therapeutic target in various intestinal diseases, including IBD.[10] GLP-2 is known to have a number of additional effects, many of which overlap with other enteric hormones, such as regulation of GI transit time and nutrient absorption. Understanding hormone function, production, and secretion is imperative to exploring the use of GLP-2 in the treatment of IBD and other GI diseases.

1.2 Enteroendocrine Cell Structure and Regulation

Enteric hormones are primarily responsible for post-prandial function of the GI system and the main role of the enteroendocrine system is to sense and respond to luminal contents. From the time that food enters the stomach, enteric hormones are secreted. There are enteroendocrine cells located in the proximal intestine that respond to the immediate presence of food, but there is also secretion of enteric hormones by ileal and colonic enteroendocrine cells prior to ingesta reaching these intestinal segments. The stimulus and regulation for these hormones is controlled by a complex combination of neurogenic, physical, and chemical stimuli. Vagal stimulation, endocrine and paracrine signaling, and direct luminal sensing all contribute to hormonal regulation.[11] Studies in cells expressing CCK and PYY have revealed the connection between enteroendocrine cells and the nervous system by demonstrating cellular processes that extend from the basal aspect of the enteroendocrine cell to the lamina propria to form a direct synapse-like contact onto neurons associated with the vagus nerve.[12] These cellular processes,

termed neuropods, utilize excitatory neurotransmitter signaling to the central nervous system via glutamate and contain both pre-and post-synaptic proteins implying that enteroendocrine cells both transmit and receive signals, synapsing with both afferent and efferent nerves of the vagus nerve.[13] This allows for rapid cross-talk between the brain and the GI system, forming a gut-brain neural circuit. As shown in a series of experiments by Kaelberer et al in 2018, vagal firing increased when intestines were presented with luminal glucose but did not respond when the glucose transporter SGLT1 was antagonized. When vagal neurons were cultured by themselves, there was no response to D-glucose; however, when co-cultured with enteroendocrine cells, D-glucose elicited excitatory currents in the neurons.[14] These experiments imply that the vagal excitation must be mediated through the epithelial connection. Communication between the nervous system and the enteroendocrine cells is thought to be one mechanism responsible for initial hormonal responses to ingestion of a meal, despite the fact that many of the responding cells are in the distal GI tract.

Another method of enteroendocrine regulation is via paracrine and endocrine effects from the release of other enteric hormones. In mice, GIP released from the proximal intestine stimulates GLP release from the ileum and colon in an endocrine manner.[15] In dogs, GIP is released from K cells found throughout the jejunum, stimulating GLP-1 release in a paracrine manner.[16,17] Somatostatin receptors are found on neighboring L cells less than three cells away, activation of which inhibits the secretion of GLP-1, proving a paracrine regulatory effect of one hormone on another.[18] Mice also have a pathway in which ghrelin, an orexigenic hormone released from the gastric mucosa, enhances GLP-1 release in the face of an oral glucose challenge when injected intraperitoneally compared to a saline injection.[19] These mice also had a blunted GLP-1 response to oral glucose when pretreated with a ghrelin receptor antagonist. Most of these examples are species specific, but they illustrate the multifactorial regulation of enteric hormone secretion.

Arguably, the most important role of the enteroendocrine cell is its ability to sense and respond to an ingested meal, the luminal environment, and the GI microbiome. The structure of the cell is vital in this role of nutrient sensing. There are two types of enteroendocrine cells, “open-type” cells and “closed-type” cells. Open-type cells have a slender, apical process that extends to the surface of the intestinal epithelium, allowing the apical surface to directly contact the lumen of the intestines. Closed-type cells lack luminal contact.[7] The histamine-producing

enterochromaffin-like cells are a classic example of a closed-type cell. Most other enteroendocrine cells, including GLP-2 secreting L cells, are open-type cells, and the luminal contact allows them to respond to the digested food and microbial by-products.

The apical membranes of the open-type cells contain the chemoreceptors responsible for the ability to respond to nutrients in the intestines; often these sensors are membrane-bound G protein-coupled receptors (GPCRs).[20] Foods are digested down to their macronutrient components, glucose, fatty acids, and amino acids or peptides by gastric acid and pancreatic enzymes. These macronutrients are further digested or absorbed via apical receptors or endocytic processes on the surface of enterocytes.[21] A different set of specific chemosensory machinery located on the surface of enteroendocrine cells is also able to detect these nutrients separate from the digestive and absorptive process of enterocytes. These apical transporters and receptors, when activated, trigger activation of calcium dependent cellular pathways that lead to hormone secretion at the basolateral aspect of the cell.[7]

1.3 Mechanisms of Enteroendocrine Nutrient Sensing

Glucose is a known major stimulator of multiple enteroendocrine hormones, especially GLP-1, GLP-2, and GIP, in many species.[11] Enteroendocrine cells, especially those located in the proximal intestine, are thought to sense or "taste" the luminal contents in order to provide the response necessary for hormone release.[5] The sodium-glucose transporter, SGLT1 is expressed on the apical surface of murine L cells.[22] It has been shown that mice lacking the SGLT1 gene have a significantly diminished incretin hormone response.[23] Inhibitors of SGLT1, such as phlorizin, block release of GLP-1, and activation of SGLT1 in the absence of absorbable glucose induces GLP-1 release.[24] This has led to one theory of intestinal nutrient sensing where SGLT1 plays a major role in stimulating the release of incretin hormones. This has not been consistent in all studied species. In canine L cell cultures, glucose did not directly stimulate GLP-1 secretion; instead, GLP-1 secretion was found to be mediated by GIP concentrations, implying that nutrient sensing by L cells themselves may not drive this specific hormonal response.[25] Yet many other species' L cells show a direct correlation between carbohydrate exposure and GLP-1 release, resulting in research aiming to elucidate the mechanisms behind the enteroendocrine cell response to glucose. There is evidence that dietary carbohydrates regulate SGLT1, with high sugar diets inducing increased expression and activity of SGLT1.[26] In an ovine model,

introduction of glucose into the small intestine after bypassing the rumen induced a 50-fold increase in SGLT1 expression.[27] This upregulation also occurred in the face of a membrane-impermeable glucose analogue that was not a substrate of SGLT1, implying the presence of an apical glucose sensor independent of SGLT1.[28] In a series of experiments, this glucose sensor was shown to be a GPCR that upregulated SGLT1 via cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA).[28] Therefore, it was hypothesized that another major player in carbohydrate detection in the intestines is the sweet taste receptor family, T1R1-T1R3. This family of GPCRs was first characterized in the oral cavity, in the epithelium of the tongue, as the primary method of tasting flavors. It has been located in many extraoral tissues and was also localized to the apical border of murine enteroendocrine cells, accompanied by the intracellular machinery necessary for its function, α-gustducin.[29] The functionality of these sweet taste receptors is still under investigation; however, there is supporting evidence for the sweet taste receptors' roles in carbohydrate sensing. For example, transgenic mice lacking the α-gustducin gene fail to secrete GLP-1 in the face of oral glucose.[30] Research has also shown that other substrates for the sweet taste receptors, specifically sucralose, induce GLP-1 release, and T1R3 inhibition blocks GLP-1 release otherwise seen after oral glucose administration.[31]

Dietary proteins are enzymatically digested by gastric and pancreatic secretions into small peptides, which are further broken down into tri- and di-peptides or individual amino acids, any of which may serve as the trigger for enteroendocrine cell secretion.[21] Some proposed mechanisms of enteroendocrine sensing of ingested proteins include a hydrogen-peptide co-transporter, PEPT1, a calcium sensing receptor, CaSR, and various GPCRs. PEPT1 is a dipeptide transporter which, when activated, increases intracellular calcium, contributing to exocytosis of hormones. *In-vitro* knockout models of this transporter demonstrated failed secretions of enteroendocrine hormones in response to dipeptides, providing evidence of its possible protein-sensing role.[32] *In-vivo* studies verifying this role are lacking. The amino acid-responsive CaSRs are important for release of CCK from I cells in response to ingested proteins, and these receptors are also expressed in L cells with a possible role in GLP-1 release.[33] Umami taste receptors are GPCRs made up of the T1R1/T1R3 subunits, similar to the sweet taste receptors. These receptors have been implicated in amino acid-induced GLP-2 secretion.[34]

Fat ingestion is a well-known stimulator of multiple enteric hormones, especially CCK, as well as the incretin hormones, GIP and GLP-1. The free fatty acid receptor family (FFAR) is a

GPCR involved in lipid chemosensing by enteroendocrine cells. FFAR1 and FFAR4 are particularly important receptors on enteroendocrine cells. They are activated by medium to long chain fatty acids, leading to increased intracellular calcium and subsequent hormone release.[32] Nutrient-derived endogenous lipids may also activate another GPCR, GP119, leading to increased post-prandial GIP, GLP-1, CCK, and PYY secretion.[35]

Other luminal contents in addition to digested nutrients provide stimuli for enteroendocrine hormone release. Many of the enteroendocrine cells located in the colon respond to various luminal molecules, notably by-products of bacterial metabolism such as short chain fatty acids (SCFA), indole, luminal fiber, and secondary bile acids. Colonic formation of SCFA leads to activation of FFAR2 on enteroendocrine cells. Bile acid-stimulated GLP-1 secretion appears to rely on various receptors, including GPBAR1 and the bile acid transporter ASBT.[32] Enteroendocrine cells can also be stimulated by lipopolysaccharide or other aspects of the intestinal microbes themselves via toll-like receptors (TLRs) and other receptors that have been identified on some enteroendocrine cells, illustrating how these cells may be major players in intestinal mucosal immunity against bacteria.[36]

Chapter 2 - Physiology of Glucagon-like peptide-2 (GLP-2)

2.1 GLP-2 Structure and Production

GLP-2 is a 33-amino acid peptide hormone secreted by L cells under the regulation of ingested nutrients. Preproglucagon, the prohormone of GLP-2 and related peptides, was first discovered in 1983 as the precursor to glucagon, a well-studied counterregulatory hormone for glucose homeostasis. The prohormone contained the sequence for glucagon, along with two GLPs and other peptide sequences.[37] The production of these peptides from the larger prohormone sequence were found to be tissue specific and processed post-translationally.[38] Preproglucagon mRNA is expressed in only three cell types, enteroendocrine cells, pancreatic alpha cells, and cells of the brainstem and hypothalamus. Preproglucagon mRNA is transcribed from the *Gcg* gene, and then the translational product, the preproglucagon protein, is differentially processed in each tissue type, first to proglucagon and then to the tissue-specific proglucagon-derived peptides.[38] In pancreatic alpha cells, proglucagon is cleaved by an enzyme called prohormone convertase-2 (PC2) to produce the hormone glucagon. In intestinal L cells, the enzyme prohormone convertase 1/3 (PC1/3) cleaves proglucagon into a number of different proglucagon-derived peptides, mainly GLP-1 and GLP-2, as well as other hormones such as oxyntomodulin, PYY, and glicentin.[16] There are minor species differences in distribution of L cells along the GI tract. In humans and rodents, L cells are located mainly in the ileum and colon.[39] L cells in canines were localized throughout the mid-jejunum in higher quantities compared to other species, but the majority were still located in the ileum and colon.[17] In cats, the majority of GLP-2 secreting cells appear to be localized to the ileum and colon.[40]

GLP-1 and GLP-2 are the most thoroughly studied products from the intestinal L cell. GLP-1 and GLP-2 are produced and co-secreted in equimolar amounts from L cells.[37] They are presumed to have similar stimuli for secretion, leading to frequent extrapolation from one to the other about timing of secretion and regulatory pathways; however, increasing evidence of divergence from the classic L cell behavior emphasizes regional differences within the intestine influencing enteroendocrine secretion more so than cell type. In a rat small intestine, L cells in the distal intestine had higher immunoreactivity to the hormone PYY than those in the proximal intestine, showing regional differences in L cell hormone production.[41] In a study evaluating L cell and K cell distribution in cats, GLP-1 positive cells were found in the duodenum

independent from GLP-2 positive cells, challenging the classic L cell definition and providing evidence that intestinal region is more important in classifying enteroendocrine behavior.[40] The mechanism underlying regional differences in GLP expression remains unknown, but a recent study of enteroendocrine mRNA and gene expression in humans showed changes to the expression of PC1/3 along the length of the GI tract, suggesting variation in activity of this enzyme may contribute to variations in L cell activity.[42] A similar example of variable PC expression is also seen in the dog, where PC2 is expressed in intestinal L cells, however the activity is different than in pancreatic alpha cells, as glucagon is not produced.[16]

Once released from L cells, GLP-2 has two predominant mechanisms for elimination from the body. Studies in nephrectomized rats provide evidence for renal excretion of GLP-2, similar to other major enteroendocrine hormones.[43] However, the principal driving mechanism of GLP-2 metabolism is rapid degradation by a proteinase called dipeptidyl peptidase IV (DPP-IV). DPP-IV is a transmembrane protein found in endothelium, epithelial cells, and lymphocytes; although, a form of the enzyme has also been discovered free in plasma.[44] This enzyme degrades proteins at the N-terminus and cleaves GLP-2 between alanine in the second position and histidine in the third position to remove the first two amino acids. This action converts the bioactive GLP-2 (1-33) to the shorter, inactive form of GLP-2 (3-33) effectively preventing signal transduction.[45] GLP-2 (1-33) has a half-life of 7 minutes in humans before enzymatic degradation[46] but there are documented species differences. Specifically, DPP-IV activity in rats is increased compared with mice which decreases the response to injected GLP-2 in rats.[45] GLP-2 (3-33) has a much longer clearance rate than bioactive GLP-2 (1-33), with a half-life of 27 minutes in humans.[47] GLP-2 (3-33) is largely inactive and likely has no major physiologic role, but when administered at supraphysiologic doses has been shown to be a partial agonist of the GLP-2 receptor with the potential to be a competitive antagonist of the receptor at lower doses.[48] The swift degradation of GLP-2 creates a challenge when studying this protein, requiring methods to preserve the intact GLP-2 (1-33) upon acquisition of samples, as well as assays necessary to differentiate between the active and inactive forms. The addition of proteinase inhibitors to plasma samples has aided the recovery of bioactive GLP-2.[49] DPP-IV activity has also been evaluated as a therapeutic target, with the goal that decreased degradation of the GLP products would lead to longer activity in-vivo. There have been two methods of preventing the rapid degradation of GLP-2 in human and animal subjects: administration of DPP-IV inhibitors

and administration of DPP-IV-resistant GLP-2. Pharmaceutical DPP-IV inhibitors are called gliptins and have the potential to prolong the half-life of GLP-2 and other incretin hormones. In fact, they have been extensively studied in the context of type II diabetes mellitus where increases in active GLP-1 concentrations improve disease control. DPP-IV inhibitors improve glycemic control in animal models of diabetes as well as human diabetic patients and this class of medication is already an approved treatment of type II diabetes mellitus.[50] These medications must have high specificity for the type of DPP-IV that degrades GLP-1, GLP-2, and PYY, or risk far-reaching effects and the potential to cause severe adverse events.[51] A GLP-2 analogue that is resistant to DPP-IV degradation was developed by replacing the alanine in the second position with glycine. This compound, Gly^2-GLP-2, has been widely used in research to study the effects of GLP-2 and has been developed into a U.S. Food and Drug Administration- approved medication called teduglutide to treat short bowel syndrome (SBS).[45,52]

2.1 GLP-2 Functions

Bioactive GLP-2 has multiple intestinotrophic and immune modulating activities in the intestines as well as some activity in remote organs, such as the lungs and brain.[10] The predominant roles of GLP-2 in the intestines appear to be intestinal repair, growth, and enhanced absorptive capabilities. Subsequently, GLP-2 is widely studied for its therapeutic implications in intestinal pathology and failure.[3] Glucagon-like peptides were first implicated in intestinal growth when various proglucagon-derived peptide-producing tumors were associated with small intestinal hypertrophy.[53] This association with proglucagon-producing tumors was repeatable throughout the 1970's and 1980's. It was not until 1996 that the specific fraction of the proglucagon-derived peptides called GLP-2 was found to be the proglucagon fragment responsible for this GI growth. Varied concentrations of isolated GLP-2 injected into 8-week-old mice consistently increased the small bowel weight up to 2-fold compared to mice injected with GLP-1.[53] This trophic effect was specific for the intestines, occurred along the length of the small intestine, had a more modest effect on colonic mucosa, and was reversible when GLP-2 treatment was discontinued.[54] GLP-2 has since been implicated as a major driver in the GI adaptive response after bowel resection. In multiple rat models of small bowel resection, endogenous GLP-2 plasma levels increased up to 130% compared to subjects that did not have bowel removed. This increase in GLP-2 was associated with an increase in thickness of all layers

of the intestines, especially the jejunal and ileal epithelium where an increase in luminal surface area was observed.[55,56]

Gastrointestinal growth models utilizing the DPP-IV resistant analogue Gly^2-GLP-2 demonstrated a longer half-life, as well as enhanced biologic activity, when administered subcutaneously compared to native GLP-2.[57] On a microscopic level, GLP-2 increases small intestinal villus height and crypt depth with an increase in epithelial cellularity caused by a combination of decreased enterocyte apoptosis and increased crypt cell proliferation[54]. Electron microscopy revealed that GLP-2 treated enterocytes have a longer and more narrow morphology, with longer microvilli compared to control cells.[58] Ultimately, these adaptations occur concurrently with an increase in functionality of the small intestinal mucosa. GLP-2 treatment increased apical nutrient transport systems in treated enterocytes and increased hexose and glucose absorption in piglets, mice, and rats.[59-61] GLP-2 is also protective against mucosal atrophy in animals deprived of enteral nutrition. When piglets were treated exclusively with total parenteral nutrition (TPN), they developed shortened villi, thin mucosa, and decreased protein expression compared to control piglets treated with enteral nutrition. Concurrent infusion with GLP-2 in the TPN-fed piglets led to maintained villus height, mucosal weight and thickness, and protein concentrations.[62]

GLP-2 administered to mice with induced and spontaneous colitis has helped solidify its anti-inflammatory and healing actions in the intestines. In murine models of dextran-sulfate (DS) induced colitis, GLP-2 administration reduced the severity of weight loss in the mice with colitis and increased the intestinal weight and crypt cell proliferation. Treated mice also had decreased local expression of the cytokine interleukin-1 (IL-1).[63] When indomethacin was used to induce enteritis in mice, treatment with GLP-2 decreased severity of the pathology and improved survival.[64] Similarly, administration of sitagliptin, a DPP-IV inhibitor, also decreases the severity of DS-induced colitis in mice and decreases colonic expression of inflammatory cytokines IL-6 and IL-1β.[65] In a mouse model of spontaneous intestinal inflammation, GLP-2 treatment improved histological lesions, as well as abolished the local expression of the cytokines, tumor necrosis factor (TNF)-α and interferon (IFN)-γ.[66] *In-vivo* rat models of damaged intestinal mucosa show increased healing and absorptive capabilities post ischemic insult when treated with GLP-2.[67] In a mechanically damaged small intestinal cell monolayer, treatment with GLP-2 increased cell migration across the wound edge, mediated by transforming growth factor (TGF)-

β.[68] GLP-2 treatment has been shown to ameliorate ischemia-reperfusion injury in murine small intestines, even increasing the absorptive capacity of the intestines 72 hours after an insult.[67] Intestinal tissue damaged *in-vitro* with hydrochloric acid had faster healing and greater cellular coverage of the superficial injury than non-treated tissue during the three hour experiment.[69]

GLP-2 administration is also associated with increased small intestinal blood flow and increased absorptive capacity of glucose secondary to upregulation of the glucose transporter SGLT1 in the brush border of the intestines.[59,70-72] Based on a study of TPN-fed piglets, GLP-2 administration acutely increased proximal intestinal portal blood flow up to 25% over baseline and this effect was dependent on nitric oxide, specifically mediated through the nitric oxide synthase (NOS) isoform called endothelial NOS.[73] Humans with SBS and healthy human volunteers administered exogenous GLP-2 subcutaneously at a supraphysiologic dose experienced increases in mesenteric arterial blood flow similar to changes seen after ingestion of a meal. This increase in arterial blood flow was independent of systemic blood pressure changes but was accompanied by mild, compensatory increases in cardiac output. The blood flow changes were postulated to be stimulated by the increased metabolic demands of the intestines based on the observation that increased blood flow was correlated with the length of the remaining intestine in patients with SBS.[70,74] GLP-2 has also been shown to regulate intestinal barrier function by affecting intestinal permeability and tight junctions. In one study, jejunal segments of mice treated with GLP-2, both wild-type murine GLP-2 and the DPP-IV resistant Gly^2-GLP-2, were used to assess transcellular and paracellular permeability. The jejunal segments were mounted in Ussing chambers and various experiments revealed reduced paracellular flux of ions and large molecules, for example chromium ethylene diamine tetra-acetic acid (Cr-EDTA).[58] The experiments also demonstrated reduced endocytic transcellular uptake of horseradish peroxidase (HRP), which mimics a luminal protein antigen. The enhanced barrier function occurred quickly, within four hours of a mouse being treated with GLP-2 and was observed prior to the change in enterocyte morphology. These findings supported the role of GLP-2 mediated enhancement of the epithelial barrier independent of its intestinotrophic actions, likely through modulation of intestinal tight junction proteins and inhibition of apoptosis.[58] A later study subsequently revealed that GLP-2 treatment increases expression of the tight junction proteins zonulin-1, occludins, and claudin proteins.[75] Treatment with GLP-2 decreased the loss of these proteins in the face of the damaging cytokine, TNF-α, as well as decreased the uptake of

dextran and increased the resistance across intestinal membranes.[57,75] Further intestinal barrier protection may come from GLP-2 dependent stimulation of Paneth cells to produce antimicrobial substances and increased immunoglobulin A (IgA) secretion.[57] Knockout mice lacking the GLP-2 receptor (GLP-2R) demonstrated reduced gene products of antimicrobial defensins produced by Paneth cells, as well as *in-vivo* small bowel microbiome dysbiosis.[76] In a rat model with a ligated common bile duct, GLP-2 treatment delayed the onset of severe clinical signs associated with obstructive biliary disease and increased the IgA staining.[77]

Further GLP-2 actions include gastric acid suppression, stimulation of glucagon secretion from the pancreas, and neuronally mediated effects on gastric motility and appetite. GLP-2 functions have also been reported in the brain, bone, lung, and liver. Both the human and rat pancreas express GLP-2R on alpha-cells. The perfused rat pancreas secretes glucagon in response to infusion with relatively high concentrations of GLP-2 with no effect on somatostatin or insulin secretion. GLP-2 infusion also counteracted the glucagon-inhibiting actions of GLP-1.[78] In humans treated with a supraphysiologic dose of GLP-2, marked increases in plasma glucagon concentrations were observed, further supporting the counter-effect of GLP-2 and GLP-1 on glucagon secretion. GLP-2 treatment in these human subjects also demonstrated a 15% reduction in gastric acid secretion and chloride secretion after a meal or pentagastrin administration, as well as increased post-prandial serum concentrations of free fatty acids and triglycerides.[79] That study also showed no effect of GLP-2 treatment on human gastric emptying after a solid meal;[79] however, increased intestinal transit times are frequently described in reports of neuroendocrine tumors that overproduce GLP-1, GLP-2, and PYY.[80] GLP-2 effects on gastric emptying remain contradictory and may vary depending on the location of the activated GLP-2R. Animal studies in pigs and mice revealed evidence of delayed gastric emptying with decreased post-prandial gastric contractions and muscle relaxation. Similarly, studies looking at GLP-2R in the brain reveal the importance of GLP-2 on the neurologic regulation of feed intake in mice, intestinal motility, and hepatic gluconeogenesis. GLP-2 receptor activation by direct GLP-2 infusion into the fourth ventricle in the brain decreased gut motility, decreased overall food intake, and decreased hepatic gluconeogenesis.[81] Further contradictory information came from a 2014 study looking at peripheral treatment with teduglutide, a DPP-IV resistant, recombinant human analogue of GLP-2, showing no effect on gastric emptying times after a liquid meal when using acetaminophen absorption as a marker.[82] Extra-GI effects of GLP-2 are also reported.[83,84]

GLP-2 treated mice were shown to have decreased oxidative damage in a model of lung injury induced by TNF-α.[83] GLP-2 treatment has also been associated with increased bone density, leading to studies evaluating its role in bone density in patients with and without diabetes mellitus.[84]

2.2 The GLP-2 Receptor

The widespread research on GLP-2 function is accompanied by a similar breadth of study on the mechanism of action and location of its receptor, GLP-2R. Remarkably, the GLP-2R has not been localized to effector cells of intestinal growth and proliferation- enterocytes or intestinal stem cells. The GLP-2R is a G-protein coupled receptor that is found on enteric neurons, other enteroendocrine cells, and intestinal subepithelial myofibroblasts. GLP-2 receptors have also been localized to gallbladder, vagus nerve, lung, cervix, and brain stem. As mentioned, these receptors have not been found on non-endocrine enterocytes or intestinal crypt cells, indicating that GLP-2 acts indirectly to carry out its role in intestinal growth. The results of GLP-2R activation appear to be carried out by several, complex intracellular signaling pathways. In many models, GLP-2R increases intracellular cAMP; however, in the absence of increases in cAMP, phosphatidylinositol 3-kinase-protein kinase B (P13K-Akt) and mitogen-activated extracellular signal-regulated kinase (Mek1/2-Erk1/2) pathways have also been implicated in GLP-2 signaling.[10] Overall, the GLP-2R appears highly specific for GLP-2, with no cross reaction with other proglucagon derived peptides. When activated, GLP-2R on intestinal subepithelial myofibroblasts increases expression of intestine-derived insulin-like growth factor-1 (IGF-1) which along with its receptor, IGF-1R, is necessary for the intestinal trophic effects of GLP-2 in mice. IGF-1 signaling in the GI tract activates the β-catenin pathway, promoting translocation of β-catenin into the cell nucleus leading to increased translation of *c-myc*, a factor necessary for crypt cell proliferation. Ultimately, β-catenin signaling is responsible for sustaining cells in an actively proliferating state. In mice lacking expression of IGF-1 or during IGF-1R inhibition, GLP-2 was unable to promote β-catenin translocation and subsequent epithelial proliferation was diminished.[85] However, the epithelial proliferation was not abolished. This observation led to the understanding that GLP-2 was still able to perform some of its proliferative roles without IGF-1 signaling and that the alternative cell survival signaling via P13K-Akt phosphorylation occurred in the absence of IGF-1. Other putative growth factors that might mediate GLP-2 actions in the

GI tract include keratinocyte growth factor (KGF), which was shown to colocalize frequently with the GLP-2R and be involved in colonic enterocyte growth, and epidermal growth factor (EGF), along with other ErbB ligands.[86] Independently of IGF-1, EGF reverses mucosal hypoplasia associated with fasting in *Glp2r* knockout mice. GLP-2 treatment upregulates expression of ErbB ligand receptors, and inhibition of ErbB modified the adaptive mucosal response in re-fed mice.[87]

Chapter 3 - Regulation of GLP-2 Secretion

Meal-induced GLP-2 release is well documented. In animal models as well as in humans, plasma GLP-2 levels rise soon after ingestion of food. Mechanisms for sensing and responding to luminal nutrients is complex and multifactorial, requiring an interaction between GI neural circuitry, enteroendocrine signaling, and direct sensing of the luminal content, as mentioned above. The timing of GLP-2 secretion is similar across species with a few key differences. Studies in humans, rats, and pigs have shown an immediate post-prandial increase in GLP-2, with an early peak around 15 to 30 minutes post-ingestion in rats and humans and within an hour in piglets.[88,89] It has been shown in humans that there is also a second peak in one to two hours making GLP-2 release biphasic.[49] As most species' GLP-2 producing L cells are located in the distal small intestine and colon, the early peak is thought to be stimulated by indirect mechanisms. Interest in the mechanisms underlying the early release of GLP-2 has led to studies examining the connections between enteroendocrine cells and the enteric nervous system as well as endocrine signaling between the proximal and distal intestine. Some evidence suggests the initial stimulus for GLP-2 release is neurologically mediated via the vagus nerve[14] and other evidence suggests it is endocrine mediated via GIP release from the proximal K cells.[90] Bilateral vagotomy in rats and administration of anticholinergics can prevent the immediate increase in plasma GLP-2 concentrations after a meal, while glucose stimulated release of GIP from the proximal intestine is known to induce GLP-1 and -2 secretion in rats and dogs.[17,91] In contrast, some reports point to the smaller population of L cells in the proximal intestine as the major source of the rapid post-prandial rise in GLP-1 and -2.[31,92] The later peak in GLP-2 concentration is thought to be stimulated by direct contact of the luminal nutrients with the L cells of the distal intestine.[93]

GLP-2 secretion after a meal depends on the nutrient content of the food. In healthy human subjects, plasma GLP-2 concentrations are particularly increased by the ingestion of meals high in carbohydrates and fats.[49] One of the earliest reports of this phenomenon looked at 6 healthy adult men who consumed liquid meals of carbohydrates, fats, or protein. The meal of carbohydrates stimulated a spike in active GLP-2 concentrations, and the meal of fat stimulated a spike in total GLP-2 concentrations while the protein meal did not seem to stimulate GLP-2 concentrations. This study also showed that meals less than 400 calories did not stimulate GLP-2

secretion.[49] These findings fit with the known behavior of GLP-1, which is strongly stimulated by ingestion of carbohydrates in humans. Thus, it provided supporting evidence that GLP-1 and GLP-2 are co-secreted by L cells in response to nutrients, especially carbohydrates in humans. As such, much of what we know about GLP-1 secretion and its triggers is extrapolated to GLP-2 in the literature. Other rodent models found nutrient composition was also important in GLP-1 and -2 secretion and that fat and glucose administered into the duodenum of mice and rats were able to stimulate secretion of these hormones.[91]

Further variability in GLP secretion as a response to nutrients is known to be species specific. For example, glucose does not directly stimulate L cell secretion of GLP-1 in a canine culture of intestinal L cells. However, GIP, independent of the concentration of glucose, was able to stimulate GLP-1 in the L cell culture.[24] Bombesin was also able to independently stimulate GLP-1 and somatostatin was able to inhibit the GLP-1 promoting effect of GIP. Immunohistochemistry of canine intestine segments showed GIP-secreting cells located throughout the intestines were spatially close to L cells in the jejunum, unlike other studied animals, suggesting that GIP release could stimulate L cell secretion via a paracrine effect.[39] However, similar to other species, peak GLP-1 plasma concentrations appear to occur between 15- and 30-minutes post-meal in healthy, with increased plasma concentrations in dogs fed diets high in fermentable fiber compared to dogs fed diets low in fermentable fiber.[94] Ruminants have a different nutrient profile for maximal stimulation of GLP-2 release. As glucose is almost completely fermented in the rumen prior to ingesta entering the small intestine, GLP-2 is stimulated by microbial fermentation products such as short chain fatty acids, especially butyrate. In one experiment with sheep, when the rumen was infused with either butyrate by itself or a mixture of all three fatty acids (acetate, propionate, and butyrate), GLP-2 plasma concentrations were much higher than after infusions with saline, propionate, or acetate.[95] In the same study, GLP-1 concentrations were also measured after butyrate administration and were found to be unassociated with GLP-2 concentrations, challenging the traditional thought that GLP-1 and GLP-2 are co-secreted by the same stimuli.[95] Butyrate-supplemented calf starter promotes an increased reticulorumen weight in calves with larger rumen papillae, and butyrate infused into the rumen of mature calves increased plasma GLP-2 concentrations.[96] In calves, GLP-2 was shown to be secreted in a biphasic pattern in response to butyrate infused directly into cannulated rumens, with the first peak within 15 minutes and the second, larger peak 30-60

minutes later. These calves were followed out to 300 minutes post-infusion. Notably, measured GLP-2 was highly variable, unlike the uniformly measured GLP-1 concentrations observed at the same time points.[97] These ruminant studies provide supporting evidence for the nutrient-induced secretion patterns of GLP-2 also seen in humans, pigs, and rodents. They provide further examples of how species differences effect GLP-2 measurements post-prandially.

Feline GLP-2 secretion has yet to be studied. The natural diet of cats is comprised entirely of prey, with all of their nutrients obtained from animal tissues. They are considered obligate carnivores and have evolved to consume a diet that is high in proteins and fats with minimal carbohydrates. Cats have a relatively unique carbohydrate metabolism, showing decreased activity of intestinal amylase and disaccharidases necessary for complex and simple carbohydrate digestion. They also have reduced sodium-dependent glucose uptake throughout the small intestine.[98] Cats also appear to have greatly diminished to absent hepatic glucokinase activity compared to non-carnivores and therefore, must have alternative pathways of intracellular glucose phosphorylation.[99] Via other mechanisms, cats are still observed to absorb and metabolize high levels of dietary carbohydrates. However, cats have a higher protein requirement than omnivores and have a limited ability to adjust enzymatic activity associated with protein oxidation when dietary protein requirements are not met, particularly for the amino acids, methionine, cysteine, taurine, and arginine.[100] A study evaluating incretin hormone activity in cats has shown that glucose is not the main stimulator of GLP-1 or GIP secretion. In this study, GLP-1 and GIP plasma concentrations were measured after a nasogastric (NG) tube bolus of carbohydrates, amino acids, and lipid solution. GLP-1 plasma concentration increased 30 minutes after the oral bolus of carbohydrates, peaked at 75 minutes, and was back to baseline by 120 minutes. GIP did not change during that experiment. After the NG bolus of lipids, GLP-1 and GIP peaked in 15 minutes. GLP-1 remained elevated until 60 minutes after the bolus and GIP remained elevated for 195 minutes. After the amino acid bolus, GLP-1 peaked in 30 minutes and remained elevated until 75 minutes, and GIP peaked at 15 minutes and remained elevated for 150 minutes.[101] There was also an observed difference in strength of stimulation of these hormones as interpreted by their fold increase after the nutrient bolus. GIP was more strongly stimulated by amino acids and lipids administered via the NG tube, and GLP-1 was slightly more strongly stimulated by amino acids.[101] Based on these results, it may be reasonable to hypothesize that GLP-2 could have similar stimuli. If GLP-2 is, in fact, created in equimolar

amounts as GLP-1, and co-secreted as traditionally thought, GLP-2 secretion in cats might be more stimulated by protein and fat content than carbohydrates.

The goals of the following experiments were to explore the ability to measure GLP-2 in feline plasma, define post-prandial GLP-2 secretion patterns in healthy cats, and characterize the change in plasma GLP-2 after meals of varying nutrient compositions.

Chapter 4 - Effect of Dietary Nutrient Profile on GLP-2 Concentrations in Cats

4.1 Introduction

To date, circulating GLP-2 has not been measured in cats, and the exact feline amino acid sequence is unknown. However, GLP-2 amino acid sequences have high homology among vertebrate species (88-97%) and the N terminus sequence is 100% conserved across all mammals reported.[102] A study using immunohistochemistry to detect GLP-2 expression in feline GI cells was successful in using antibodies based on the human GLP-2 sequence.[40] Therefore, using an assay based on the human GLP-2 protein is likely to detect GLP-2 in the plasma of cats, allowing the study of GLP-2 secretion patterns in a feline study population. The following experiments used such an assay to study the plasma concentrations of feline GLP-2, the first step in characterizing this enteroendocrine hormone in cats.

4.2 Objectives and Hypothesis

The following experiments had two objectives. Objective 1 was to determine the feasibility of detecting GLP-2 in the plasma of cats, and this experiment was labeled the feasibility study. A secondary objective of the feasibility study was to evaluate if adding proteinase inhibitors, blocking DPP-IV and trypsin activity, to the plasma samples improved recovery of GLP-2. Objective 2 was to determine the maximal post-prandial plasma concentration of GLP-2 and the time post-meal at which it occurred. This experiment was labeled the nutrition study. A secondary objective of the nutrition study was to determine if meal nutrient composition affected the maximal post-prandial concentration and the timing at which the maximum concentration was observed. Based on relative GLP-2 sequence conservation among species, the hypotheses of the feasibility study were that GLP-2 would be successfully measured in feline plasma via ELISA and that inclusion of proteinase inhibitors in collected samples would result in higher detected concentrations. Based on previous studies measuring GLP-1 and GIP plasma concentrations in cats, the hypotheses of the nutrition study were that maximal plasma GLP-2 would be detected 30 minutes after eating a meal and that measured concentrations would be higher following ingestion of a high-fat diet compared to high-protein or high-carbohydrate

diets. This study was reviewed and approved by the Kansas State University IACUC (4275 and 4385).

4.3 Feasibility Study Materials and Methods

Study Population

For the feasibility study, six client-owned cats were prospectively recruited. Inclusion criteria were a body condition score (BCS) between 4 and 6 on a scale of 9[103] and determination of health based on the results of a physical exam, complete blood count, and serum biochemistry. Exclusion criteria were cats less than one year of age, use of medications other than routine preventatives, and any lifetime history of systemic or GI illness, including any chronic disease controlled with medication or diet.

Study Design and Sample Collection:

Food was withheld 10 to 15 hours prior to sampling. A 1.5 mL pre-prandial blood sample was obtained from venipuncture of the jugular or lateral saphenous vein of each cat. The sample was then split between two chilled tubes containing EDTA and placed on ice. One of the samples was immediately mixed with two proteinase inhibitors, a trypsin inhibitor, aprotinin (3-8 trypsin inhibitory units/mg; Sigma Aldrich, St. Louis, MO), and a DPP-IV inhibitor, diprotin A (0.1 mM; ILE-PRO-ILE) (Sigma Aldrich, St. Louis, MO). They were each added at a 3% volume per sample volume.[3] Immediately following collection, samples were centrifuged at *3,000 rpm* at 4°C for 20 minutes and separated using transfer pipettes. The resulting plasma aliquots were stored at -80°C and analyzed in bulk at study completion, within four months of collection.
The cats were then fed a commercial diet (Purina Beyond Grain Free Wild Salmon Pate, Societé de Produits Nestlé S.A., Vevey, Switzerland) at ¼ resting energy requirement (RER). The nutritional components of the diet included protein at 10.4 g/100 kcal, fat at 7.25 g/100 kcal, and carbohydrates at 0.12 g/100 kcal. RER was calculated based on the predictive resting energy requirement equation 70 x [(body weight in kilograms)$^{0.75}$].

Cats were allowed approximately 10 minutes to eat the offered meal. A 1.5 mL post-prandial blood sample was obtained 1 hour after the meal was consumed and collected, processed, and stored in the same manner as the pre-prandial sample.

Plasma GLP-2 Concentrations:

Determination of plasma GLP-2 concentration was performed by using a commercially available glucagon-like peptide-2 sandwich ELISA (Cat Glucagon Like Peptide 2, MyBioSource, San Diego, CA) marketed for cats. The ELISA was run exactly according to manufacturer instructions. In brief, the samples were allowed to thaw for one hour. 50 µL of sample was used for each well. The plates were incubated twice at 37°, once for 60 minutes following the addition of the HRP-Conjugant Reagent, and then for 15 minutes following addition of the Chromagen solutions. The optical density was read at 450 nm at exactly five minutes using an ELISA plate reader. The ELISA was based on the known human sequence of 1-33 GLP-2 (HADGSFSDEMNTILDNLAARDFINWLIQTKITD) with monoclonal mouse anti-human GLP-2 (1-33) capture antibodies and polyclonal rabbit anti-mouse detection antibodies. The manufacturer reported detection limit of this assay is between 0.96 – 2.14 ng/ml with a sensitivity of 0.1 ng/ml in feline samples. The manufacturer reported no cross-reaction with GLP-1 or glucagon; however, results using this ELISA have not yet been published. All samples were run in duplicate, including pre-prandial with and without proteinase inhibitors and post-prandial samples with and without proteinase inhibitors. Three sample duplicates were repeated on the same plate for intra-assay variability. Three sample duplicates were repeated on a different plate for calculation of inter-assay variability. There were at least four rows of blank wells to account for background absorbance.

4.4 Nutritional Study Materials & Methods

Study Population

To study the effect of dietary nutrient composition on plasma GLP-2 concentrations, 9 healthy adult research cats were prospectively included with similar criteria (normal physical exam, BCS 4-6/9, normal complete blood count, normal serum biochemistry profile). Cats were housed in an AAALAC- accredited facility in compatible groups but housed individually for the 24 hours during which each experiment was performed. Cats are normally fed Purina Lab Diet 5003 (protein: 6.6 g/100 kcal, fat: 5.6 g/100 kcal, carbohydrate: 8.2 g/100 kcal; Société des Produits Nestlé S.A., Vevey, Switzerland) with caloric allotment calculated based on body weight and

feeding label and adjusted for weight gain and loss. The food is normally shared amongst group-housed cats and divided into two daily feedings provided at 8 AM and 4 PM.

Sample Collection

Blood samples were collected via 21-gauge butterfly catheter or tuberculin syringe from the medial saphenous or lateral saphenous vein. Samples were collected, processed, and stored as described above. No more than 6% of each cat's blood volume, based on 55 ml/kg circulating volume, was drawn in a week and each sample was <1 mL.

Food was withheld between 12 and 17 hours prior to obtaining a pre-prandial blood sample. Cats were then offered the assigned study diet instead of their normal morning meal. They were fed a meal containing ¼ RER with a diet that was high in one of three different nutrients: fat, protein, or carbohydrate. Post-prandial blood samples were obtained at 30 minutes, 60 minutes, 75 minutes, 90 minutes, and 120 minutes after feeding. The nine cats were allocated via simple randomization to receive one of the three diets first, with subsequent simple randomization of the second diet. The third diet fed was assigned by default to the diet not yet received. Cats were randomly divided into two sampling day groups to minimize differences in fasting times among cats. Each cat received each diet on a rotating schedule, with only one diet per day, and one rest day in between each sampling day (**Figure 1**). The amount of each offered meal ingested was recorded by estimating the meal volume leftover at the end of the 10-minute meal time to the nearest 25% of the total volume fed.

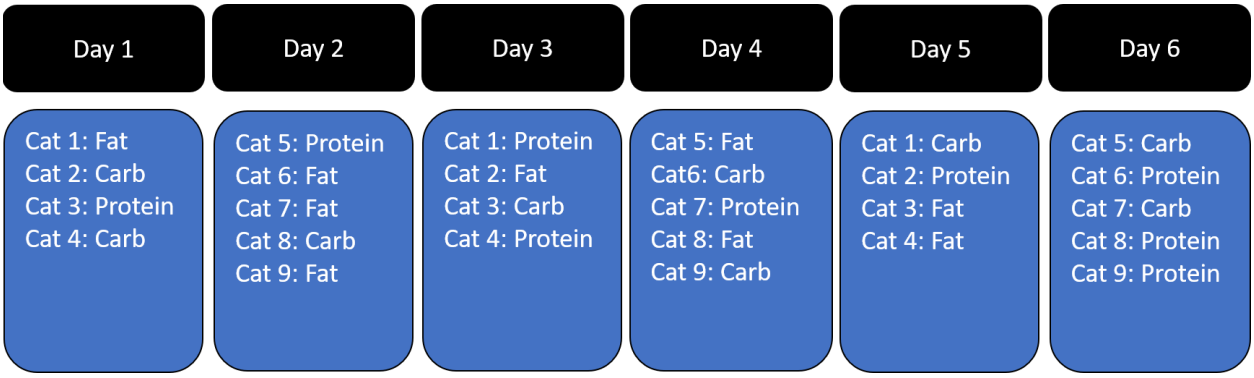

Figure 1. Study timeline

Timeline of the diets offered to each cat during the course of the study. The cats were randomly assigned to receive a high-fat diet, high-carbohydrate diet, or high-protein diet

first, with a different diet randomly assigned second. Sampling days were assigned at random. ***Carb; Carbohydrate***

Diet and Feeding

Diets were chosen to provide a specific nutrient profile providing a high-protein, high-carbohydrate, or high-fat meal (**Table 1**). The enriched nutrient was high relative to the other nutrients in the diet on a g/100 kcal basis. Each diet was a canned diet. The high-protein diet was Purina Pro Plan Tuna Entrée in Sauce (Société des Produits Nestlé S.A., Vevey, Switzerland). The high-carbohydrate diet was Hill's Science Diet Perfect Weight Adult Roasted Vegetable and Chicken Medley (Hill's Pet Nutrition Inc., Topeka, KS). The high-fat diet was Purina Pro Plan Prime Adult 7+ Turkey and Giblet (Société des Produits Nestlé S.A., Vevey, Switzerland).

Diet Composition	High-Protein Meal	High-Carbohydrate Meal	High-Fat meal
Protein (g/100 kcal)	16.7	10.9	8.6
Carb (g/100 kcal)	3.4	9.3	0.9
Fat (g/100 kcal)	3.7	3.4	7.7
kcal per 3 oz	68	62	108

Table 1. Diet composition

Nutrient composition of the diets used for evaluation of post-prandial plasma GLP-2 concentrations in 9 healthy research cats.

Plasma GLP-2 Concentrations

The ELISA was performed exactly as described for the feasibility study. All samples were run in duplicate, including pre-prandial with and without proteinase inhibitors and post-prandial samples with and without proteinase inhibitors. At least two sample duplicates were repeated on the same plate for intra-assay variability. One - three sample duplicates were repeated on alternate plates for calculation of inter-assay variability. There were at least six blank wells on each ELISA plate.

4.5 Statistical Analysis

For both the feasibility study and nutritional study, statistical analysis was performed using the computer software program GraphPad Prism (GraphPad Version 9.1.2 Software Inc, San Diego, CA, USA). 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. Intra-assay variability was calculated by dividing the standard deviation of the duplicates by the mean of the duplicates and averaging this for every sample on the plate. Average inter-assay variability was calculated by dividing the standard deviation of the replicates on other plates by the mean of the replicates and averaging this for every inter-assay variation calculated amongst all plates containing replicates.

Feasibility study

Pre- and post-prandial GLP-2 concentrations were compared using a paired t test. The concentrations of GLP-2 with and without proteinase inhibition were compared using a paired t test.

Nutritional Study

Nine cats were enrolled based on a power calculation for a repeated measures study based on a mixed model for treatment and time (https://glimmpse.samplesizeshop.org/design/STATISTICAL_TESTS), with a goal of power = 0.8, alpha = 0.05, using the standard deviation of 0.47 from the preliminary healthy cat data and estimating changes from baseline at 1.5x (carbohydrate), 1.8x (fat), and 1.9x (protein) based on described post-prandial changes in GLP-1 in cats.[101] Effect of proteinase inhibition on GLP-2 concentrations was compared using a mixed analysis of variance accounting for repeated measures within-subject effects (time, interaction of treatment and time) and between-subject effects (treatment [i.e. proteinase inhibition]) for each diet type (two-way repeated measures ANOVA). Because there was no significant effect of proteinase inhibitor, data from non-proteinase inhibitor samples were used for the remainder of the analysis.

For each diet, the Friedman test (non-normally distributed data) or one-way ANOVA of repeated measures (normally distributed data) was used to evaluate for maximal concentration of GLP-2

For statistically significant models, Dunn's multiple comparisons test or Tukey's multiple comparisons test were used for post-hoc pairwise testing, respectively.

Effect of diet on GLP-2 concentrations was compared using a mixed analysis of variance accounting for repeated measures accounting for within-subject effects (time, interaction of treatment and time) and between-subject effects (treatment [i.e. diet]). For statistically significant models, post-hoc pairwise testing was performed between time-points using Sidak's multiple comparisons test.

4.6 Results

Feasibility Study Population:

Six healthy client-owned cats were enrolled following physical exam and screening bloodwork. The median age was 3.5 years (Range, 1.5 – 7 years), median weight was 4.35 kg (Range, 3.8 - 4.96 kg) and median BCS was 5.5/9 (Range, 4 - 6). There were five spayed female domestic short hair cats and one castrated male Sphinx cat.

GLP-2 Concentrations:

GLP-2 was detected in all samples. Mean intra-assay variability was 2.8%. Inter-assay variability was 7.83%. The standard curves, as second order polynomials, are reported in **Figure 2** showing excellent agreement of the standards with all r^2 values >0.99.

There was no significant difference in GLP-2 concentration between samples with and without proteinase inhibitors. Mean GLP-2 concentration in pre-prandial samples with proteinase inhibitors was 1.92 ± 0.47 ng/mL and without proteinase inhibitor was 1.93 ± 0.48 ng/mL (P = 0.96). For the post-prandial samples, mean concentration of GLP-2 with proteinase inhibitors was 1.97 ± 0.58 ng/mL and without proteinase inhibitors was 1.92 ± 0.55 ng/mL (P = 0.55). There was no difference between mean pre- versus post-prandial GLP-2 concentrations regardless of proteinase inhibition (P = 0.46). (**Figure 3**)

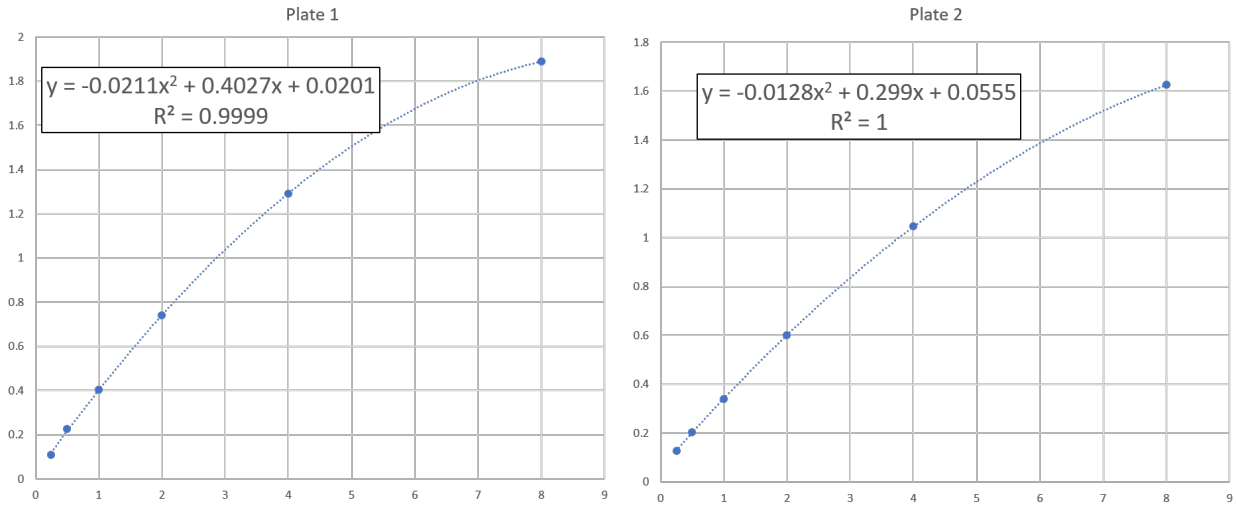

Figure 2. Feasibility study standard curves

Standard curves and r^2 values for the GLP-2 ELISA data used in the feasibility study.

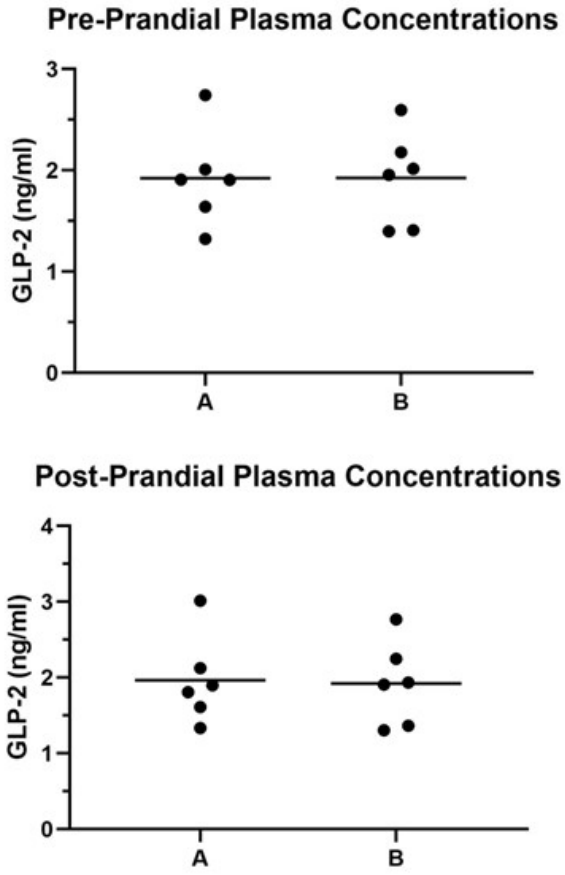

Figure 3. Plasma GLP-2 concentrations in the feasibility study

Individual cat GLP-2 concentrations in pre-prandial (top) and one-hour post-prandial (bottom) plasma samples from six healthy cats. The horizontal line represents the median GLP-2 concentration. On the x-axis, A represents samples collected with proteinase inhibition, and B represents samples collected without proteinase inhibition.

Nutrition Study Population

Nine healthy domestic short hair research cats were enrolled following screening physical examination and blood work. The median age was 1.83 years (Range, 1.67-5.33 years). The median weight was 5.2 kg (Range, 4.4-7.7 kg). The median BCS was 5.5/9 (Range, 5-6.5). There were 8 neutered males and 1 spayed female.

Meal Ingestion

Each cat consumed the entire portion of the offered high-fat meal. Five cats ate all of the offered high-carbohydrate meal, three cats ate at least 75% of the offered meal, and one cat only ate 50%

of the offered meal. For the high-protein meal, five cats ate all of the offered meal, one cat ate 75% of the meal, and three cats ate 50% of the meal. The volume of food consumed by the cats who ate half of the high-carbohydrate and -protein meal was similar to that of the cats who ate all of the high-fat meal (**Table 1**).

GLP-2 Concentration Measurements

GLP-2 was detected in all samples. Standard curve data are reported in **Table 2** as second order polynomials with all r^2 values >0.99. Mean intra-assay variability was 6.1%. Inter-assay variability was 13.2%. There was no difference between GLP-2 concentration measured with proteinase inhibitor versus without proteinase inhibitor for any of the three diet types at any timepoint (Table 3). Median fasting GLP-2 concentration across all cats and over all days was 1.56 ng/mL (Range, 0.66 - 1.98 ng/mL).

ELISA Plate (by cat)	Standard Curve	R^2
1	$y = 0.007x^2 + 0.1551x + 0.0353$	0.9997
2	$y = 0.009x^2 + 0.1189x + 0.04701$	0.999
3	$y = 0.008x^2 + 0.124x + 0.0309$	0.999
4	$y = 0.0074x^2 + 0.1148 + 0.0353$	0.999
5	$y = 0.0064x^2 + 0.07 + 0.0173$	0.998
6	$y = 0.0049x^2 + 0.1007x + 0.0148$	0.999
7	$y = 0.0069x^2 + 0.1311x + 0.0234$	1.000
8	$y = 0.00804x^2 + 0.118x + 0.026$	0.999
9	$y = 0.00798x^2 + 0.1205x + 0.021$	0.999

Table 2. Nutrition study standard curves

Standard curve equations and r^2 values for the GLP-2 ELISA data of each of the nine cats used in the nutrition study. Each ELISA plate represents samples from one cat. R^2 values show good agreement for the standards.

Diet	GLP-2 With Proteinase Inhibitor (ng/mL)	GLP-2 Without Proteinase Inhibitor (ng/mL)	Standard Error of difference	*P*-value
High-Fat	1.55	1.50	0.13	0.68
High-Carbohydrate	1.49	1.47	0.16	0.89
High-Protein	1.50	1.46	0.18	0.85

Table 3. Mean GLP-2 concentrations with and without addition of proteinase inhibitors Across all time-points, there was no difference in measured concentration of GLP-2 between samples with the addition of proteinase inhibitors versus without.

Serum GLP-2 Concentration Over Time

There was no difference between baseline GLP-2 concentrations and any post-prandial time-point after ingestion of a high-carbohydrate or high-protein meal. Following ingestion of the high-fat meal, GLP-2 concentration measured 30 minutes post meal (1.64 ± 0.23 ng/mL) was higher than at 90 minutes (1.39 ± 0.31 ng/mL; $P = 0.029$) or 120 minutes (1.44 ± 0.27 ng/mL; $P = 0.031$) post meal. GLP-2 concentration was numerically higher at 30 minutes post high-fat meal than at baseline (1.49 ng/mL; $P = 0.085$), but this was not statistically significant.

Effect of Diet Composition on GLP-2 Concentration

Serial GLP-2 concentrations at baseline and following ingestion of each diet are shown in **Figures 4-6**. Variability between individual cats after ingesting a high-fat meal is shown in **Figure 7**. There was no difference in GLP-2 concentrations amongst the three diets (P = 0.969).

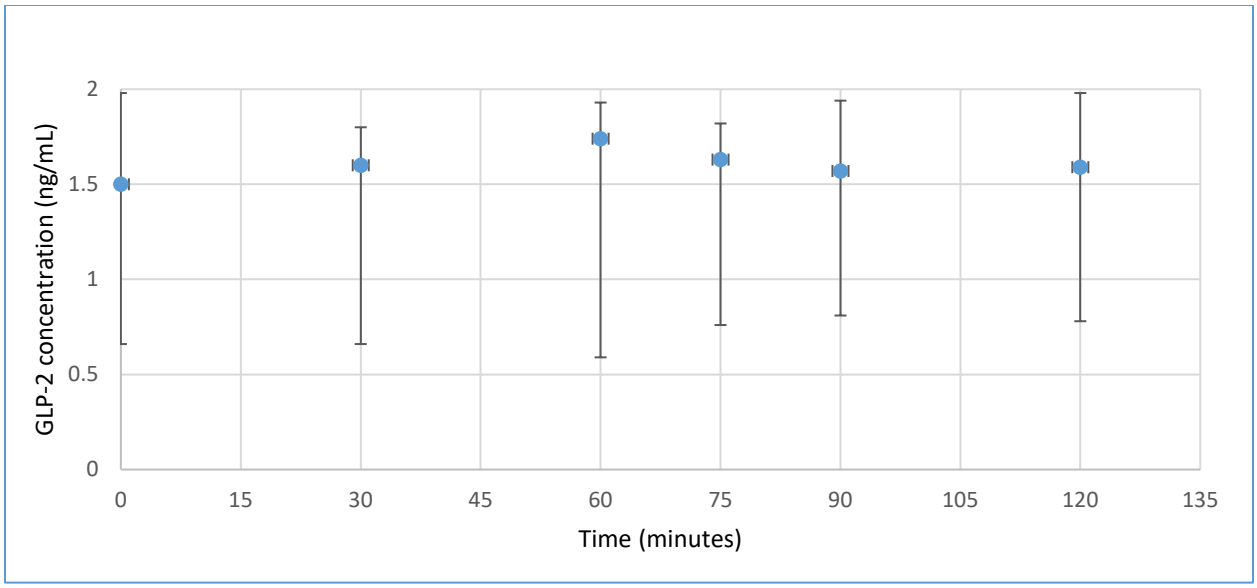

Figure 4. Plasma GLP-2 concentrations after a high-protein meal

Plasma concentration of GLP-2 at baseline and post high-protein meal in 9 healthy research cats. Values are depicted as median ± range. There was no significant change between pre-prandial and post-prandial measurements.

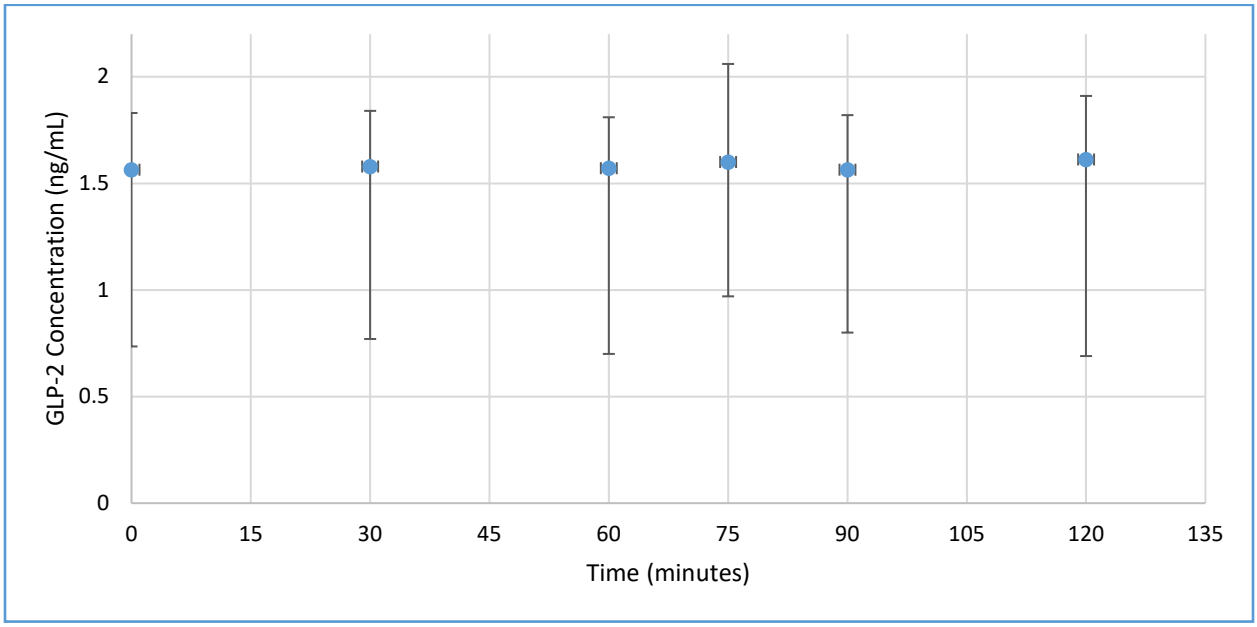

Figure 5. Plasma GLP-2 concentrations after a high-carbohydrate meal

Plasma concentration of GLP-2 at baseline and post high-carbohydrate meal in 9 healthy research cats. Values are depicted as median ± range. There was no significant change between pre-prandial and post-prandial measurements.

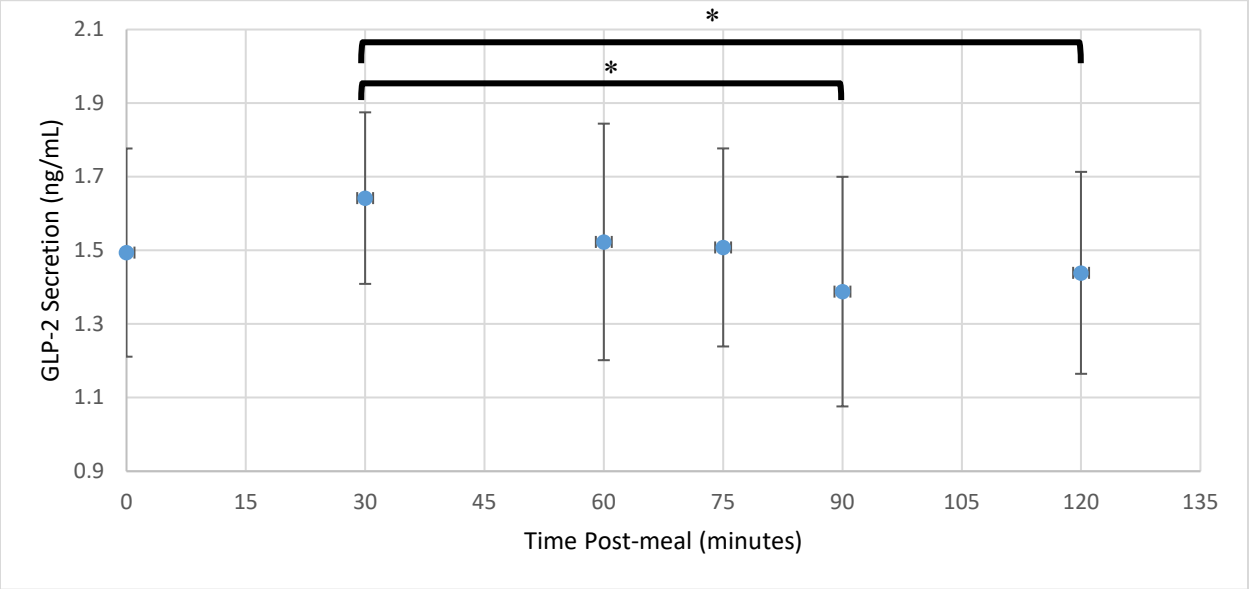

Figure 6. Plasma GLP-2 concentrations after a high-fat meal

Plasma concentrations of GLP-2 at baseline and post high-fat meal in 9 healthy research cats. Data are reported as mean ± standard deviation. * denotes $P < 0.05$.

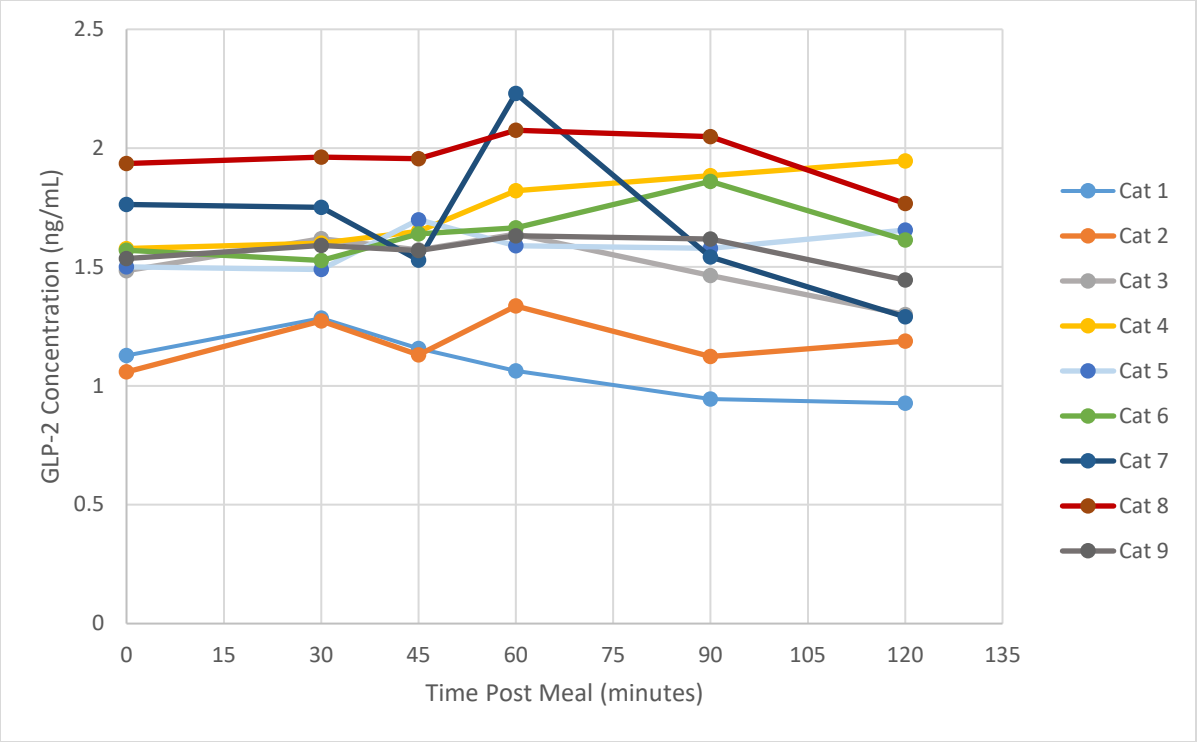

Figure 7. Individual plasma GLP-2 concentrations

Baseline and post meal pattern of plasma GLP-2 concentration following ingestion of a high-fat meal in 9 healthy research cats.

Chapter 5 - Discussion of Study Results

To the author's knowledge, this is the first study to report fasting plasma GLP-2 concentrations in cats and the GLP-2 response to nutrient intake. GLP-2 was successfully detected in all samples, demonstrating feasibility of GLP-2 measurements in feline plasma via ELISA. Results after meal ingestion show that plasma GLP-2 concentration is increased following a meal with a high-fat content, but not by meals with a high-protein or -carbohydrate content.

Only a few studies have characterized enteroendocrine activity in cats and previous research has evaluated incretin hormones, including GLP-1. GLP-1 secretion may be a marker for GLP-2 release due to the reported equimolar production of the molecules and co-secretion from the same enteroendocrine cell.[36] Therefore, when discussing the regulation of GLP-2 secretion, it is necessary to incorporate previous findings from studies focused on GLP-1 secretion. In fact, previous results looking at GLP-1 release in cats helped shape the hypothesis for this study, specifically the timing of maximal secretion and the response to a high-fat meal. Earlier studies showed that cats administered glucose via NG tube had only a 1.3-fold increase in plasma GLP-1 and no increase in plasma GIP.[101] Lipids, however, are a potent stimulator of GIP in cats. When administered a mixed solution of lipids via NG tube, cats experienced a 20-fold increase in plasma concentrations of GIP within 15 minutes, while GLP-1 only increased 1.8-fold.[101] In the current study, after ingestion of balanced commercial diets enriched in either carbohydrate, protein, or fat content, only the high-fat diet stimulated a detectable change in plasma GLP-2. GLP-2 was higher 30 minutes following ingestion of a meal compared to measurements taken at 90 minutes and 120 minutes. Although GLP-2 plasma concentration at 30 minutes trended higher than baseline, it did not reach statistical significance. It is possible that a significant GLP-2 peak could have occurred 15 minutes after ingestion of the high-fat meal, especially if GIP is an endocrine stimulator for GLP-2 release. However, this study did not obtain a measurement at this time. These findings still support that nutrient-stimulated changes in GLP-2 plasma concentration mirror those of GLP-1 and GIP. It is possible that GIP is an endocrine stimulator of GLP-2, as it appears to be for GLP-1. Further studies exploring the effect of GIP on feline L cells, including concurrent measurement of post-prandial GLP-2 and GIP, would be necessary to answer that question.

GIP is strongly implicated in endocrine stimulation of GLP-1 in other species[15,24]. If carbohydrates are unable to stimulate GIP release in cats, then there may be an absent or stunted stimulatory effect on the proglucagon-derived peptides GLP-1 and GLP-2. The present study found no change in GLP-2 in the face of a high-carbohydrate diet, consistent with this theory. This is consistent with the fact that as obligate carnivores, the natural feline diet is rich in fats and proteins but low in carbohydrates. Thus, it can be argued that carnivores do not require an enteroendocrine response to dietary sugars.[98] However, in the Gilor et al study, cats treated with NG administered glucose had an increased secretion of insulin compared to intravenous administration of glucose. This implies the existence of an incretin effect, although minimal and not mediated by GIP.[101] This would suggest that either GLP-1 is directly stimulated by enteral glucose or there are other incretin hormones not yet known. Based on the current study, GLP-2 is not stimulated by oral carbohydrates. This discrepancy could be caused by differences in the study design or reflect true differences in nutrient regulation of GLP-1 and GLP-2 secretion.[42]

The present study showed that GLP-2 did not increase in response to a high-protein meal. This was an unexpected finding since previous work by others showed that an amino acid solution administered via NG tube stimulated GLP-1 and GIP release in cats.[101] The cause for this discrepancy is not apparent but may be a result of methodology or study design (direct gastric delivery of amino acids versus voluntary intake of a high-protein meal) or differences in physiologic mechanisms for protein-mediated GLP-1 and GLP-2 stimulation.

There are several differences in study design which could account for the discrepancies of the enteroendocrine responses reported in the present study and previous studies. Cats in the present study ate a mixed nutrient and balanced meal versus NG administration of solutions containing pure glucose, amino acids, and lipids.[101] Voluntarily eating a meal may trigger enteroendocrine secretion differently than a meal administered via an NG tube, as taste and swallowing could affect vagal responses. A liquid meal may also be emptied from the stomach at a faster rate than a solid meal, such as what the cats in the present study ate. This could lead to a different time course of GLP-2 secretion. Similarly, a meal with complex carbohydrates and complex proteins, as found in the commercial diets fed in this study, may require longer time for nutrient breakdown into products that stimulate enteroendocrine hormone release. Furthermore, in the Gilor *et al* 2011 paper, a fifteen-minute delay was applied after administering the lipid solution before obtaining the blood samples to account for longer gastric emptying and digestion

of the meals.[101] This could also affect comparison of the two studies and the timing of maximal secretion.

There may also be variation in enzymes responsible for the post-translational processing of proglucagon which could lead to divergence in GLP-1 and GLP-2 secretion and expression. While GLP-1 and GLP-2 are classically thought to be co-secreted, there is evidence in both cats and humans that there may be variation in production of these hormones in different regions of the intestines.[40] One theory behind this observed difference is variation in the post-translational processing of proglucagon and variable expression of the prohormone convertase enzymes.[42]

The meals fed in this study were not purely made of one component, so feeding a mixed meal, even if it is higher in one nutrient than another, may blunt or change the GLP-2 response compared to a single nutrient. The meals were chosen to provide the highest amount of the macronutrient of interest relative to the other nutrients in the meal, while maintaining the other macronutrient content as similar as possible on a g/100 kcal basis. Commercially available meals were chosen with the goal of voluntary consumption by the cats. A meal comprised exclusively of one nutrient would likely be unpalatable to cats, necessitating delivery that would be more stressful to the cats, such as NG tube feeding, and that would less accurately mimic GLP-2 secretion following natural food intake. However, this means that the meals had a mixture of protein, fat, and carbohydrates, which could have affected the overall secretion of GLP-2. Additionally, because the cats were free to eat the meals, some of them more readily finished the meals than others. All cats finished most (>50%) or all of the offered meal; although some cats did not finish the whole meal immediately and had to be encouraged. It appeared the high-fat diet was more palatable than the other diets and it was eaten in a shorter period of time. There was also variation in the volume of food for the number of calories offered; the high-fat diet ultimately had less overall volume than the high-carbohydrate and protein diets while remaining calorically equivalent. The cats fed via NG tube were only given 3.4 kcal/kg, which calculates to less calories than ¼ RER, but the volume fed each cat was around 45 mLs of fluid (9 mL/kg in total administered to each cat).[101] The meals fed in the current study would have been less volume than 45 mLs. It is not known whether meal volume or time to consume the meal affects GLP-2 secretion; however, these may be further variables that contributed to the between-cat variation in measured GLP-2 levels and the response difference between the current study and that by Gilor et al in 2011.[101]

There may also be variations based on the source of the nutrient as well. As demonstrated in ruminants, different sources of carbohydrate fermentation variably stimulate GLP-2, with butyrate promoting higher GLP-2 concentrations than other SCFA.[104] Finally, there may be divergent stimulation for GLP-2 versus GLP-1 despite previous reports of the co-secretion of the two hormones. As mentioned, in humans, PC1/3 has variable expression along the length of the intestines, affecting production of GLP-1 and GLP-2.[42] Alternatively, it has been shown that GLP-1 is more susceptible to DPP-IV degradation than GLP-2 and has a shorter half-life, which could account for differences in molar concentrations when measured at the same time-point. However, this does not explain why plasma GLP-1 concentrations would increase without concurrent increase in GLP-2 if there is the same stimulus for secretion.[105]

Another proposed stimulus for the first phase of GLP secretion within 30 minutes of a meal is direct stimulation of the small population of L cells located in the proximal duodenum.[31,92] It is thought that this population of cells could release enough GLP-2 as food enters the proximal duodenum soon after a meal to account for the rise in GLP-2 seen within 15-30 minutes after a meal in other species.[31] However, a study evaluating feline GLP-1 and GLP-2 intestinal expression via immunohistochemistry demonstrated very low GLP-2 staining in the proximal duodenum compared to other species.[40] In contrast, there was a much higher level of GLP-1 expression seen in duodenal cells, which could account for the small rise in GLP-1 seen in Gilor et al 2011[101] and the lack of GLP-2 response seen in the present study. Again, it is possible that GLP-1 and GLP-2 are not co-secreted, and that variation in PC1/3 activity in different regions of the GI tract of cats could be the cause. Further studies to evaluate the contribution of proximal L cells to the early post-prandial secretion of GLP-1 and GLP-2, as well as studies evaluating PC1/3 expression in cats, are warranted.

The receptors responsible for responding to intestinal glucose, subsequently upregulating GLP-2 secretion are not well-defined, but there is evidence to suggest that SGLT1 is responsible for absorption of glucose, signaling GLP-1 release, as SGLT1 inhibitors blunt the GLP-1 response.[23] There is also evidence to suggest that sweet taste receptors, the T1R2/T1R3 dimer, located both on the tongue and in the brush border of the GI tract are responsible for the initial sensing of intestinal glucose.[106] The contribution of these receptors to enteroendocrine signaling is still under investigation. One possible mechanism to explain why cats may not respond to ingested nutrients in a similar manner to other mammals is because they lack the same taste

receptors as other mammals.[107] The cat genome contains a pseudogene of the sweet taste receptor, T1R2, with a large deletion in the gene. This leads to a complete absence of expression of the protein. Because of this pseudogene, cats are unable to taste sweetness, observed as a general lack of interest in high-carbohydrate meals.[107] Sweet taste receptors are known as a possible stimulator of L cell secretion. If feline enteroendocrine cells lack expression of a functional sweet taste receptor, this may explain a blunted incretin response and lack of GLP-2 response to glucose in cats. It does not explain the lack of response to amino acids or proteins.

It is possible that this study was underpowered to find a significant difference in GLP-2 concentrations between nutrient composition, especially when using mixed nutrient meals. In the high-fat meal group there was a trend towards a peak plasma GLP-2 concentration at 30-minutes post-prandially that never reached significance. It is possible that a larger population of cats eating the high-fat diet would have revealed a stronger peak at 30 minutes. However, the first study demonstrating differential nutrient regulation of GLP-2 secretion in humans used 6 subjects,[49] and the power calculation supported the use of 9 cats. When considering the power calculation, it was considered that differences in GLP-2 concentration compared to baseline could be less using the mixed diets in this study compared to pure-nutrient solutions; however, it was anticipated that the 27 total measurements, when accounting for the crossover design, would be sufficient. Post hoc sample size calculation using the observed GLP-2 concentrations and standard deviation demonstrated that 21 cats would have been needed to observe a difference from baseline following ingestion of the high-fat diet. Another possibility is that the ELISA could be detecting both the active GLP-2(1-33) and inactive GLP-2(3-33) rather than just the active form. Previous reports in humans show that even if bioactive GLP-2 concentrations increase, total GLP-2 concentrations are unchanged. Specifically, bioactive GLP-2 increases after a meal of carbohydrates, while total GLP-2 does not increase.[49] If the ELISA is capturing total GLP-2, meaning both GLP-2 (1-33) and GLP-2 (3-33), the full response to the ingested food may not be detected by this study. Furthermore, the addition of proteinase inhibitors did not affect the measured GLP-2 concentrations in this study, which could be explained by this lack of specificity.

There is also a discrepancy between previously reported values of plasma GLP-2 concentrations in multiple other species such as human, pigs, rats, and mice and those in this study. In humans, total plasma GLP-2 concentrations have been reported to be between 300-600

pg/mL and bioactive GLP-2 even lower at about 100-200 pg/mL.[3,49] Non-fasted weaned piglets had a mean plasma bioactive GLP-2 concentration of 34 pmol/L (128 pg/mL) and rats have a resting bioactive GLP-2 concentration of about 10 pmol/L (37.7 pg/mL).[56,108] Compared to these previously reported GLP-2 concentrations as well as previously reported GLP-1 concentrations, the concentrations detected by the assay used in this study are higher by an order of magnitude.[101] This may reflect a naturally higher resting GLP-2 concentration in cats, differences in the study design or assay used to detect GLP-2 in these different studies. However, most previous studies used radioimmunoassay (RIA) to detect GLP-2. The current study used an ELISA and previous publications measuring GLP-2 with an ELISA have shown serum or plasma GLP-2 concentrations within the same order of magnitude as detected here.[109,110]

There is high sequence homology of the proglucagon derived peptides among mammalian species, but the exact feline sequence is unknown. The ELISA used in these experiments is based on the human sequence which may be a limitation to the sensitivity or specificity of the measured GLP-2 concentrations. Discovery of the specific amino acid sequence of feline GLP-2 would help solidify the results of this study, as well as allow further recovery of the different fractions of GLP-2, both active and inactive.

Ultimately this study shows that cats have a mild GLP-2 response to a high-fat meal but lack response to meals high in carbohydrates or protein. Further research directions include using more exclusive nutrient profiles as the test meal to see if the behavior of enteroendocrine secretion changes when the meal is not balanced. Another future goal is to differentiate bioactive GLP-2 from inactive, possibly using high performance liquid chromatography to separate the two fractions. This would be accompanied by sequencing of the GLP-2 protein of cats to ensure the specificity of the ELISA and to prove the high homology between species. Finally, characterizing GLP-2 plasma concentrations in cats with intestinal disease compared to heathy cats will help elucidate its potential as a biomarker or therapeutic agent in these patients.

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