

Gastrointestinal Sensorimotor
Dysfunctions in Diabetes
Mellitus

NCT 02170870

June 16, 2017

PROTOCOL

Title of the study: Gastrointestinal Sensorimotor Dysfunctions in Diabetes Mellitus
IRB Number 14-002098
Principal investigator: Adil E. Bharucha, MD
Co-investigator(s): Ananda Basu, MD, Michael Camilleri, MD., Magnus Halland, MD, Yogish Kudva, MD, Alan Zinsmeister, PhD, Phillip Low MD, Wolfgang Singer MD, Lawrence Szarka MD, Tonette Gehrking, Jade Gehrking, Gopanandan Parthasarathy, Thomas Smyrk, Pratyusha Tirumanisetty
Study Coordinator: Bridget Neja, Kelly Feuerhak
Study Technicians: Duane Burton, Michael Ryks, Debbie Rhoten
Version 6/16/17

ABSTRACT

Upper gastrointestinal symptoms (early satiety, pain, nausea, and vomiting) are not uncommon in diabetic (DM) enteropathy. While these symptoms are often attributed to accelerated or delayed gastric emptying, the precise contribution of abnormal gastric emptying to symptoms in patients with DM gastroparesis is often unclear.

We recently observed that approximately 50% of patients with functional dyspepsia have increased sensation to duodenal nutrient (carbohydrate and lipid) perfusion. Another recent study suggests that patients with functional dyspepsia have low-grade mucosal inflammation, abnormalities of cell-to-cell adhesion proteins which predispose to increased epithelial permeability, and a leaky epithelial barrier. Type 1 DM is associated with increased small intestinal permeability even in subjects who do not have celiac disease.

Hence, we propose to evaluate the overall hypothesis that intestinal chemosensitivity related to increased epithelial permeability and GLP-1 explains symptom severity in patients with functional dyspepsia and in patients with DM and dyspepsia. 24 healthy subjects, 40 patients with DM and GI symptoms, and 40 patients with functional dyspepsia will undergo assessment of intestinal chemosensitivity during duodenal nutrient perfusion, gastric emptying (by scintigraphy), cardiovascular and GI vagal functions (plasma pancreatic polypeptide response to sham feeding and a comprehensive autonomic reflex screen), in vivo assessment of small intestinal permeability (urinary lactulose:mannitol ratio), and upper endoscopy with assessment of epithelial tight junction proteins and permeability on small bowel biopsies. During the nutrient infusion, subjects in each group (ie, healthy subjects, functional dyspepsia and DM) will be randomized to lipid infusion and placebo or lipid infusion {66.7 mL Microlipid (0.5 gm/mL diluted in water to 222 ml)} and exendin 9-39 in the ratio of 1:1. Hormonal responses (i.e., GLP-1, CCK, GIP, glucagon, PYY, C-peptide, and insulin) and plasma glucose will also be evaluated during enteral nutrient infusion. GI symptoms during each perturbation (meal, nutrient infusion) will be evaluated by validated questionnaires. Blood will be collected for DNA-based genetic analyses, initially to assess the relationship of GI sensorimotor dysfunctions and symptoms with SNPs affecting CCK and GLP-1 receptors. The analysis will assess for disturbances in these parameters in functional and DM dyspepsia, investigate associations between symptoms during enteral infusion and hormonal-epithelial functions, and evaluate relationships between daily symptoms and results of testing.

Table of Contents

ABSTRACT.....	1
1 SPECIFIC AIMS	3
2 BACKGROUND AND SIGNIFICANCE.....	5
3 RESEARCH DESIGN AND METHODS	7
3.1 Study Design.....	7
3.2 Study Subjects.....	7
3.3 Details of Study Assessments	8
3.4 Gene and Epigenetic Studies	15
3.5 Data Analysis	16
3.6 Potential Pitfalls	17
4 HUMAN SUBJECTS	17
5 SAFETY AND ADVERSE EVENTS	19
5.1 Definitions.....	19
5.2 Recording of Adverse Events	21
5.3 Reporting of Serious Adverse Events and Unanticipated Problems.....	22
5.3.1 Sponsor-Investigator reporting: notifying the Mayo IRB.....	22
5.3.2 Sponsor-Investigator reporting: Notifying the FDA.....	22
5.4 Stopping Rules.....	23
5.5 Medical Monitoring	23
APPENDIX.....	23
REFERENCES	26

1 SPECIFIC AIMS

Upper gastrointestinal symptoms (early satiety, pain, nausea, and vomiting) are not uncommon in diabetes mellitus (DM) ¹. These symptoms are often attributed to delayed gastric emptying or gastroparesis. Over a 10-year time period, the risk of developing gastroparesis was 5% in type 1 DM (HR 33, 95% CI 4.0, 274 adjusted for age and gender versus controls), 1% in type 2 DM (HR 7.5 (95% CI: 0.8, 68) adjusted for age and gender versus controls and 1% in controls ². However, similar to functional dyspepsia ³, the precise contribution of abnormal gastric emptying to symptoms in patients with DM gastroparesis is unclear. Indeed, a majority of patients with DM and delayed gastric emptying are asymptomatic. In our cohort of DM patients with GI symptoms who underwent scintigraphy, a substantial proportion, i.e., 55 of 129 patients (42%) had normal gastric emptying; the remainder had had delayed (36%) or rapid GE (22%) ⁴. Moreover, symptoms did not predict abnormal (i.e., delayed or rapid) gastric emptying. Other, small, studies reported impaired gastric accommodation and increased gastric sensation in DM ^{5,6} but did not describe the relationship of these abnormalities to upper GI symptoms.

Nutrients and enteral hormones (e.g., CCK, GLP-1) released in response to nutrients also stimulate small intestinal mechano and chemoreceptors or tastant receptors, which are located on or activate vagal afferents. Few studies have explored the contribution of duodenal sensitivity to symptoms in functional dyspepsia. Duodenal acidification induces proximal gastric relaxation, increases sensitivity to gastric distension, and inhibits gastric accommodation to a meal ⁷. Duodenal lipid infusion increased the sensitivity to gastric distention in functional dyspepsia ⁸⁻¹¹. This sensitizing effect was blocked by a lipase inhibitor or a CCK-A receptor antagonist ^{12,13}. We recently observed increased sensitivity to duodenal nutrient (carbohydrate and lipid perfusion) in approximately 50% of patients with functional dyspepsia during lipid and separately carbohydrate infusion, without gastric distention (unpublished observations). Moreover, increased sensitivity was associated with higher plasma concentrations of CCK during lipid infusion and more severe dyspeptic symptoms during daily life. However, the contribution of intestinal chemosensitivity to symptoms in DM is unknown. We recently observed that patients with functional dyspepsia reported more severe symptoms during enteral lipid and carbohydrate infusion. Second, this increased sensitivity was associated with higher plasma concentrations of enteral hormones (e.g., GLP-1 during lipid and carbohydrate infusion), more severe dyspeptic symptoms, and a greater impact on quality of life.

Vagal dysfunction has been implicated as a mechanism for GI sensorimotor dysfunctions in DM ¹⁴. The plasma pancreatic polypeptide (PP) response to sham feeding evaluates GI vagal function ¹⁵. In clinical practice, assessment of cardiovascular vagal functions is used as a surrogate measure of GI vagal dysfunction. Only 1 study evaluated the relationship between cardiovascular and GI vagal dysfunction (i.e., gastric acid secretion, PP response to sham feeding) and observed a modest correlation ¹⁵. The utility of the sham feeding response and cardiovascular measures to predict upper GI sensorimotor dysfunctions has not been evaluated.

A recent study suggests that patients with functional dyspepsia have low-grade mucosal inflammation, abnormalities of cell-to-cell adhesion proteins which predispose to increased epithelial permeability, and a leaky epithelial barrier ¹⁶. Type 1 DM is associated with increased small intestinal permeability even in subjects who do not have celiac disease ^{17,18}. Moreover, a comparison of 339 type 1 DM patients and 89 first degree relatives observed higher serum zonulin levels in type 1 DM patients than controls or relatives; patients with increased zonulin had increased epithelial permeability ¹⁹. The only study which evaluated small intestinal permeability in type 2 DM used suboptimal techniques and reported normal permeability ²⁰.

Our *overall hypothesis* is that intestinal chemosensitivity related to increased epithelial permeability and CCK explains symptom severity in patients with DM with upper GI symptoms.

The **specific aims** of this study are to address the following hypotheses in 24 healthy subjects, 40 patients with DM and GI symptoms, and 40 patients with functional dyspepsia:

- 1) **A) Patients with DM and GI symptoms have increased intestinal chemosensitivity compared to controls and B) Intestinal chemosensitivity predicts severity of ongoing upper gastrointestinal symptoms independent of gastric emptying.** Intestinal chemosensitivity will be recorded by evaluating symptoms during duodenal lipid infusion (222 kcal, 222 ml) and placebo or the GLP-1 receptor antagonist exendin 9-39 over 2 hours; plasma hormones (i.e., glucose, CCK, GLP-1, PYY, C-peptide, will also be measured. Delivering nutrients into the duodenum will ensure that hormonal responses are not affected by gastric emptying disturbances. Day-to-day symptoms will be evaluated by the Nepean dyspepsia severity index and the GCSI. The relationship between daily symptoms and intestinal chemosensitivity will be assessed in a model which also incorporates gastric emptying and accommodation.
- 2) **Intestinal chemosensitivity will be A) associated with higher plasma CCK and GLP-1 concentrations B) reduced by the GLP-1 antagonist exendin 9-39 and C) inversely associated with impaired abdominal vagal function.** This hypothesis will be evaluated by comparing intestinal chemosensitivity (a) with plasma CCK and GLP-1 concentrations (b) with and without exendin 9-39 and (c) in patients with and without and GI vagal dysfunction as assessed by the plasma pancreatic polypeptide response to sham feeding.
- 3) **A) Patients with DM and GI symptoms have abnormal epithelial tight junction proteins which predispose to increased epithelial permeability and increased small bowel permeability and B) These structural and functional markers of epithelial permeability are associated with intestinal chemosensitivity.** Intestinal permeability will be measured in vivo by two sugar urine excretion after oral ingestion. Biopsies from the distal second or third portion of the duodenum will be obtained endoscopically, to measure various markers of epithelial structure and function.

2 **BACKGROUND AND SIGNIFICANCE**

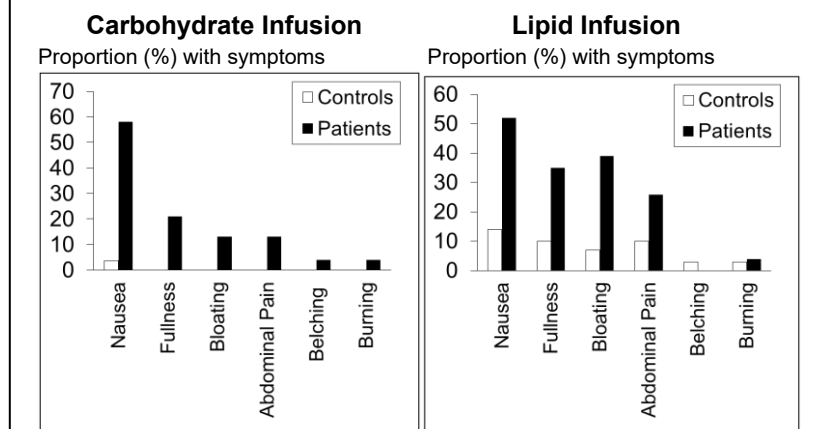
Diabetic enteropathy

The term diabetic *enteropathy* implies that diabetes mellitus (DM) can affect the entire gastrointestinal (GI) tract ¹. Diabetic enteropathy may be asymptomatic or manifest with upper (i.e., heartburn, dysphagia, dyspepsia, gastroparesis) or lower GI symptoms (diarrhea, constipation, and fecal incontinence). Gastrointestinal dysmotility in DM is multifactorial: extrinsic and intrinsic (i.e., enteric) neural dysfunction, hyperglycemia, and hormonal disturbances have been implicated.

Patients with DM may have accelerated or delayed gastric emptying (GE), increased and reduced gastric sensation, and impaired gastric accommodation. Antral hypomotility and/or pylorospasm, which can result from a vagal neuropathy, can delay GE ²¹. Much attention has focused on delayed gastric emptying or gastroparesis as an explanation for upper GI symptoms in DM. Similar to idiopathic gastroparesis, the contribution of delayed gastric emptying to symptoms in patients with DM gastroparesis is unclear for several reasons. A majority of patients report symptoms within 1 hour of eating. However, even normally, only approximately 25% of solids have emptied from the stomach by then. Hence, delayed gastric emptying is unlikely to explain early postprandial symptoms. Second, a majority of patients with DM and delayed gastric emptying are asymptomatic. Third, a substantial proportion of DM patients with GI symptoms who underwent scintigraphy, i.e., 55 of 129 patients (42%) in our cohort, had normal gastric emptying; the remainder had had delayed (36%) or rapid GE (22%) ⁴. Symptoms did not predict abnormal (i.e., delayed or rapid) gastric emptying in patients with DM and GI symptoms ⁴. Even in functional dyspepsia, gastric emptying and accommodation only explained one-third of the variance in postprandial symptoms ^{22, 23}.

Impaired gastric accommodation and increased gastric sensation have been implicated to cause early satiety in functional dyspepsia ²⁴. Our understanding of gastric accommodation and gastric sensation in DM is limited to 3 studies, which assessed a total of 37 patients. While gastric sensation was increased in both studies, gastric compliance in DM was increased in 1 study ⁵ and reduced in 2 studies ^{6, 25}. Another study, with 10 healthy subjects and 10 DM patients, observed an association between a lack of symptoms in diabetic gastrointestinal motility disorders and visceral afferent neuropathy identified by esophageal electrical stimulation ²⁶. Thus, while sensory disturbances – increased or decreased – are recognized in diabetic peripheral neuropathy, there are minimal data on GI sensation. Conceivably, GI sensory disturbances influence the expression of GI motor dysfunctions in DM. For example, asymptomatic delayed gastric emptying may be explained by vagal neuropathy. Conversely, in symptomatic patients, delayed gastric emptying is likely explained by enteric dysfunction rather than vagal neuropathy.

Figure 1. Gastrointestinal symptoms during enteral nutrient infusion. A higher proportion of patients with functional dyspepsia (n=25) than controls (n=33) reported symptoms of moderate severity or worse during enteral carbohydrate ($p \leq 0.01$) and lipid ($p \leq 0.01$) infusions.



Small intestinal dysmotility, more frequently characterized by reduced than by increased motility²⁷, may also contribute to gastric stasis in DM. We recently observed increased sensitivity during duodenal carbohydrate and separately lipid perfusion in approximately 50% of patients with functional dyspepsia (Figure 1). Moreover, increased sensitivity was associated with higher plasma concentrations of GLP-1 during lipid infusion and more severe dyspeptic symptoms during daily life (unpublished data). Previous

studies have evaluated the effects of duodenal nutrient infusion on glycemia, hormonal responses, glucose absorption, and gastroduodenal motility but not sensation in DM²⁸⁻³¹. Moreover, no studies have integrated findings from assessments of gastric emptying, accommodation, and sensation. This is a major limitation since the expression (symptoms) resulting from a disturbance may depend on other coexisting disturbances. For example, it is conceivable that intestinal hypersensitivity may amplify the consequences of rapid gastric emptying. Doing so is necessary to better understand the pathogenesis of symptoms and devise therapies targeted to the underlying disturbance in DM.

Vagal neuropathy

Vagal dysfunction has been implicated as a mechanism for GI sensorimotor dysfunctions in DM¹⁴. The plasma pancreatic polypeptide (PP) response to sham feeding evaluates GI vagal function¹⁵. In clinical practice, assessment of cardiovascular vagal functions is used as a surrogate measure of GI vagal dysfunction. Only 1 study evaluated the relationship between cardiovascular and GI vagal dysfunction (i.e., gastric acid secretion, PP response to sham feeding) and observed a modest correlation¹⁵. The utility of the sham feeding response and cardiovascular measures to predict upper GI sensorimotor dysfunctions is unknown and will be evaluated in this study.

Small intestinal epithelial permeability and mucosal inflammation

A recent study suggests that patients with functional dyspepsia have low-grade mucosal inflammation, abnormalities of cell-to-cell adhesion proteins which predispose to increased epithelial permeability, and a leaky epithelial barrier¹⁶. Type 1 DM is associated with increased small intestinal permeability even in subjects who do not have celiac disease^{17, 18}. Moreover, a comparison of 339 type 1 DM patients and 89 first degree relatives observed higher serum zonulin levels in type 1 DM patients than controls or relatives; patients with increased zonulin had increased epithelial permeability¹⁹. The only study which evaluated small intestinal permeability in type 2 DM used suboptimal techniques and reported normal permeability²⁰. However, a high fat diet induced glucose intolerance, decreased intestinal tight junction proteins, increased epithelial permeability, and plasma concentrations of circulating cytokines in OLETF mice, which is a

spontaneous CCK-1 receptor knockout mouse and an animal model of obesity³². Given the similarities between this mouse model and type 2-DM in humans, it is conceivable that epithelial permeability is also increased in type 2 DM.

3 RESEARCH DESIGN AND METHODS

3.1 Study Design

In addition to questionnaires, the primary study procedures include assessment of:

- i) Screening visit – The participant will undergo a physical examination if one has not been completed within the last 3 months.
- ii) Study day 1 - GI vagal function by sham feeding, autonomic functions, small intestinal permeability test and enteral nutrient infusion of lipids, to be performed in that order, on another day (Charlton 7 CRU)
- iii) Study day 2 – Women of child bearing potential will have a urine pregnancy test within 48 hours before the GI Transit study, Gastric emptying of solids and liquids and small intestinal transit on 1 day (Charlton 7 CRU)
- iv) Study day 3 - Upper gastrointestinal endoscopy with a small amount of fluid and intestinal biopsies

These tests will preferably be performed in the order listed above. (ii) must always be performed before (iv). Ancillary procedures include collection of blood for genetic and epigenetic studies and a stool sample for microbial assessment. Vital signs and nurse assessment will be performed at each visit.

3.2 Study Subjects

We plan to accrue up to 115 subjects to ensure that 104 subjects complete the study in its entirety. 40 patients with DM and GI symptoms and 40 patients with functional dyspepsia will be recruited from the clinical practice; 24 healthy subjects will be recruited by public advertisement. To reduce the possibility of demographic differences between controls and patients, we will "frequency match" on age and gender by recruiting 5 controls after each set of 5 patients is studied. Doing so will provide similar age and gender distribution in each type. We will also attempt to recruit an even number of subjects with type 1 and 2 DM.

Inclusion criteria for controls

1. Healthy male or non-pregnant, non-breastfeeding female volunteers;
2. 18-70 years old;
3. Able to provide written informed consent before participating in the study;
4. Able to communicate adequately with the investigator and to comply with the requirements for the entire study

Additional inclusion criteria for patients

1. Symptoms of dyspepsia (i.e., early satiety, postprandial discomfort, nausea, vomiting, regurgitation)

2. Patients in the DM group will also require Type 1 or 2 DM of ≥ 3 years duration; in patients with type 2 DM, the dyspepsia symptoms should have begun or worsened after DM was diagnosed

Exclusion criteria – for patients and controls

1. Major abdominal surgery (i.e., appendectomy, cholecystectomy, tubal ligation, hysterectomy, and limited colonic resection are permissible)
2. Clinical evidence (including physical exam and EKG) of significant cardiovascular, respiratory, renal, hepatic, gastrointestinal, hematological, neurological, psychiatric or other disease that may interfere with the objectives of the study and/or pose safety concerns
3. Opiates, alpha adrenergic agonists, metoclopramide, and high doses of anticholinergic agents (eg, amitriptyline greater than 50 mg daily). If medically safe, these drugs may be discontinued for four half lives prior to study assessments
4. Treatment with GLP-1 agonists and amlyin which cause vagal blockade and may affect central processing of pain
5. Use of tobacco products within the past 1 week or NSAIDs or aspirin within the past week (since they all may affect intestinal permeability)
6. Bleeding or clotting disorders or medications that increase risk of bleeding from mucosal biopsies
7. Positive tissue transglutaminase antibodies (TTG),
8. For two days prior to studies, subjects will be instructed to avoid ingestion of artificial sweeteners such as Splenda™ (sucralose), Nutrasweet™ (aspartame), foods containing lactulose or mannitol
9. Pregnant or breast-feeding females
10. Known intolerance or allergy to eggs
11. Poor peripheral venous access, if central venous access is not available
12. Any other condition or prior therapy that, in the opinion of the investigator, would make the patient unsuitable for the study

Exclusion criteria for controls only

1. Current symptoms of a functional gastrointestinal disorder assessed by questionnaire

Exclusion criteria for patients only

1. Severe vomiting that would preclude tube placement or participation in the study
2. Structural cause for symptoms by endoscopy within the past 48 months
3. Patients with gastric pacemakers

3.3 Details of Study Assessments

In patients with DM, fingerstick bedside reflectance meter blood glucose concentrations will be assessed during enteral infusion and gastric emptying studies at time 0, every 30 minutes for 2h, then at 120 and 180 minutes. For patients with type 1 and type 2 DM who are treated with insulin, insulin will be given according to the patient's sliding scale. For type 2 DM patients who are not on insulin, no insulin will be given.

i) Screening items include questionnaires (for overall functional GI symptoms [Rome III criteria], severity of dyspepsia [Nepean severity index], gastroparesis cardinal symptom index [GCSI], anxiety and depression [HAD], and autonomic symptoms), blood tests (CBC, glycosylated hemoglobin, fasting glucose, sodium, potassium, bicarbonate, calcium, chloride, phosphorous, AST, total bilirubin, alkaline phosphatase, creatinine, high sensitive C-reactive protein, tissue transglutaminase antibodies, and lipase), and EKG (to be reviewed by study physician). Some patients with diabetes mellitus have low serum lipase. Patients with serum lipase < lower limit of normal (i.e., < 10 IU/ml) will not be eligible to participate in the study.

Study procedures will be conducted over 3 days. Items (ii), (iii), (iv), and (v) below will be performed on the same day preferably before other study procedures. A tentative schedule is as follows: (ii) between 8 and 8.45 am, (iii) between 8.45 am and 11 am, (iv) can start while (iii) is in progress and (v) between 12.30 pm and 3 pm.

ii) Plasma pancreatic polypeptide (PP) response to sham feeding. Pancreatic polypeptide (PP) is secreted by the pancreas in response to hypoglycemia, ingestion of food or sham feeding secondary to vagal nerve stimulation. Vagal nerve injury blocks secretion. This assessment will be performed in the Charlton 7 CRU. Subjects will be fasting with NPO for 8 hours prior to this test. They must abstain from coffee or for 12 hours and stop all cholinergic agents for 48 hours before the test. During the sham feeding test, food is chewed and spit out (not swallowed) . After placement of an IV in the hand or forearm vein for blood draws, this hand will be placed in a hotbox heated to 55°C. Blood will be drawn in a lavender-top (EDTA) tube(s) at -5, 0, 5, 10, 15, 20, 25, and 30 minutes for PP. Spin down, keep specimen cold, and send 1.0 mL (0.5 mL minimum) of EDTA plasma frozen in plastic vial.

iii) Small intestinal permeability in vivo. As in prior studies^{33,34}, lactulose, 1000 mg, and mannitol, 200 mg (L7877 and M8429 from Sigma-Aldrich, St. Louis, MO 63103), will used to determine the urine sugar excretions at different times as markers of small bowel mucosal permeability after oral ingestion of the sugars in aqueous solution. 500 mL of tap water will be given 30 minutes after the start of the small bowel permeability test. Because colonic permeability will not be assessed, urine will be collected every 30 minutes for the first 2 hours (when the participant was able to provide a specimen, and cumulated for the entire 2 hours). The total volume of each collection is measured, and an aliquot from each collection will be obtained to estimate the total content of each sugar for the different time intervals. The urine aliquot will be stored at -20° Celsius until it was thawed for analysis. Cumulative and ratio excretions of the two sugars at 0-2 hours will be used to estimate small bowel and colonic mucosal permeability, respectively, based on recent validation studies. Urinary saccharide concentrations will be measured by high performance liquid chromatography-tandem mass spectrometry. Details of this method were previously described elsewhere; the assay was adapted from the method of Lostia et al.³⁵.

The cumulative excretion = [concentration of sugar (µg/mL)]* total urine volume (mL).

The lactulose:mannitol ratio (L:M ratio) was calculated by:

$$L:M \text{ ratio} = 0.2 \times (\text{cumulative excretion lactulose}) / (\text{cumulative excretion mannitol})$$

iv) Autonomic reflex screen. Patients will complete a standardized autonomic symptom questionnaire developed by Dr. Low and undergo a standardized evaluation of autonomic functions

in Dr. Low's laboratory (Charlton CRU). This assessment is routinely used in clinical practice and comprises: 1) the Quantitative Sudomotor Axon Reflex Test (QSART) at proximal and distal standard sites to assess sympathetic postganglionic sudomotor axon function ³⁶; 2) heart rate responses to deep breathing and the Valsalva maneuver to assess cardiovagal function ³⁷; and 3) beat-to-beat blood pressure responses to the Valsalva maneuver and head-up tilt (HUT) to assess sympathetic adrenergic function ³⁸. Subjects will be studied under standardized conditions.

v) Hormonal responses and symptoms during duodenal infusions. A small bore nasogastric tube will be inserted through the nostril into the stomach over a guidewire and advanced under fluoroscopic guidance until the infusion ports are in the 2nd part of the duodenum.

Thereafter, subjects in each group (ie, functional dyspepsia and DM) will be randomized to lipid infusion {66.7 mL Microlipid (0.5 gm/mL diluted in water to 222 ml)} together with placebo or exendin 9-39 in the ratio of 1:1. Lipids will be delivered into the duodenum at an infusion rate which matches the rate at which glucose enters the systemic circulation after oral glucose. After placement of an IV in the hand or forearm vein for blood draws, this hand will be placed in a hotbox heated to 55°C. We have considerable preliminary data for symptoms and hormonal responses with this paradigm. Plasma glucose, insulin, C-peptide, GLP-1, CCK, ghrelin, and PYY will be collected at 5 minute intervals for 30 minutes, 10 minute intervals from 30 – 60 minutes, and at 15 minute intervals from 60 – 180 minutes (18 times including 0 minutes).

vi) GLP-1 antagonist exendin 9-39 will be sourced from C.S. Bio, reconstituted in the Mayo Pharmacy by an established process used in previous studies, and undergo endotoxin testing, sterility testing and bacterial testing, and held in quarantine before it will be used in humans. Research compounded sterile preparations (CSP) are prepared and assessed for end preparation testing according to USP <797> in the hospital pharmacy services production lab. Each batch is tested for sterility according to USP <71> utilizing membrane filtration. The sterility testing is validated by a formulation Bacteriostasis Fungistasis test. The amount of each batch tested is determined by USP <71>. Each batch is batch is tested for endotoxin according to USP <85>. High risk CSP are quarantined for 14 days and when all end preparation testing is completed.

Exendin 9-39 will be administered intravenously at the same dose (1,200 pmol/kg bolus followed by infusion at 300 pmol/kg/min) as previously described at Mayo Clinic (Dr. A Vella) ^{39, 40}. This dose has previously been shown to block the effects of GLP-1 infused at supraphysiologic doses and the effects of endogenous GLP-1 on gastrointestinal motility and insulin secretion ⁴¹⁻⁴³. The effects of this dose are qualitatively similar to studies using 2.5- to 3.0-fold higher infusion rates ⁴⁴⁻⁴⁶. It is possible that endogenous GLP-1 still contributes to insulin secretion in the presence of exendin-9,39; however the residual effect is likely to be small ⁴⁵. For example, the 25% increase in integrated glucose concentrations with an infusion of 900 pmol/kg/min ⁴⁶ is comparable to the change in glucose concentrations in other studies ⁴⁰. The half life of exendin 9-39 is 33 minutes. Exendin 9-39 is very safe and well tolerated and the increase in blood glucose concentration with exendin 9-39 infusion is modest. For example, in 1 study blood glucose concentrations after a duodenal meal were on average only 6 mg/dl higher in healthy subjects and 19 mg/dl higher in T2DM for exendin 9-39 versus saline ⁴⁷. Even in type 1DM, the effects of exendin 9-39 on blood glucose are modest ⁴⁸. [Blood glucose data was provided as AUC rather than mg/dl]. We will monitor blood glucose by fingerstick and administer insulin as necessary (Section 3.3).

vii) Upper GI Endoscopy and Biopsies. Upper GI endoscopy will be performed in the endoscopy lab of Clinical Research Unit, Charlton 7. Patients will receive conscious sedation as required for the procedure to be conducted with comfort. A full endoscopy report will be placed in the medical record. There are 3 changes in this revision (6/15/15). Because electron microscopy studies suggest that duodenal epithelial integrity is impaired in diabetes, we propose to (i) assess mucosal impedance during endoscopy and (ii) assess routine histology with mucosal biopsies. Also, as explained below, we propose to evaluate the mucosal microbiome with biopsies

During endoscopy, mucosal impedance will be measured with a new endoscopically placed probe that measures epithelial impedance over a 2 cm area⁴⁹, and is or has being used to measure transepithelial resistance in the esophagus and duodenum in other IRB-approved research protocols at Mayo Clinic⁵⁰. By measuring current generated by ion flow, this device provides a measure of transepithelial resistance and permeability. The soft mucosal impedance (MI) catheter will be advanced through the working channel of an upper endoscope. Measurements will be obtained until approximately 4 optimal recordings are obtained from the mucosa in the second part of the duodenum. The electrodes are connected to an impedance voltage transducer at the bedside via thin wires, which ran the length of the catheter. The voltage generated by the transducer was limited to produce at most 2.5 microamps RMS of current. The frequency for the measuring circuit will be set at 2 kHz. Mucosal impedance will be expressed in ohms as the ratio of voltage to the current, according to Ohm' law ($V=IR$). Data will be acquired with a stationary impedance data acquisition system (InSight; Sandhill Scientific, Inc) and were viewed and analyzed on BioView Analysis software (Sandhill Scientific, Inc). A small amount of fluid and 15 biopsies will be collected from the second part of the duodenum for the following assessments:

- a) ICS diameter using transmission electron microscopy, performed at the Optical Imaging Core (5-10 micron sections, 2 bites, separate medium (Trump solution) or even formalin)
- b) Transepithelial resistance (TER) and fluorescein flux across the squamous epithelium, using mini-Ussing chambers, in collaboration with Dr.Farrugia's laboratory

TER: Four biopsies will be mounted in 4 ml Ussing chambers (Physiological Instruments, San Diego, CA) exposing 0.03 cm² area. Using a pair of Ag/AgCl electrodes with agar-salt bridges current-giving platinum electrodes, TER will be measured. These assessments need to be performed within 30 minutes of collecting samples (3 bites, Krebs's solution).

Paracellular flux: Macromolecular flux across biopsies will be studied using FITC Dextran (4 kDa, Molecular Probes, NY) added to the mucosal compartment. At 30 min intervals for a total of 3 hrs, fluorescence in the basal compartment will be analyzed using a Synergy Multi-Mode Microplate Reader (BioTek, VT, USA). The same specimen used for TER can be used for this.

- c) Expression of tight junction gene expression with quantitative real-time RT-PCR will be assessed using Taqman gene expression assay. The following mRNA levels will be measured: ZO-1, ZO-2, ZO-3, occludin, and Claudins-1-4. The expression of each gene will be normalized to housekeeping genes beta-actin, beta-2-microglobulin, and GAPDH. Protein concentrations will be determined by Pierce bicinchoninic acid assay as previously described. Primary immunoglobulin (Ig)G antibodies directed against ZO-1, ZO-2, ZO-3, occludin and claudins 1-4 will be used. Corresponding secondary antibodies will be used and densitometric comparison will be carried out on the immunoblot. In non-post-infectious IBS, occludin and claudin-1 were found to be "internalized"

into the cytosolic space. Similar changes have been seen with occludin expression in the jejunal tissue from non-post-infectious IBS making distribution of TJ proteins an important component of studying expression. Immunohistochemistry will be used to determine expression and distribution of ZO-1, occludin, phosphorylated occludin and claudin-1. This requires 2 tissue biopsies (fresh frozen in liquid nitrogen at -80).

d) Small bowel inflammation: will be assessed with RT-PCR for global markers of macrophages (CD68), pro-inflammatory macrophage (M1) markers: TNF α , IL1 β IL6 and anti-inflammatory (M2) macrophage markers (CD206, CD36) using previously described methods. This requires 2 tissue biopsies (fresh frozen in liquid nitrogen at -80).

e) Assessment of microbiome (2 specimens snap frozen in liquid nitrogen at -80C) – Currently, we are collecting stool but not mucosal biopsy specimens for microbial analysis. Our recent studies (IRB 12-6091) indicate that the mucosal microbiome discriminated between health and constipation with 94% accuracy independent of colonic transit. In contrast, the fecal microbiome predicted colonic transit and breath hydrogen production. Hence, and given documented associations between the gut microbiota and diabetes mellitus cited below, we propose to collect 2 mucosal biopsies for this purpose.

f) Three specimens for mRNA expression to be collected in RNA later [No change].

g) Routine histology – Because preliminary analysis of electron microscopy analysis demonstrates widening of intercellular spaces and because there is evidence for low grade intestinal mast cell and eosinophil infiltration in functional disorders⁵¹, we propose to evaluate routine histology in 2 mucosal biopsies collected in formalin; Dr. Thomas Smyrk will examine the same.

viii) Gastric emptying and small intestinal transit of a mixed solid-liquid meal. Using established techniques, gastric emptying of solids and liquids and small bowel transit will be simultaneously assessed by scintigraphy.⁵² ^{99m}Tc-sulfur colloid (1 mCi) will be added to 2 raw eggs during the scrambling and cooking process. The eggs will be served on 1 slice of bread and with 240 mL of 1% milk (296 kcal, 32% protein, 35% fat, 33% carbohydrate) labeled with ¹¹¹In-diethylenetriaminepentaacetate (0.1 mCi). During the gastric emptying study, patients will complete VAS scales for severity of 6 symptoms (nausea, fullness, bloating, abdominal pain, belching and burning), at 15 minute intervals, on a Likert scale with the descriptors (absent, light, moderate, severe, and intolerable). **During the study**, patients will eat a standardized meal, a lunch of chicken breast, during this study visit this meal is not radioactive. Data will be analyzed as in previous studies.

ix) Stool Collection. Two recent metagenome-wide association studies have highlighted associations between specific gut microbiota and type 2 diabetes mellitus^{53, 54}. To build on these observations and extend our ongoing studies evaluating the relationship between gastrointestinal transit and gut microbiome (IRB 12-6091) we will collect a stool sample in each subject. Using a stool kit and standardized instructions, patients will collect stool a sample according to the procedure in the Appendix. The stool sample will be frozen and stored in a -20°C freezer. The stool specimen will be collected without a laxative.

x) Symptom diary. Participants will complete a validated daily diary (i.e., Gastroparesis Cardinal Symptom Index-Daily Diary (GCSI-DD) for 2 weeks at home^{55, 56}. This will probably require 5 minutes daily.

xi) Immunochemical Analysis. Arterialized venous plasma samples will be placed in ice, centrifuged at 4°C, separated and stored at -20°C until assay. Glucose will be measured by the Hitachi 912 (Roche Diagnostics, Indianapolis, IN), hexokinase catalyzes the phosphorylation of glucose by ATP. G-6-P is oxidized to 6-phosphogluconate in the presence of NADP by the enzyme glucose-6-phosphate dehydrogenase. No other carbohydrate is oxidized. The amount of NADH formed during the reaction is equivalent to the amount of D-glucose in the specimen and can be measured photometrically by the increase in absorbance at 340nm.

GLP-1 has been developed to measure biologically active GLP-1(7–36, 7-37) amide level will be measured by enzyme linked immunosorbent assay (Linco Research), with the lowest levels of detection 3 pM with no cross reactivity to GLP-1-(9–36) amide, GLP-2 and glucagon also have no cross reactivity.

CCK – concentrations will be measured by an immunoassay (Alpco Diagnostics) which utilizes rabbit antiserum to a synthetic cholecystokinin 26-33 sulphate (CCK 8 sulphate) and binds to most biological active forms with nearly equimolar potency.⁵⁷ It has essentially no cross-reactivity with gastrin and plasma CCK immunoreactivity did not increase during gastrin-17 infusion into healthy subjects. With this RIA, plasma CCK concentrations averaged ~ 1 pmol/L under basal conditions and increased to ~ 5 pmol/L after a meal.

PYY will be measured by radioimmunoassay (Linco Research, Inc.). PYY exists in at least 2 molecular forms, 1-36 and 3-36, both of which are physiologically active. There is no measureable crossreactivity to glucagon, ghrelin, insulin, GLP-1.

C-peptide levels will be measured by a 2-site immunometric (sandwich) assay using electrochemiluminescence detection (Cobas e411, Roche Diagnostics Indianapolis, IN). Patient specimen, biotinylated monoclonal C-peptide specific antibody, and monoclonal C-peptide-specific antibody labeled with ruthenium react to form a complex. Streptavidin-coated micro particles act as the solid phase to which the complex becomes bound. Voltage is applied to the electrode inducing a chemiluminescent emission from the ruthenium. (red top, needs 0.5 ml serum, 3 ml tube)

Plasma insulin concentrations will be measured using a chemiluminescence assay (Access Assay; Beckman Coulter, Chaska, MN). Established approaches will be used to estimate β cell responsiveness and insulin sensitivity from plasma glucose, insulin, and C-peptide concentrations.

Total ghrelin will be measured by a radioimmunoassay technique (Linco Research, Inc.). The assay uses 125I-labeled ghrelin and a ghrelin antiserum to determine the level of total ghrelin in plasma. There is no measurable crossreactivity to glucagon, GLP-1(7-36), insulin. (0.8 microL)

Summary of Blood Collections. The total blood collected for this study is 403 ml.

Item	Test ID	Lab	Tube (amount required)
Complete blood count [#]	9109	Central Clinical Lab	3 ml EDTA
Tissue transglutaminase antibody [#]	83671	Immunology Lab	5 ml red top (0.5 ml serum)*
High sensitive CRP [#]	82044	ICL	1 ml serum
Fasting glucose [#]	7702	ICL	2 ml Sodium fluoride (0.5 ml)
Lipase [#]	8328	Central Clinical Lab	5 ml SST (1 ml serum)
Glycosylated hemoglobin [#]	82080	Central Clinical Lab	3 ml EDTA (2 ml whole blood)
Sodium	81692	Central Clinical Lab	5 ml SST **
Potassium	81390	Central Clinical Lab	5 ml SST **
Bicarbonate	876	Central Clinical Lab	5 ml SST **
Calcium	8432	Central Clinical Lab	5 ml SST **
Chloride	8460	Central Clinical Lab	5 ml SST **
Phosphorus	8029	Central Clinical Lab	5 ml SST **
AST	8360	Central Clinical Lab	5 ml SST **
Total bilirubin	81785	Central Clinical Lab	5 ml SST **
Alkaline phosphatase	8340	Central Clinical Lab	5 ml SST **
Glucose ¹	7702	ICL	2 ml Sodium fluoride (0.5 ml)

GLP-1 ¹	80075	ICL	3 ml EDTA (1 ml plasma)
CCK ¹	90162	ICL	4 ml EDTA (2 ml plasma)
PYY ¹	7670	ICL	3 ml EDTA (1 ml plasma)
Total ghrelin ¹	83334	ICL	1.6 ml EDTA (0.8 ml plasma)
C-peptide ¹	7653	ICL	5 ml red top (0.5 ml serum)*
Insulin ¹	7661	ICL	5 ml red top (0.8 ml serum) *
Plasma PP ²		ICL	3 ml EDTA (1 ml plasma)
Epigenetic analysis (RRBS) ³	31669	BAP Lab	500 ng DNA (EDTA tube buffy coat - snap-frozen in liquid nitrogen)
DNA for SNPs ³	31669	BAP Lab	Use buffy coat from EDTA tubes
RNA analysis ⁴	31669	BAP Lab	3 PAX gene tubes (2.5 ml each, total = 7.5 ml)

* Same tube for these specimens; ** Same tube for these specimens; [#] Safety and screening assessments, one time only.

¹ Collected 18 timepoints during each study; ² Sham feeding test – 8 collections;

³ Requires DNA. Will be obtained from EDTA tubes for ghrelin and a separate 10 ml tube;

³ PAX gene tube

3.4 Gene and Epigenetic Studies

i) Towards our long-term objective of uncovering associations between SNPs and dyspepsia and gastroparesis, we propose to extract DNA from blood, to be drawn from study participants. Genome-wide analysis will be conducted using Illumina 610 QUAD microarray or comparable approaches. Genotype-phenotype correlations will be examined using these patients and other patients in ongoing studies.

ii) Epigenetic studies. The association of differentially methylated CpG or genomic regions with gastroparetic symptoms, abnormal (delayed or rapid) gastric emptying, intestinal chemosensitivity, presence or absence of autonomic neuropathy, nephropathy, and retinopathy, will be evaluated; age, gender, glycosylated hemoglobin, and duration of DM are potential covariates.

Reduced representation bisulfite sequencing (RRBS) and analysis will be performed. RRBS is base resolution methylation sequencing to assess genome-wide DNA methylation enriched in CpG rich regions in the genome, particularly in the coding and its neighboring regions. We will use Next Gene Sequencing to determine methylation status as outlined by Gu et al ⁵⁸ and Illumina. Briefly, DNA is digested with Msp1 and the digested DNA is purified. Purified DNA is end repaired and adenylated. DNA is ligated to Illumina adapters using T4 DNA ligase. Ligated DNA is purified and

size selected using Ampure beads. Purified size selected DNA undergoes bisulfite modification and clean-up. Modified DNA is PCR amplified and purified using Ampure beads. This RRBS library DNA will be run on the Illumina HiSeq2000, indexing 4 samples per lane, using a standard operating procedure based on Illumina's protocol.

An additional 7.5 ml of blood will be collected for mRNA-sequencing and miRNA sequencing in 3 Paxgene tubes (2.5 ml each).

3.5 Data Analysis

Chemosensitivity will be measured using a 100mmVAS anchored at each end (left end:No symptoms, right end: Severe symptoms). The distribution of this measure will be checked for its approximation to a gaussian distribution. An analysis to provide preliminary data for a new grant submission will be examined after approximately 15 subjects (5 DM, 10 controls) have completed the studies, but there is no intention of stopping the study based on the preliminary results. We anticipate conducting this analysis in July 2014 or thereafter.

Question	Summary Parameter	Statistical analysis
Sensation (symptoms) during enteral infusion (Aim 1)	VAS score	i) compare VAS scores in response to lipid infusion + exendin vs. lipid infusion + placebo separately for each group using a 2-sample t-test or Wilcoxon rank sum test ii)compare the delta VAS scores (exendin – placebo) to assess the association with subject status (FD,DM, and controls) using an ANOVA model (adjusting for covariates, eg, type of DM) or Kruskal-Wallis test.
Relationship between intestinal chemosensitivity and severity of ongoing upper GI symptoms	Mean symptom score and QoL defined by the Nepean dyspepsia index and GCSI diary	Multiple linear regression with the dependent variable = mean symptom score, and separately the QoL score (possibly after rank transformation). Potential predictor variables = VAS score during enteral lipid + placebo infusion, gastric emptying, age, gender, BMI
Relationship between sensation and plasma hormones during lipid infusion (Aim 2)	Using the VAS score from the enteral infusions and for hormones, the AUC values	Spearman correlations between the VAS score and the AUC values for the various hormones, overall and separately by group (FD,DM, controls)
Small intestinal tight junction proteins,	Protein expression, lactulose:mannitol	Assess the associations between permeability and protein expression, vs. the VAS score, based on Spearman

permeability, intestinal chemosensitivity	ratio, VAS score	correlations.
---	------------------	---------------

The sample size estimate is based on our previous study which evaluated intestinal chemosensitivity in functional dyspepsia in which the proportions with severe symptoms were assessed. [In the current proposal, symptoms during lipid infusion will be evaluated on a continuous VAS scale, likely providing the ability to detect smaller effect sizes]. The effect of lipid plus

Group	Proportions (%) with severe symptoms			
	exendin+lipid vs. placebo+lipid	Controls vs. FD (exendin+lipid)	Controls vs. DM (exendin+lipid)	FD vs. DM (exendin+lipid)
Controls(n=12 vs n=12)	10% vs. 68%	10% vs. 60%	10% vs. 60%	30% vs. 73%
functional dyspepsia (n=20 vs. 20)	10% vs. 50%			
DM (n=20 vs. 20)	10% vs. 50%			

placebo vs. lipid plus exendin in response to enteral infusion can be based on a comparison of the proportions with severe symptoms using a two sample t-test. The association of group status with response to lipid plus exendin can also be assessed using a two-sample t-test for proportions. There is ~80% power (alpha =0.05, 2-sided) to detect the specified differences listed in the table below.

3.6 Potential Pitfalls

To reduce the likelihood of patient intolerance, a smaller diameter tube will be used for enteral nutrient infusion. Only lipid and not carbohydrate infusions will be given; hence the duration of this study is shorter and hyperglycemia during nutrient infusion is less likely.

4 HUMAN SUBJECTS

Description: Studies will be conducted over 3 days. Subjects will be remunerated \$ 600. A screening assessment will be performed to ensure subjects fulfill entry criteria. Subjects will fast overnight prior to study days. Study procedures will be performed using established techniques by experienced technicians in our programs.

Research Materials: Blood samples (hormones, glucose, extract DNA and RNA) and stool samples will be obtained for this study.

Population and Recruitment of Subjects: Healthy subjects will be recruited from the community and patients with DM and dyspepsia will be recruited from the clinical practice and to patients who have provided research authorization. The Institutional Review Board will approve all recruitment materials. After discussing the study procedure and its risks, informed consent will be obtained, before study procedures. No children or prisoners will be recruited.

Potential Risks: Blood sampling. Blood samples are collected by venipuncture for this study. Assays for measuring blood glucose and plasma hormone will require 19ml blood at each of 18 time points during the enteral infusion study. The total blood drawn is 408 ml. Bruising can occur with venipuncture, as can fainting, etc. Risk Monitoring/Risk Reduction: The samples are collected using aseptic technique in the venipuncture area of the Clinical Research Unit where facilities are available should untoward reactions (fainting, etc.) occur. Given the aseptic nature of the sample collection and the small risk of bruising, the monitoring plan is focused on advising volunteers to call the co-investigators (physician) should they have unusual pain or discomfort from the venipuncture site.

Enteral tube placement This will require exposure to radiation and may be associated with discomfort. The risk of perforation with a soft-tipped feeding tube is very low. Risk Monitoring / Risk Reduction: The radiation safety committee will review all projected exposure to radiation prior to approval of the protocol by the IRB. The small bore soft-tipped feeding tube will be positioned by trained personnel under fluoroscopic guidance in the CRU. Subjects will be closely monitored for tolerance.

Enteral nutrition. This may be associated with nausea and abdominal cramping symptoms. Risk Monitoring/Risk Reduction: Subjects will fast overnight prior to the lipid infusion. To minimize the effect on blood glucose, lipid instead of glucose infusion is being administered. They will be closely monitored by trained personnel.

Exendin 9-39 infusion. The effects of exendin infusion on postprandial blood glucose concentrations is modest. For example, in 1 study blood glucose concentrations after a duodenal meal were on average only 6 mg/dl higher in healthy subjects and 19 mg/dl higher in T2DM for exendin 9-39 versus saline ⁴⁷. Risk Monitoring / Risk Reduction: Blood glucose concentrations will be monitored regularly and insulin will be administered if necessary.

Gastric emptying. Gastric emptying and small intestinal transit will be measured by scintigraphy. This will require exposure to radiation. Risk Monitoring / Risk Reduction: The radiation safety committee will review all projected exposure to radiation prior to approval of the protocol by the IRB. Pregnancy tests will be checked within 48 hours prior to each study day. Vital signs will be recorded before the study.

Dosimetry and Organ Exposure in mrad (see attached under APPENDIX)

In view of the radiation exposure, all females of childbearing age will be required to have a negative urine pregnancy test within 1 week of this study.

Protection: The key personnel in this application have completed the required education on the protection of human research participants. The institution has established a formal program entitled the Mayo Investigator Training Program or MITP. The MITP is a web based educational course designed to provide all personnel involved in human subject research with training about human subject protection. All Mayo personnel engaged in human subject research are required to complete the course.

Data Safety and Monitoring Plan. The ultimate goal of this application is to further our understanding of GI symptoms in patients with dyspepsia and diabetes. The DSMP utilized will adhere to the protocol approved by the Mayo Clinic IRB. We propose the following plan: -

Data quality and management: The principal investigator will review all data collection forms on a three-monthly basis for completeness and accuracy of the data as well as protocol compliance.

Adverse events grading: The common grading scale listed below will be used to grade AEs:

0 No adverse event or within normal limits or not clinical significant

- 1 Mild AE, did not require treatment
- 2 Moderate AE, resolved with treatment
- 3 Severe AE, resulted in inability to carry on normal activities and required professional medical attention
- 4 Life threatening or disabling AE
- 5 Fatal AE

Attribution scale: An adverse event includes both, an expected side effect that is of a serious nature, or an unexpected side effect/ event regardless of severity. All events will be graded as to their attribution (unrelated to protocol, or possibly, probably, or definitely related to protocol). Any event that is reported to either the principal investigator or his designated research associates by the subject or medical staff caring for the subject and which meets the criteria will be documented as such.

Data Monitoring. The majority of data generated from these protocols will be from analyses performed in our laboratory or the immunochemical core laboratory. Standard quality control procedures are in place for each assay. The frequency of data review for this study differs according to the type of data and can be summarized in the following table:

Data type	Frequency of review
Subject accrual (adherence to protocol regarding demographics, inclusion/exclusion)	Weekly
Adverse event/safety rates (injuries)	Weekly
Annual report	Yearly for IRB

Informed Consent. Written informed consent will be obtained from all individuals who participate in the study. The principal investigator or his co-investigators meet with each participant, review the consent form in detail and confirm the subjects understanding of the study. They answer all questions posed by the participants and when convinced that the subject verbally demonstrates understanding of the protocol obtains a signed consent. Only designated staff are authorized to obtain informed consent.

Benefits: This study exposes subjects to risks detailed above. However, it will advance our knowledge of the mechanisms of GI sensorimotor dysfunctions in patients with DM.

5 SAFETY AND ADVERSE EVENTS

5.1 Definitions

Unanticipated Problems Involving Risk to Subjects or Others (UPIRTSO)

Any unanticipated problem or adverse event that meets the following three criteria:

- **Serious:** Serious problems or events that results in significant harm, (which may be physical, psychological, financial, social, economic, or legal) or increased risk for the subject or others (including individuals who are not research subjects). These include: (1) death; (2) life threatening adverse experience; (3) hospitalization - inpatient, new, or prolonged; (4) disability/incapacity - persistent or significant; (5) birth defect/anomaly; (6) breach of

confidentiality and (7) other problems, events, or new information (i.e. publications, DSMB reports, interim findings, product labeling change) that in the opinion of the local investigator may adversely affect the rights, safety, or welfare of the subjects or others, or substantially compromise the research data, **AND**

- Unanticipated: (i.e. unexpected) problems or events are those that are not already described as potential risks in the protocol, consent document, not listed in the Investigator's Brochure, or not part of an underlying disease. A problem or event is "unanticipated" when it was unforeseeable at the time of its occurrence. A problem or event is "unanticipated" when it occurs at an increased frequency or at an increased severity than expected, **AND**
- Related: A problem or event is "related" if it is possibly related to the research procedures.

Adverse Event

An untoward or undesirable experience associated with the use of a medical product (i.e. drug, device, biologic) in a patient or research subject.

Serious Adverse Event

Adverse events are classified as serious or non-serious. Serious problems/events can be well defined and include;

- death
- life threatening adverse experience
- hospitalization
- inpatient, new, or prolonged; disability/incapacity
- persistent or significant birth defect/anomaly

and/or per protocol may be problems/events that in the opinion of the sponsor-investigator may have adversely affected the rights, safety, or welfare of the subjects or others, or substantially compromised the research data.

Other important medical events are those that may not be immediately life threatening, but are clearly of major clinical significance. They may jeopardize the subject, and may require intervention to prevent one of the other serious outcomes noted above. For example, drug overdose or abuse, a seizure that did not result in in-patient hospitalization or intensive treatment of bronchospasm in an emergency department would typically be considered serious.

All adverse events that do not meet any of the criteria for serious, should be regarded as **non-serious adverse events**.

Withdrawal criteria

- Inability to place nasoduodenal feeding tube
- Inability to obtain intravenous access
- Severe abdominal discomfort, nausea or vomiting during enteral nutrient infusion
- Severe hyperglycemia (> 400 mg/dl) unresponsive to insulin therapy

Adverse Event Reporting Period

Example

For this study, the study treatment follow-up period is defined as 1 days following the last administration of study treatment. The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up.

Preexisting Condition

A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

General Physical Examination Findings

At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

Post-study Adverse Event

All unresolved adverse events should be followed by the sponsor-investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the sponsor-investigator should instruct each subject to report, to the sponsor-investigator, any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study.

Hospitalization, Prolonged Hospitalization or Surgery

Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for an adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

- Hospitalization or diagnostic or elective surgical procedures for a preexisting condition. Surgery should not be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study (e.g., diabetes mellitus), unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

5.2 Recording of Adverse Events

At each contact with the subject, the study team must seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events should be recorded immediately in the source document, and also in the appropriate adverse event section of the case report form (CRF). All clearly related signs, symptoms, and abnormal diagnostic, laboratory or procedure results should be recorded in the source document.

All adverse events occurring during the study period must be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been ultimately determined that the study treatment or participation is not the probable cause. Serious adverse events that are still ongoing at the end of the study period must be followed up, to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be at least possibly related to the study treatment or study participation should be recorded and reported immediately.

5.3 Reporting of Serious Adverse Events and Unanticipated Problems

When an adverse event has been identified, the study team will take appropriated action necessary to protect the study participant and then complete the Study Adverse Event Worksheet and log. The sponsor-investigator will evaluate the event and determine the necessary follow-up and reporting required.

5.3.1 Sponsor-Investigator reporting: notifying the Mayo IRB

This section is written with the intent that a specifically designed adverse event worksheet will be completed for any SAE. The information on that worksheet will be reported to the IRB in a de-identified manner..

The IRB requirements reflect the guidance documents released by the Office of Human Research Protections (OHRP), and the Food and Drug Administration (FDA) in early 2007 and are respectively entitled “Guidance on Reviewing and Reporting Unanticipated Problems Involving Risks to Subjects or Others and Adverse Events” and “Guidance for Clinical Investigators, Sponsors, and IRBs: Adverse Event Reporting – Improving Human Subject Protection.”

(Refer to the IRB Policy and Procedure on Reporting Unanticipated Problems Involving Risks to Subjects or Others to the IRB, on the IRB web site. Describe how this will be carried out by the study team and investigator. Also address notification of other investigators if necessary.
<http://mayocontent.mayo.edu/irb/DOCMAN-0000047812>

The sponsor-investigator will report to the Mayo IRB any UPIRTSOs and Non-UPIRTSOs according to the Mayo IRB Policy and Procedures. According to Mayo IRB Policy any serious adverse event (SAE) which the Principal Investigator has determined to be a UPIRTSO must be reported to the Mayo IRB as soon as possible but no later than 5 working days after the investigator first learns of the problem/event.

The sponsor-investigator will review all adverse event reports to determine if specific reports need to be made to the IRB and FDA. The sponsor-investigator will sign and date the adverse event report when it is reviewed. For this protocol, only directly related SAEs/UIPRTSOs will be reported to the IRB.

5.3.2 Sponsor-Investigator reporting: Notifying the FDA

The sponsor-investigator will report to the FDA all unexpected, serious suspected adverse reactions according to the required IND Safety Reporting timelines, formats and requirements.

Unexpected fatal or life threatening suspected adverse reactions where there is evidence to suggest a causal relationship between the study drug/placebo and the adverse event, will be reported as a serious suspected adverse reaction. This will be reported to the FDA on FDA Form 3500A, no later than 7 calendar days after the sponsor-investigator's initial receipt of the information about the event.

Other unexpected serious suspected adverse reactions where there is evidence to suggest a causal relationship between the study drug/placebo and the adverse event, will be reported as a serious suspected adverse reaction. This will be reported to the FDA on FDA Form 3500A, no later than 15 calendar days after the sponsor-investigator's initial receipt of the information about the event.

Any clinically important increase in the rate of serious suspected adverse reactions over those listed in the protocol or product insert will be reported as a serious suspected adverse reaction. This will be reported to the FDA on FDA Form 3500A no later than 15 calendar days after the sponsor-investigator's initial receipt of the information about the event.

Findings from other studies in human or animals that suggest a significant risk in humans exposed to the drug will be reported. This will be reported to the FDA on FDA Form 3500A, no later than 15 calendar days after the sponsor-investigators initial receipt of the information about the event.

5.5 Stopping Rules

All study procedures (blood draws, upper endoscopy, enteral nutrient infusion, exendin 9-39) are very safe. If any subject develops an intestinal perforation or clinically significant bleeding during endoscopy or intestinal lipid infusion, the study will be placed on hold until the cause of the complication is determined.

5.6. Medical Monitoring

It is the responsibility of the Principal Investigator to oversee the safety of the study at his/her site. This safety monitoring will include careful assessment and appropriate reporting of adverse events as noted above, as well as the construction and implementation of a site data and safety-monitoring plan (see section 10 "Study Monitoring, Auditing, and Inspecting"). Medical monitoring will include a regular assessment of the number and type of serious adverse events.

APPENDIX

Stool Specimen Collection Instructions

Attaching the collection container

1. Open the kit

2. Lay the collection container on a flat surface with the labeled side facing up. Fold up the two cardboard sides (Figure 1).

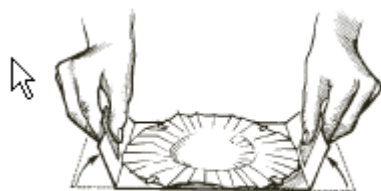


Figure 1

3. Remove the backing from the tape on each of the cardboard sides.
4. Insert the collection container into the toilet bowl and attach the tape to the top of the toilet seat toward the back half of the bowl (Figure 2). The cardboard sides should be up against the bottom of the toilet seat.



Figure 2

5. Shape the paper dish (middle part of the collection container) into a bowl by gently pushing down the center.

Collection a sample

1. Do not urinate into the collection container. (You may wish to urinate before attaching the collection container to the toilet seat.)
2. Have a bowel movement into the paper dish
3. Take out the collection tube and unscrew the cap. Use the spoon attached to the cap to scoop a marble-sized sample. Insert filled spoon back into the tube and tightly screw the cap onto the collection tube (Figure 3).

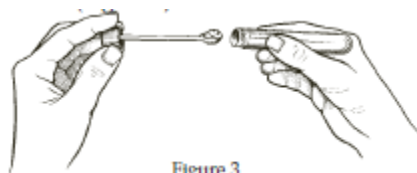


Figure 3

Disposal

1. Remove the paper dish holding the stool by gently lifting up the four attachment sites. Flush the paper dish and stool.
2. Remove the cardboard frame from the toilet seat and discard it in the wastebasket.
3. Wash your hands thoroughly with soap and water.

Returning the sample to Mayo Clinic

1. Be sure the collection tube cap is tightly fastened.
2. Place the collection tube containing your sample into the small white bag.



Collection tube

If you are at Mayo Clinic:

At your earliest convenience, return the white bag containing your specimen to: Station S/Specimen Collection Cart
Monday through Friday, 7 a.m. to 5 p.m.

If you are mailing your stool specimen to Mayo Clinic:

At your earliest convenience, mail your specimen to Mayo Clinic using the prepaid mailer provided.



Mailing tube

Dosimetry and Organ Exposure in mrad

Gastric and Small Bowel Transit

^{99m}Tc-sulfur colloid

Effective Dose Equivalent (He)

organ doses in mrad

organ doses in mrad		Organ/Wt											
	Administered	Body	Gonads	Breast	RBM	Lung	Thyroid	Bone	O#1: ULI	O#2: LLI	O#3: SI	O#4: Stomach	O#5: Uterus
RAM	Activity (mCi)		0.25	0.15	0.12	0.12	0.03	0.03	0.06	0.06	0.06	0.06	0.06
Tc-99m Non- Absorbable Markers (solids)	1	21	96	2	19	4	0	17	444	307	226	218	59
Effective Dose			24	0	2	0	0	1	27	18	14	13	4
												He (mrem):	103

¹¹¹In-DTPA

Effective Dose Equivalent (He)

organ doses in mrad

organ doses in mrad		Organ/Wt											
	Administered	Body	Gonads	Breast	RBM	Lung	Thyroid	Bone	O#1: ULI	O#2: LLI	O#3: SI	O#4: Stomach	O#5: Bladder
RAM	Activity (mCi)	0.25		0.15	0.12	0.12	0.03	0.03	0.06	0.06	0.06	0.06	0.06
In-111 Non-Absorbable Markers (solids)	0.1	16	155	2	36	3	0	11	407	740	185	93	41
Effective Dose			39	0	4	0	0	0	24	44	11	6	2
												He (mrem):	132

REFERENCES

1. Camilleri M, Bharucha AE, Farrugia G. Epidemiology, mechanisms, and management of diabetic gastroparesis. *Clinical Gastroenterology & Hepatology* 2011;9:5-12; quiz e17.
2. Choung RS, Locke GR, 3rd, Schleck CD, et al. Risk of gastroparesis in subjects with type 1 and 2 diabetes in the general population. *Am J Gastroenterol* 2012;107:82-88.
3. Lacy BE. Functional dyspepsia and gastroparesis: one disease or two? *Am J Gastroenterol* 2012;107:1615-1620.
4. Bharucha AE, Camilleri M, Forstrom L, et al. Relationship between clinical features and gastric emptying disturbances in diabetes mellitus. *Clin Endocrinol (Oxf)* 2008;70:415-420.
5. Samsom M, Salet GA, Roelofs JM, et al. Compliance of the proximal stomach and dyspeptic symptoms in patients with type I diabetes mellitus. *Digestive Diseases & Sciences* 1995;40:2037-2042.
6. Kumar A, Attaluri A, Hashmi S, et al. Visceral hypersensitivity and impaired accommodation in refractory diabetic gastroparesis. *Neurogastroenterol Motil* 2008;20:635-642.
7. Ajaj W, Goehde SC, Papanikolaou N, et al. Real time high resolution magnetic resonance imaging for the assessment of gastric motility disorders. *Gut* 2004;53:1256-1261.
8. Barbera R, Feinle C, Read NW. Nutrient-specific modulation of gastric mechanosensitivity in patients with functional dyspepsia. *Dig Dis Sci* 1995;40:1636-1641.
9. Barbera R, Feinle C, Read NW. Abnormal sensitivity to duodenal lipid infusion in patients with functional dyspepsia. *Eur J Gastroenterol Hepatol* 1995;7:1051-1057.
10. Feinle C, Meier O, Otto B, et al. Role of duodenal lipid and cholecystokinin A receptors in the pathophysiology of functional dyspepsia. *Gut* 2001;48:347-355.
11. Bjornsson E, Sjoberg J, Ringstrom G, et al. Effects of duodenal lipids on gastric sensitivity and relaxation in patients with ulcer-like and dysmotility-like dyspepsia. *Digestion* 2003;67:209-217.
12. Feinle C, D'Amato M, Read NW. Cholecystokinin-A receptors modulate gastric sensory and motor responses to gastric distension and duodenal lipid. *Gastroenterology* 1996;110:1379-1385.
13. Faas H, Hebbard GS, Feinle C, et al. Pressure-geometry relationship in the antroduodenal region in humans. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2001;281:G1214-1220.
14. Bharucha AE, Camilleri M, Low PA, et al. Autonomic dysfunction in gastrointestinal motility disorders. *Gut* 1993;34:397-401.
15. Buysschaert M, Donckier J, Dive A, et al. Gastric acid and pancreatic polypeptide responses to sham feeding are impaired in diabetic subjects with autonomic neuropathy. *Diabetes* 1985;34:1181-1185.
16. Vanheel H, Vicario M, Vanuytsel T, et al. Impaired duodenal mucosal integrity and low-grade inflammation in functional dyspepsia. *Gut* 2014;63:262-271.
17. Damci T, Nuhoglu I, Devranoglu G, et al. Increased intestinal permeability as a cause of fluctuating postprandial blood glucose levels in Type 1 diabetic patients. *Eur J Clin Invest* 2003;33:397-401.
18. Secondulfo M, Iafusco D, Carratu R, et al. Ultrastructural mucosal alterations and increased intestinal permeability in non-celiac, type I diabetic patients. *Digestive & Liver Disease* 2004;36:35-45.

19. Sapone A, de Magistris L, Pietzak M, et al. Zonulin upregulation is associated with increased gut permeability in subjects with type 1 diabetes and their relatives. *Diabetes* 2006;55:1443-1449.
20. Secondulfo M, de Magistris L, Sapone A, et al. Intestinal permeability and diabetes mellitus type 2. *Minerva Gastroenterol Dietol* 1999;45:187-192.
21. Feldman M, Corbett DB, E.J. R. Abnormal gastric function in longstanding, insulin-dependent diabetic patients. *Gastroenterology* 1979;77:12-17.
22. Delgado-Aros S, Camilleri M, Castillo EJ, et al. Effect of gastric volume or emptying on meal-related symptoms after liquid nutrients in obesity: a pharmacologic study. *Clinical Gastroenterology & Hepatology* 2005;3:997-1006.
23. Delgado-Aros S, Camilleri M, Cremonini F, et al. Contributions of gastric volumes and gastric emptying to meal size and postmeal symptoms in functional dyspepsia.[see comment]. *Gastroenterology* 2004;127:1685-1694.
24. Vanheel H, Farre R. Changes in gastrointestinal tract function and structure in functional dyspepsia. *Nature Reviews Gastroenterology & Hepatology* 2013;10:142-149.
25. Bredenoord AJ, Chial HJ, Camilleri M, et al. Gastric accommodation and emptying in evaluation of patients with upper gastrointestinal symptoms. *Clinical Gastroenterology and Hepatology* 2003;1:264-272.
26. Rathmann W, Enck P, Frieling T, et al. Visceral afferent neuropathy in diabetic gastroparesis. *Diabetes Care* 1991;14:1086-1089.
27. Camilleri M, Malagelada JR. Abnormal intestinal motility in diabetics with gastroparesis. *Eur J Clin Invest* 1984;420-427.
28. Rayner CK, Schwartz MP, van Dam PS, et al. Small intestinal glucose absorption and duodenal motility in type 1 diabetes mellitus. *Am J Gastroenterol* 2002;97:3123-3130.
29. Rayner CK, Schwartz MP, van Dam PS, et al. Upper gastrointestinal responses to intraduodenal nutrient in type 1 diabetes mellitus. *Eur J Gastroenterol Hepatol* 2004;16:183-189.
30. Gentilcore D, Chaikomin R, Jones KL, et al. Effects of fat on gastric emptying of and the glycemic, insulin, and incretin responses to a carbohydrate meal in type 2 diabetes. *J Clin Endocrinol Metab* 2006;91:2062-2067.
31. Pilichiewicz AN, Chaikomin R, Brennan IM, et al. Load-dependent effects of duodenal glucose on glycemia, gastrointestinal hormones, antropyloroduodenal motility, and energy intake in healthy men. *American Journal of Physiology - Endocrinology & Metabolism* 2007;293:E743-753.
32. Suzuki T, Hara H. Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. *Nutr Metab* 2010;7:19.
33. Camilleri M, Nadeau A, Lamsam J, et al. Understanding measurements of intestinal permeability in healthy humans with urine lactulose and mannitol excretion. *Neurogastroenterol Motil* 2010;22:e15-26.
34. Rao AS, Camilleri M, Eckert DJ, et al. Urine sugars for in vivo gut permeability: validation and comparisons in irritable bowel syndrome-diarrhea and controls. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2011;301:G919-928.
35. Lostia AM, Lionetto L, Principessa L, et al. A liquid chromatography/mass spectrometry method for the evaluation of intestinal permeability. *Clin Biochem* 2008;41:887-892.

36. Low PA, Caskey PE, Tuck RR, et al. Quantitative sudomotor axon reflex test in normal and neuropathic subjects. *Ann Neurol* 1983;14:573-580.
37. Low PA, Denq JC, Opfer-Gehrking TL, et al. Effect of age and gender on sudomotor and cardiovagal function and blood pressure response to tilt in normal subjects. *Muscle Nerve* 1997;20:1561-1568.
38. Low PA. Autonomic nervous system function. *J Clin Neurophysiol* 1993;10:14-27.
39. Sathananthan M, Farrugia LP, Miles JM, et al. Direct effects of exendin-(9,39) and GLP-1-(9,36)amide on insulin action, beta-cell function, and glucose metabolism in nondiabetic subjects. *Diabetes* 2013;62:2752-2756.
40. Shah M, Law JH, Micheletto F, et al. Contribution of endogenous glucagon-like peptide 1 to glucose metabolism after Roux-en-Y gastric bypass. *Diabetes* 2014;63:483-493.
41. Schirra J, Sturm K, Leicht P, et al. Exendin(9-39)amide is an antagonist of glucagon-like peptide-1(7-36)amide in humans. *Journal of Clinical Investigation* 1998;101:1421-1430.
42. Schirra J, Nicolaus M, Roggel R, et al. Endogenous glucagon-like peptide 1 controls endocrine pancreatic secretion and antro-pyloro-duodenal motility in humans.[see comment]. *Gut* 2006;55:243-251.
43. Schirra J, Nicolaus M, Woerle HJ, et al. GLP-1 regulates gastroduodenal motility involving cholinergic pathways. *Neurogastroenterol Motil* 2009;21:609-618, e621-602.
44. Ionut V, Liberty IF, Hucking K, et al. Exogenously imposed postprandial-like rises in systemic glucose and GLP-1 do not produce an incretin effect, suggesting an indirect mechanism of GLP-1 action. *American Journal of Physiology - Endocrinology & Metabolism* 2006;291:E779-785.
45. Jimenez A, Casamitjana R, Viaplana-Masclans J, et al. GLP-1 action and glucose tolerance in subjects with remission of type 2 diabetes after gastric bypass surgery. *Diabetes Care* 2013;36:2062-2069.
46. Jorgensen NB, Dirksen C, Bojsen-Moller KN, et al. Exaggerated glucagon-like peptide 1 response is important for improved beta-cell function and glucose tolerance after Roux-en-Y gastric bypass in patients with type 2 diabetes. *Diabetes* 2013;62:3044-3052.
47. Woerle HJ, Carneiro L, Derani A, et al. The role of endogenous incretin secretion as amplifier of glucose-stimulated insulin secretion in healthy subjects and patients with type 2 diabetes. *Diabetes* 2012;61:2349-2358.
48. Kielgast U, Holst JJ, Madsbad S. Antidiabetic actions of endogenous and exogenous GLP-1 in type 1 diabetic patients with and without residual beta-cell function. *Diabetes* 2011;60:1599-1607.
49. Weijenborg PW, Smout AJPM, Verseijden C, et al. Hypersensitivity to acid is associated with impaired esophageal mucosal integrity in patients with gastroesophageal reflux disease with and without esophagitis. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2014;307:G323-329.
50. Katzka DA, Ravi K, Geno DM, et al. Endoscopic Mucosal Impedance Measurements Correlate With Eosinophilia and Dilation of Intercellular Spaces in Patients With Eosinophilic Esophagitis. *Clinical Gastroenterology and Hepatology*.
51. Walker MM, Warwick A, Ung C, et al. The role of eosinophils and mast cells in intestinal functional disease. *Current Gastroenterology Reports* 2011;13:323-330.
52. Camilleri M, Zinsmeister AR, Greydanus MP, et al. Towards a less costly but accurate test of gastric emptying and small bowel transit. *Digestive Diseases & Sciences* 1991;36:609-615.

53. Karlsson FH, Tremaroli V, Nookaew I, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* 2013;498:99-103.
54. Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 2012;490:55-60.
55. Revicki DA, Camilleri M, Kuo B, et al. Development and content validity of a gastroparesis cardinal symptom index daily diary. *Aliment Pharmacol Ther* 2009;30:670-680.
56. Revicki DA, Camilleri M, Kuo B, et al. Evaluating symptom outcomes in gastroparesis clinical trials: validity and responsiveness of the Gastroparesis Cardinal Symptom Index-Daily Diary (GCSI-DD). *Neurogastroenterol Motil* 2012;24:456-463.
57. Rehfeld JF. Accurate measurement of cholecystokinin in plasma.[see comment]. *Clin Chem* 1998;44:991-1001.
58. Gu H, Smith ZD, Bock C, et al. Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. *Nature Protocols* 2011;6:468-481.