Effects of Glucagon-like Peptide-1-(7-36)amide (GLP-1) on Gastric Motor Function in Health and Diabetes: Potential Mechanism of Action

Doctoral thesis submitted to the Autonomous University of Barcelona by

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1. General Introduction

__Glucagon-like peptide-1__

Glucagon-like peptide-1 (GLP-1) is a peptide derived from a specific proteolytic processing of the proglucagon molecule in L cells of the jejunum and colon [1, 2]. Different forms of this peptide are generated from this proteolysis: GLP-1-(1-37), which is biologically inactive, and GLP-1-(7-37) or GLP-1-(7-36)amide, which are the biologically active forms [3-5]. GLP-1 will be used in this text to refer to the short, active forms of the molecule.

This peptide is being proposed as a new treatment in people with type II diabetes since it stimulates insulin gene expression [3] and potentiates glucose-induced insulin release from pancreatic β cells [6]. It has been shown that exogenous administration of GLP-1 helps to control postprandial hyperglycemia in type II diabetes [7].

Besides its role regulating endocrine secretion from the pancreas, GLP-1 has been shown to regulate several other gastrointestinal functions, for example it decreases gastric acid secretion [8], gastric emptying rate [9, 10] and intestinal transit [11].

GLP-1 may also play a role in the regulation of food intake. There is evidence in animal models and humans that administration of exogenous GLP-1 decreases food consumption over the short term and increases postprandial satiety [12-16]. Abdominal symptoms, such as nausea, have also been described after high doses of exogenous GLP-1 infusion [17, 18].

It is still not clear how these effects of GLP-1 are mediated. *In vitro* studies have demonstrated that direct binding of the peptide to the GLP-1 receptor in the pancreatic β cells is followed by insulin secretion [19]. Nevertheless, GLP-1 is rapidly inactivated when it is released into the circulation [20], being the *in vivo* half life of the active GLP-1 limited to approximately 1 minute. The rapid inactivation of GLP-1 in the circulation makes it unlikely that active GLP-1, released from the intestinal tract, can reach distant organs such as the pancreas. It is more likely that effects of GLP-1 on distant organs are neurally mediated. *In vitro*, binding of GLP-1 to receptors in gastric parietal cells stimulates acid production [21]. In contrast, *in vivo* administration of GLP-1 produces inhibition of gastric acid secretion [8]. The effects of GLP-1 on gastric acid and pancreatic secretions are abolished after vagotomy in animals and humans [22, 23]. These observations suggest that

the effects of GLP-1 are most likely to be vagally mediated. It has also been shown that GLP-1 modulates the vagally-mediated delay in gastric emptying in response to intraluminal nutrients [24]. Overall, these data suggest an inhibitory effect of GLP-1 on vagally-mediated reflexes in the upper gastrointestinal tract.

Gastric Accommodation and Vagal Function

The vagus nerve is known to modulate most of gastrointestinal functions, including secretion, absorption or motor functions. The abdominal vagus is known to carry mostly afferent (sensory) fibers whereas only 10% are efferent motor fibers [25]. Cell bodies of visceral vagal fibers lie in vagal ganglia and their central processes terminate predominantly in the nucleus of the solitary tract (NST). From the NST afferent fibers connect to several central regions (such as the hypothalamus, amygdala and limbic cortex). They also convey signals to the dorsal motor nucleus of the vagus (DMNV) from which efferent fibers convey messages to the NST and to the gut wall [26]. These anatomical connections provide the basis for the vago-vagal reflexes that are crucial for the adequate control of upper gastrointestinal functions. (See Introductory Schema)

The accommodation or relaxation of the stomach in response to meal ingestion has been shown to involve the vagus nerve since vagal denervation impairs gastric relaxation in animals [27-29] and humans [30]. This vagally-mediated reflex prevents the increase in intragastric pressure when food and fluid enter in the stomach [31], avoiding the development of postprandial symptoms such as nausea, bloating, pain or vomiting in health. There is also evidence suggesting that this gastric postprandial reflex plays an important role in the regulation of satiation [32, 33], one of the final signals that arise during meal ingestion and that contribute to meal termination [34].
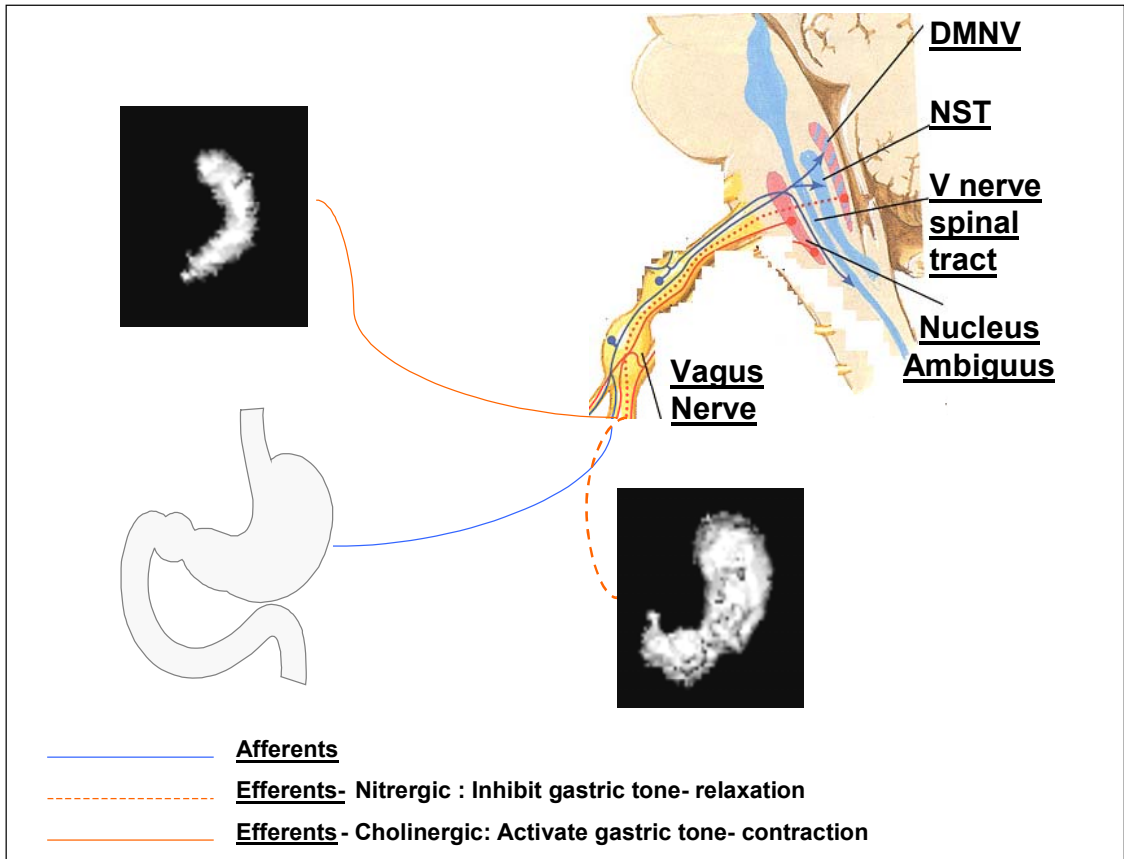
Study Hypotheses

Our first hypothesis was that GLP-1 induces inhibition of gastric accommodation reflex, as part of its general inhibition of vagally-mediated reflexes in the gastrointestinal tract, and that this effect could partly explain the effects of GLP-1 on food consumption and postprandial symptoms and satiety. To test this hypothesis, we compared, in healthy volunteers, the effects of intravenous infusion of GLP-1 and placebo, on gastric postprandial accommodation, postprandial response of plasma human pancreatic polypeptide (a surrogate marker of vagal abdominal function), the volume of a nutrient

liquid meal (Ensure®) ingested at maximum satiation, postprandial symptoms and gastric emptying of Ensure®. Measurements of gastric emptying were included to evaluate the potential confounder of delayed gastric emptying when assessing the effects of gastric accommodation on satiation and postprandial symptoms.

The results of the first study showed that GLP-1 does not inhibit postprandial gastric accommodation; on the contrary, it increased fasting and postprandial gastric volumes. This was accompanied by a marked inhibition of the normal postprandial increase of the pancreatic polypeptide, a hormone whose release is under vagal cholinergic control [35]. Since gastric tone (contraction) is maintained by vagal cholinergic (excitatory) input [27, 36], the results observed in our first study suggested that GLP-1 could induce gastric relaxation by inhibition of vagal (excitatory) cholinergic pathways during fasting and postprandially. (See Introductory Schema)

If this hypothesis was true, one would predict that in the presence of vagal dysfunction or vagotomy, GLP-1 would not induce gastric relaxation. To test this second hypothesis we studied the effect of the same intravenous infusion of GLP-1, compared to placebo, on fasting and postprandial gastric volumes in a sample of subjects with diabetes affected with vagal neuropathy.

Introductory Schema

DMNV

NST

V nerve
spinal
tract

Nucleus
Ambiguus

Vagus
Nerve

————————	**Afferents**
- - - - - - - - - -	**Efferents-** Nitrergic : Inhibit gastric tone- relaxation
————————	**Efferents -** Cholinergic: Activate gastric tone- contraction

2.1 Effect of GLP-1 on Gastric Volume, Emptying, Maximum Volume Ingested, and Postprandial Symptoms in Humans

2.1.1. Introduction

Glucagon-like peptide-1 (7-36) amide (GLP-1) is produced by the processing of proglucagon in enteroendocrine L cells of the intestinal mucosa. It is released in response to meal ingestion [37, 38], exerting a glucose-dependent effect on β cells of the pancreas enhancing insulin release. GLP-1 also has an inhibitory effect on the pancreatic α cells, reducing glucagon release [39, 40]. These properties provide the rationale for reducing glycemia and for its use in diabetes mellitus [7, 10, 41].

However, GLP-1 also exerts several effects on the upper digestive tract: inhibition of gastric acid and pancreatic exocrine secretions [23, 42, 43], delay in gastric emptying for liquids and solids in health [9] and diabetes [10]. The latter may result from enhanced pyloric tone or diminished antroduodenal motility during the interdigestive and fed states in health [44]. Preliminary data also indicate relaxation of the proximal stomach in response to intravenousGLP-1 during fasting [45]. GLP-1 has been reported to also reduce the amount of food and fluid consumed, to reduce hunger and enhance the feeling of fullness in health [16] and diabetes [14] but its effects on postprandial symptoms are unclear.

Previous studies showed dose-related, reversible inhibition of human pancreatic polypeptide (HPP) release in response to a meal after subcutaneous or intravenous infusion of GLP-1. GLP-1 also inhibits centrally-induced pancreatic and gastric acid secretions [42], and these effects are lost after abdominal vagotomy in humans [22] and pigs [23], suggesting an inhibition of efferent vagal-cholinergic function.

Postprandial gastric accommodation is a vagally-mediated reflex [46, 47]. Impaired gastric accommodation is an important cause of postprandial symptoms [48-51]. We hypothesized that GLP-1 diminishes the postprandial gastric accommodation response by inhibition of vagal function, reducing maximum volume ingested and increasing postprandial symptoms. The aims of this study were to compare the effects of GLP-1 on postprandial gastric volumes, gastric emptying, maximum tolerated volume of a nutrient liquid meal, postprandial symptoms and vagal function in healthy volunteers.

2.1.2. Subjects and Methods

Study Population

Healthy volunteers over 18 years of age were recruited from the local community by public advertisement. Exclusion criteria included: pregnant or breastfeeding females, prior abdominal surgery other than appendectomy or tubal ligation; positive symptoms on an abridged bowel disease questionnaire; present or previous chronic gastrointestinal illness; systemic disease or use of medications that may alter gastrointestinal motility.

Study Design

This study was approved by the Mayo Institutional Review Board. Eligible volunteers gave their written informed consent and were randomized to receive either GLP-1 (Bachem, San Diego, CA) as an infusion of 1.2 pmol/kg/min over 60 minutes, or saline infusion (placebo) in a double blind design.

The study was performed on two consecutive days. On the first day (*Protocol 1*), subjects underwent assessment of gastric volumes and measurements of fasting and postprandial glucose and HPP. On the second day (*Protocol 2*), maximum tolerated volume (MTV), scintigraphic gastric emptying and postprandial symptoms were assessed. GLP-1 or saline (placebo) was infused for sixty minutes on both occasions.

GLP-1

GLP-1 was infused at a rate of 1.2 pmol/Kg/min. Previous studies have demonstrated that steady state levels are achieved within ~30 minutes from the onset of theinfusion [41]. Therefore, the physiological measurements of gastric accommodation, emptying and satiety, as well as the plasma levels of glucose and HPP, were done under steady levels for a total of at least 30 minutes.

We used an infusion rate of GLP-1 of 1.2 pmol/Kg/min since it has been previouslyshown to affect gastrointestinal function and satiety in humans [41, 43-45]. Higher rates of GLP-1 infusion may cause gastrointestinal distress [17].

99mTc-SPECT method to measure gastric volume

We used a noninvasive method to measure fasting and postprandial gastric volumes in humans using single photon emission computed tomography (SPECT) [52]. This method has been recently validated in vitro and in vivo [53]. In healthy volunteers, simultaneous measurements of postprandial gastric volume changes with SPECT and the

barostat balloon device were strongly correlated (r = 0.9) [53]. The latter is currently considered the gold standard for the measurement of gastric accommodation. We have also showed the high intraobserver reproducibility of this technique to measure gastric volumes [54]. In dyspeptic patients, the SPECT technique has reproduced the changes in gastric volume [55] obtained by using the barostat device [33].

The method has been described in detail elsewhere [52, 55]. Briefly, 10 minutes after intravenous injection of ^{99m}Tc-sodium pertechnetate, dynamic tomographic acquisition of the gastric wall was performed using a dual-head gamma camera (Helix SPECT System, Elscint, Haifa, Israel) which performs orbits of 360° around the supine participant and takes 10 minutes per orbit. Each orbit leads to a three-dimensional rendering of the stomach using the AVW 3.0 (Biomedical Imaging Resource, Mayo Foundation, Rochester, MN) image processing libraries. This was accomplished by identifying the stomach in the transaxial SPECT images and separating it from the background noise using a semi-automated segmentation algorithm (Fig. 1).

A customized algorithm was developed to estimate the volume of the proximal stomach; this algorithm estimates the longest axis of the reconstructed stomach and divides it into a proximal two-thirds and distal one-third. The volume corresponding to the length of the proximal two-thirds was then obtained.

Protocol 1: Measurement of gastric volumes, plasma levels of glucose and HPP

After an 8 hour period of fasting, patients lay down on the SPECT camera and the 10mCi ^{99m}Tc-sodium pertechnetate was injected intravenously. Ten minutes later, a first orbit (360º over 10 minutes) was performed for baseline (pre-infusion) tomographic images (Fig. 2).

Infusion of 1.2 pmol/Kg/min GLP-1 or saline placebo was started. After 30 minutes' infusion (time to achieve steady levels), a second image was obtained over 10 minutes to assess effects of the GLP-1 on fasting gastric volumes.

A nutrient liquid meal (Ensure®: 300 mL, 316 Kcal, 7.6g fat, 50.6g carbohydrate and 11.4g protein) was ingested over 3 minutes, and two further 10 minute images were obtained to measure postprandial gastric volumes.

Blood samples were taken at baseline (pre-infusion), after 30 and 40 minutes' infusion (fasting period), and 10 and 20 minutes postprandially for measurement of

glucose and HPP. Plasma glucose concentrations were measured using a glucose oxidase method (using a glucose analyzer). Plasma levels of HPP were analyzed using a radioimmunoassay kit [56, 57].

Protocol 2: Measurement of MTV, gastric emptying and postprandial symptoms

To compare effects of GLP-1 and placebo on MTV (that is, the volume ingested until maximum satiety is reached), we adapted the method used by Tack et al. [33], appending a scintigraphic evaluation of gastric emptying by radiolabeling Ensure® ingested during the test (Fig. 3).

After 30 minutes of GLP-1 or saline placebo infusion to achieve steady state, subjects were asked to ingest Ensure® at a constant rate (30 mL per minute) by refilling a glass with a perfusion pump, and drinking at the filling rate. Participants scored their level of satiety during the drink test using a graphic rating scale graded 0-5 (0 = no symptoms; 5 = maximum or unbearable satiety). Participants were told to stop meal intake when a score of 5 was reached. The total volume ingested was the MTV.

To assess gastric emptying, the second glass of Ensure® was radiolabeled with 50 µCi of ^{111}In-DTPA and 1-minute duration scans of the abdomen were obtained at 10-minute intervals for the first 30 minutes and then at 15-minute intervals until at least 50% of the meal was emptied, or for a maximum of 3 hours after the meal.

Thirty minutes after completing ingestion of the Ensure®, participants were requested to score their postprandial symptoms (nausea, bloating, fullness, pain) using a 10cm visual analog scale anchored with the words unnoticeable and unbearable at the left and right ends of the lines. This symptom assessment is consistent with previous studies in the literature [58].

Data Analysis

Gastric volumes

Total and proximal gastric volume at baseline (pre-infusion), fasting and during two postprandial periods (0-10 minutes and 10-20 minutes) were measured; the postprandial gastric volume was calculated from the average of the two postprandial volumes. Volume change from baseline (pre-infusion) to fasting and postprandial periods were assessed as differences and as ratios over baseline volumes (Fasting Difference= Fasting Volume-Baseline volume; Fasting Ratio= Fasting Volume/Baseline Volume;

Postprandial Difference= Postprandial Volume-Baseline Volume; Postprandial Ratio= Postprandial Volume/Baseline Volume).

MTV and postprandial symptoms

The total volume ingested (MTV) was recorded. The aggregate postprandial symptoms score (thirty minutes after completing ingestion of Ensure®) was calculated as the sum of VAS scores for each postprandial symptom (maximum 400).

Gastric emptying

Gastric emptying during the drink test was measured by scintigraphy by radiolabeling the second glass of Ensure for all participants and as described above. The primary endpoint for assessment of effects on gastric emptying was the proportion emptied at 30 minutes, which corresponded with the time when the GLP-1 or placebo infusion was completed. This time was selected in view of the very short half-life of infused GLP-1 estimated as ~5 minutes [59]. At this time point all the participants had ingested approximately the same volume since the rate of ingestion of the nutrient liquid meal was standardized and all the participants, except one, were still drinking at 30 minutes. Four of the participants who reached full satiety at that point did not completely drink the last glass of Ensure® (200mL) and had slightly less volume and caloric intake: 3 were in the GLP-1 group (875, 822 and 772 mL), and 1 in the placebo group (882 mL). Thus, the volume and caloric intake was identical for nineteen of the participants. The secondary endpoint was the proportion emptied at 90 minutes (one hour after the infusion ended); this was intended to determine whether there were longer lasting effects of the infused hormone.

Plasma glucose and HPP

Fasting and postprandial plasma levels of glucose and HPP were calculated from the average of the two fasting measurements and the two postprandial measurements respectively. Changes in the levels of glucose and HPP from baseline to fasting and to postprandial periods were assessed by subtracting baseline (pre-infusion) values from fasting and postprandial levels.

Statistical Analysis

Unpaired t-test was used to compare absolute gastric volumes as well as the volume differences and ratios between GLP-1 and placebo groups. Wilcoxon rank sum

test was used to compare the variables that were not normally distributed: maximum tolerable volume (MTV), the aggregate postprandial symptoms score, the gastric emptying at 30 and 90 minutes, and change in plasma levels of glucose and HPP. Prior to the study, the estimated sample size for 80% power to detect a 25% difference in the primary endpoint (postprandial gastric volume) in response to GLP-1 compared to placebo was 12 per group ($\alpha = 0.05$). All the tests were two-tailed and results are presented as medians and interquartile ranges (IQR).

2.1.3. Results

Study Conduct and Participants

Twenty-four healthy volunteers were studied (13 in the GLP-1 group and 11 in the placebo group). We were not able to obtain peripheral blood samples from two participants; accurate assessment of gastric emptying was not possible for technical reasons in one participant. Missing data excluded these individuals from specific comparisons; however, data for all 24 participants were used when available that is, in all comparisons except where indicated above. There were no statistically significant differences among demographic and baseline variables between the two study groups (Table 1).

Total Gastric Volumes

Figure 4 shows examples of the stomach reconstructions at baseline (pre-infusion), fasting and postprandially in the GLP-1 and placebo groups. Table 2 shows the data for total gastric volumes, differences in volumes and ratios. The fasting volume was significantly greater in the group that received GLP-1 (312 mL, IQR: 253 to 365) compared to the placebo group (225 mL, IQR: 185 to 239; p=0.002).

The difference between fasting and baseline volume was 80 mL (IQR: 61 to 128) for the participants who received GLP-1 and 17 mL (IQR: -21 to 25) for those who received placebo (p=0.005). The ratio of fasting over baseline volume was also greater for the GLP-1 group (1.48 IQR: 1.26 to 1.60) than for the placebo group (1.08 IQR: 0.90 to 1.12; p=0.003).

Postprandial volumes were also greater in the GLP-1 group (848 mL, IQR: 789 to 899) compared to placebo group (651mL, IQR: 602 to 801; p=0.004). The difference

between postprandial and baseline volume was 608 mL (IQR: 532 to 671) for GLP-1 group, and 435 mL (IQR: 401 to 549) for placebo group (p=0.008). No significant differences were found between groups when comparing total gastric volume ratios postprandially, 3.53 (IQR: 3.19 to 4.39) for the GLP-1 group and 3.14 (IQR: 2.79 to 3.61) for the placebo group (p= 0.15).

Proximal Gastric Volumes

Table 3 shows gastric volumes, differences and ratios for the proximal stomach in the two groups. No significant differences were found when comparing fasting absolute volumes, differences between fasting and baseline volumes or the fasting/baseline ratios.

In contrast, the postprandial proximal volumes were significantly greater in the GLP-1 group (629 mL, IQR: 581 to 647) than the placebo group (455 mL, IQR: 338 to 618) (p<0.0001). The absolute difference between postprandial and baseline volume was also significantly greater in the GLP-1 group (461 mL, IQR: 400 to 553) compared to the placebo group (302 mL, IQR: 223 to 389; p= 0.0003). There was a trend towards a greater postprandial ratio in the GLP-1 group (5.77, IQR: 3.41 to 7.45) compared to the placebo group (3.72, IQR: 2.84 to 4.81; p=0.08).

MTV and Postprandial Symptoms

As shown in Table 4, the median volume ingested to reach full satiety was 1119 mL (IQR: 874 to 1546) for GLP-1 group and 1350 mL (IQR: 1082 to 1606) for placebo group (p=0.16). The individual values are shown in figure 5.

The aggregate postprandial symptom score was 185 (IQR: 121 to 250) in the GLP-1 group and 169 (IQR: 121 to 199) in the placebo group (p=0.54). No differences were found when comparing each of the symptoms separately (nausea, bloating, fullness and abdominal pain; see Table 4).

Gastric Emptying of Nutrient Liquid

One volunteer, subsequently shown to be in the GLP-1 group, vomited after the satiety test was completed. These data were excluded from the analysis of gastric emptying.

Figure 6 shows the gastric emptying of the radiolabeled liquid nutrient meal. The proportion emptied was significantly lower for the GLP-1 group at the point the infusion ended, at 30minutes (7%, IQR: 3.5 to 19 vs. 23% IQR: 14 to 23, respectively; p=0.008).

However, this effect was transient; one hour after the infusion ended, the proportion emptied was not different for the two groups, 21% (IQR: 14.5 to 38) for the GLP-1 group vs. 35% (IQR: 21.75 to 38.25) for the placebo group (p=0.28).

Plasma Levels of Glucose and HPP

During fasting, the glucose change relative to baseline (pre-infusion) was -12.3 mg/dL (IQR: -19.1 to -8) for GLP-1 group and 0.5 mg/dL (IQR: -2.3 to 3.3) for placebo group (p=0.0002). The postprandial increase in glucose levels was -9.3 mg/dL (IQR: -18.5 to -6.4) for the GLP-1 group and 19.3 mg/dL (IQR: 15.9 to 26.6) for the placebo group (p<0.0001; Fig. 7).

The fasting HPP change relative to baseline was similar in the two groups: -12.0 pg/mL (IQR: -22.5 to -2.0) for GLP-1 and -0.25 pg/mL (IQR: -14.4 to 31.4) for placebo (p= 0.12). However, GLP-1 reduced significantly the postprandial increase in HPP levels: 6.5 pg/mL (IQR: -22.4 to 6.9) for GLP-1 compared to 119.8 pg/mL (IQR: 60.1 to 357.0) for placebo (p=0.0001).

2.1.4. Discussion

The results of the present study suggest that GLP-1 increases gastric volume during fasting and in the postprandial period and retards gastric emptying. These effects are not associated with changes in maximum volume of Ensure® tolerated or in postprandial symptoms.

In this study, we confirmed the delay of gastric emptying for liquid meals during intravenous infusion of GLP-1 in healthy subjects. Schirra et al. [9] had previously reported that an isolated subcutaneous injection of either 125 or 250 pmol/Kg of GLP-1 delays the emptying of a 300Kcal mixed liquid meal. The retarding effect of GLP-1 on gastric emptying is transient, and the post-infusion emptying of the liquid meal (as assessed by the proportion emptied at 90 minutes, that is, one hour after the infusion ended) was not different in the two groups. This observation is consistent with the short biological activity of the hormone (~5 min). The mechanism by which GLP-1 delays gastric emptying of liquids is unclear. The reported inhibition of antroduodenal motility during the postprandial state [9, 44] and the increase in isolated pyloric pressure waves (IPPWs) may contribute to delayed emptying of solids. However, gastric emptying of

liquids is thought to depend on fundic pressure [60] and to be less influenced by antral motility [61, 62]. An alternative mechanism for delayed gastric emptying of liquids is that the GLP-1-induced increase in postprandial gastric volume was associated with a decrease in fundic tone. Previous studies on GLP-1 have shown decreased fasting fundus tone [45].

GLP-1 decreases the feeling of hunger before meals and reduces food and fluid intake in healthy subjects [13, 16] and in diabetic [14] and nondiabetic [15] obese patients. However, no effects of GLP-1 on postprandial symptoms have been reported. In this study, we observed no differences in the maximum tolerated volume and aggregate postprandial symptoms scores or in individual symptoms of nausea, bloating, fullness or pain. It might be expected that increased gastric volume could allow the ingestion of a larger volume before reaching satiation and, possibly reduce the likelihood of developing postprandial symptoms. Failure to observe this could be explained by the marked inhibition of gastric emptying by GLP-1. Another possible explanation is that GLP-1 might regulate food intake independently of its motor effects. Data from animal studies suggest a central site of action of the effect of GLP-1 on reduced food consumption, unrelated to a change in gastric functions [12, 24]. Thus, we postulate that GLP-1 sensory effects might also be centrally mediated in humans.

Wank et al. [45] showed that slightly lower infusion rates of 0.3 and 0.9 pmol/Kg/min of GLP-1 diminished fasting gastric tone recorded with an electronic barostat device. We confirmed this finding in our study using SPECT and expanded the knowledge base by showing the effect is observed in both proximal and whole stomach. Prior to our study, the effects of GLP-1 on postprandial gastric volumes or accommodation had not been reported. In our study, greater postprandial gastric volumes (proximal and whole stomach) with GLP-1 were demonstrated compared to placebo, using a validated method that images the gastric wall, not the intragastric content. Hence, this method is independent of the volume and the rate of emptying of the meal. Our method does not measure tone and therefore cannot measure relaxation of the stomach. However, since the intragastric pressure is subject to the positive intra-abdominal pressure and to equilibration with atmospheric pressure via the belching reflex, and since these conditions were not altered before and after the meal, the postprandial increase in

volume, measured by SPECT, constitutes a measure of the gastric accommodation, which is enhanced by intravenous infusion of GLP-1.

The mechanisms by which GLP-1 increases gastric volume are unclear. It is known that, during fasting, gastric tone is maintained via vagal cholinergic input and that 2-adrenergic and nitrergic pathways induce gastric relaxation [47, 63]. During the fed state, gastrointestinal motility is partly controlled by nonadrenergic, noncholinergic (NANC) vagal pathways [64], and nitric oxide (NO) modulates the postprandial accommodation response [46, 65]. Our study starts to explore the mechanism for the enhanced postprandial gastric volume in response to GLP-1. Thus, we have shown that the effect of GLP-1 on postprandial gastric volume is accompanied by a marked inhibition of the normal postprandial increase of human pancreatic polypeptide. The latter is a hormone of the endocrine pancreas that is under cholinergic control [35]. The effect of GLP-1 on the human postprandial pancreatic polypeptide response has been previously shown to be independent of gastric emptying [10, 44]. This suggests that the delay in gastric emptying by GLP-1 is not the cause of the inhibition of pancreatic polypeptide release. Therefore, our data are consistent with GLP-1 inhibition of efferent vagal-cholinergic function.

The increased gastric volume observed with GLP-1 may result from inhibition of cholinergic innervation during fasting and postprandially. An alternative hypothesis is that GLP-1 enhances gastric volumes by activation of vagal nitrergic pathways, which mediate the normal postprandial accommodation response.

In conclusion, we have shown that GLP-1, a novel agent in the treatment of diabetes and obesity, increases the fasting and postprandial volume of the stomach, transiently retarding gastric emptying without increasing postprandial symptoms in healthy subjects. The current study suggests that GLP-1 induces gastric relaxation through a mechanism that involves the vagus nerve. To test this hypothesis we performed the second study presented in this thesis.

2.2 Effects of Glucagon-Like Peptide-1 and Feeding on Gastric Volumes in Diabetes Mellitus with Cardio-Vagal Dysfunction

2.2. 1 Introduction

Glucagon-like peptide-1 (GLP-1), a peptide released after meal intake [39], has several effects on gastric and pancreatic functions by a mechanism that appears to involve the vagus nerve. The presence of nutrients in the small intestine results in vagally-mediated inhibition of gastric emptying. This response is abolished by the GLP-1 receptor antagonist exendin-(9,39) [28]. Exogenously administered GLP-1 inhibits gastric acid secretion and gastric emptying [43]. These effects are respectively abolished by vagal afferent denervation in rats [24] and in vagotomized patients [22]. In the first study presented in this thesis, we showed that GLP-1 increases gastric volume during fasting and postprandially in healthy subjects. This response was associated with inhibition of the pancreatic polypeptide response over the first 20 minutes after the meal. Pancreatic polypeptide response to feeding is dependent on vagal activation since vagal blockade completely abolishes the response [66-70]. However, the effect of GLP-1 on gastric volume in the presence of vagal dysfunction is unknown.

Cholinergic and nitrergic vagal pathways are involved in the regulation of gastric tone in animals [27-29, 60, 71] and humans [30, 36, 72, 73]. Vagal participation in the gastric relaxation response to a meal is mediated through nonadrenergic noncholinergic fibers [74-76]. However, there is increasing evidence suggesting that the control of gastric tone and the gastric relaxation after a meal may recover with passage of time after vagal denervation [30, 77, 78]. In elegant animal studies by Takahashi and Owyang, it was shown that this recovery of gastric accommodation is neurally-mediated and appears not to involve sympathetic pathways [28] suggesting adaptive control of gastric tone by the enteric nervous system (ENS).

However, in a human model of vagal neuropathy such as diabetic vagal neuropathy, it is not yet known whether the gastric volume response to a meal is preserved, since previous data are controversial with some studies showing diminished mean gastric accommodation in diabetic patients with vagal neuropathy, while individual accommodation responses were within the normal range in some of the patients evaluated [50, 79, 80].

In this study, we hypothesized that gastric volume response to GLP-1 is abolished in diabetic patients with vagal neuropathy. To test this hypothesis, we studied the responses of fasting and postprandial gastric volume to placebo and GLP-1 in diabetic patients with evidence of cardio-vagal neuropathy. We also compared gastric volumes in diabetic patients on placebo to those in the healthy subjects who participated in the first study presented in this thesis.

2.2. 2 Patients and Methods

Study Population

Healthy subjects

Volunteers over 18 years of age recruited from the local community by public advertisement. Exclusion criteria included: pregnant or breast-feeding females, prior abdominal surgery other than appendectomy, laparoscopic cholecystectomy or tubal ligation; positive symptoms on an abridged bowel disease questionnaire; present or previous chronic gastrointestinal illness; systemic disease or use of medications that may alter gastrointestinal motility.

Diabetic subjects

Diabetic patients (over 18 years of age) diagnosed at Mayo Clinic, Rochester (MN) with a positive diagnosis of cardiovagal neuropathy were recruited from the local community by public advertisement. Exclusion criteria were the same as for healthy volunteers, except for the presence of diabetes.

Cardiovagal neuropathy serves as a surrogate of abdominal vagal neuropathy; pathological and functional observations show that changes in the vagus nerve start distally and progress cranially [81-83]. Thus, when the branches of the vagus that innervate the heart are affected, the more distal branches of the vagus, such as the gastric nerve, are affected [84-88].

Cardiovagal neuropathy was based on heart rate (HR) variability in response to deep breathing. Heart rate was recorded using a standard ECG monitor. The subject performed deep breathing at a respiratory rate of 6 per minute for six cycles. HR variability was analyzed as the average HR change for the six consecutive cycles. This is a widely used test to noninvasively assess the cardiovagal function in human subjects [89-91].

Study Design

The studies were approved by the Mayo Institutional Review Board, and all eligible volunteers gave written informed consent before enrollment in the study.

The study performed in healthy volunteers followed a randomized, placebo-controlled, double-blind, parallel-group design as described before. Eligible volunteers were randomized to receive either GLP-1 or saline infusion (placebo) for 60 minutes. Participants with diabetes were randomized in a placebo-controlled, double-blind, crossover design study and received GLP-1 or saline infusion (placebo) for 60 minutes on two separate days (at least 48 hours apart). Diabetic subjects underwent assessment of gastric volumes under euglycemic conditions.

GLP-1

GLP-1 (Bachem, San Diego, CA) was infused during 60 minutes at a rate of 1.2 pmol/Kg/min, a dose previously shown to affect gastrointestinal function without causing side effects in healthy volunteers [43, 44]. Gastric volume measurements were started after 30 minutes of infusion when GLP-1 steady levels in plasma are achieved [41].

Measurement of gastric volumes using ^{99}Tc-SPECT (Fig. 8)

Participants were studied after at least 6 hours of fasting. The diabetic patients were specifically asked to have their last meal in a liquid form. Blood glucose was monitored by means of a reflectance meter prior to commencing and throughout the study. Intravenous insulin was used as necessary to maintain glucose levels in the normal range (60-120 mg/dL). Patients lay down on the SPECT camera, and the 10mCi ^{99m}Tc-sodium pertechnetate was injected intravenously. Ten minutes later, a first orbit (360º over 10 minutes) was performed for baseline (pre-infusion) tomographic images.

Infusion of 1.2 pmol/Kg/min GLP-1 or saline placebo was started. After 30 minutes' infusion, a second image was obtained over 10 minutes to measure the effects of the GLP-1 compared to placebo on fasting gastric volumes.

A nutrient liquid meal (Ensure®: 300 mL, 316 Kcal, 7.6g fat, 50.6g carbohydrate and 11.4g protein) was ingested over 3 minutes, and one further 10 minute image was obtained to measure postprandial gastric volume.

Data Analysis

Gastric volumes

Total and proximal gastric volumes at baseline (pre-infusion), fasting and during 10 minutes postprandially were measured. Volume changes from baseline (pre-infusion) to fasting and postprandial periods were assessed as differences and as ratios over baseline volumes (Fasting Difference= Fasting Volume-Baseline volume; Fasting Ratio= Fasting Volume/Baseline Volume; Postprandial Difference= Postprandial Volume-Baseline Volume; Postprandial Ratio= Postprandial Volume/Baseline Volume).

Statistical Analysis

Mann-Whitney and Wilcoxon signed rank sum tests were used for inter- and intra-individual comparisons respectively. Based on the measurements of the increase in gastric volumes in healthy subjects in response to GLP-1, a sample size of 7 provided 94% power to detect the same increase in diabetic patients using a crossover design and one-side alpha of 0.05. We used a one-tailed test in this case since the null hypothesis was unidirectional, that is, that GLP-1 does not increase the gastric volume in diabetic patients. All other comparisons were based on two-tailed tests, and the significance level was set at 0.05. The results are presented as medians and interquartile ranges (IQR).

2.2. 3 Results

Study Conduct and Participants

Seven diabetic patients with a diagnosis of cardiovagal neuropathy were studied. In the healthy studies 24 healthy volunteers participated (13 in the GLP-1 group and 11 in the placebo group). Demographic variables for both study populations are presented in Table 5. Of the 7 diabetic patients, 5 had insulin-dependent diabetes mellitus (IDDM) and 2 non-insulin-dependent diabetes mellitus (NIDDM); 5 had diabetic retinopathy; 5 had peripheral somatic neuropathy; 4 had erectile dysfunction; and 3 renal impairment. One of the diabetic patients had chronic diarrhea, which was attributed to autonomic dysfunction after ruling out other possible diagnoses. No other gastrointestinal symptoms were reported by any of the patients.

Two diabetic patients received insulin before starting the placebo study and two other patients received insulin before starting the GLP-1 study to correct fasting hyperglycemia (plasma glucose >180 mg/dL). Insulin was stopped during the study in all the patients. One diabetic patient received intravenous glucose during the study to

correct insulin-induced hypoglycemia (plasma glucose <80).

Effect of GLP-1 on Gastric Volumes in Diabetes with Vagal Neuropathy

Total gastric volumes

Table 6 shows the effects of placebo or GLP-1 infusion on total gastric volumes at baseline, during fasting, and ten minutes postprandially. The differences in volumes and ratios post-drug over baseline volumes are also presented. GLP-1 did not increase fasting or postprandial gastric volumes in diabetic patients. Data demonstrating significant effects of GLP-1 in healthy volunteers from our previous study are included in the table for comparison.

Proximal gastric volumes

Table 7 shows the effects of placebo or GLP-1 infusion on proximal gastric volumes at baseline, during fasting, and ten minutes postprandially. The differences in volumes and ratios post-drug over baseline volumes are also presented. As observed when assessing total volumes, GLP-1 did not increase the fasting or postprandial volume of the proximal stomach.

Figure 9 shows gastric volume changes fasting and postprandially in diabetic patients during placebo or GLP-1 infusion and includes results in healthy individuals from our previous study for comparison.

Gastric Volumes During Fasting and After a Standard Meal in Health and Diabetes with Vagal Neuropathy

Observations during placebo treatment studies were used to compare gastric volume during fasting and the gastric volume response to a standard meal in diabetic patients with vagal neuropathy with those observed in healthy controls. The total and proximal gastric volumes measured during fasting and postprandially in diabetics with vagal neuropathy under placebo treatment (n=7) were not significantly different compared to those in healthy subjects under placebo treatment (n=11). (Table 8)

Figure 10 shows examples of the stomach reconstructions during fasting and postprandially in a healthy subject and a diabetic patient.

Plasma Glucose Levels

Plasma glucose levels in diabetic patients were similar on both study days (Fig. 11) and within the normal range during fasting (100 mg/dL IQR:89; 124) and

postprandially (111 mg/dL IQR: 90; 127).

2.2. 4 Discussion

The diabetic patients evaluated in our study had fasting gastric volumes that were not significantly different from those of healthy controls. Our study also shows that exogenous GLP-1 increases gastric volume in healthy individuals, but not in diabetic patients with vagal neuropathy who retain the ability to increase the gastric volume in response to a meal. These data strongly suggest that the effect of GLP-1 on gastric volume is dependent on vagal function. This interpretation is consistent with previous studies that suggested that GLP-1 inhibition of gastric acid secretion and emptying is mediated through the vagus [22, 24] and with our data from the first study presented in this thesis, showing that GLP-1-induced increase in gastric volume is associated with vagal inhibition.

Gastric relaxation in response to a meal results in an increase of gastric volume to accommodate ingested food, without an increase in intragastric pressure. This prevents the development of symptoms caused by the increase in intragastric hydrostatic pressure from the meal and gastric secretions. In animal and in vitro studies, this reflex response has been thoroughly characterized. When the food arrives in the stomach, vagal afferent terminals in the gastric wall convey the signal to the nucleus of the solitary tract (NST) in the dorsal vagal complex (DVC). These first order afferents synapse with neurons in the dorsal motor nucleus of the vagus (DMNV) in the DVC [92]. From the DMNV, nonadrenergic noncholinergic (NANC) vagal efferents [93] reach the stomach inducing gastric relaxation. Afferents from the NST in the DVC connect to several central regions (such as hypothalamus, amygdala and limbic cortex) [94] that project back to the DVC [95]. The DVC also binds hormones and neuropeptides such as GLP-1 [96]. All these neural and hormonal influences are important to modulate gastrointestinal function and eating behavior [88, 97].

In the denervated gut, it is well known that the enteric nervous system (ENS) can elicit the motility patterns that characterize the fasting and postprandial states [98-100], as well as intrinsic reflexes such as the peristaltic reflex stimulated by a food bolus [101, 102]. King and Szurszewski have demonstrated that a number of entero-enteric reflexes evoked

by distension relay through the prevertebral ganglia [103]. In vitro, gastric relaxation in response to distension is maintained in the denervated and vascularly-isolated stomach and is abolished by tetrodotoxin, suggesting the ENS is also capable of inducing reflex relaxation in the absence of extrinsic innervation [28, 75]. In vivo, in the presence of a longstanding vagotomy, reflex gastric relaxation is preserved in animals and humans [28, 30, 77]. In rats studied in vivo, surgical vagotomy resulted in a reduced postprandial accommodation response, but the gastric accommodation reflex was fully restored four weeks after vagotomy. This preserved response in chronically vagotomized rats was significantly reduced by tetrodotoxin, but was not affected by guanethidine or splanchnicotomy, suggesting it is a neurally-mediated response that does not involve sympathetic pathways [28].

In humans, Azpiroz and Malagelada showed that reflex gastric relaxation in response to distension was not significantly different in vagotomized patients with gastroparesis compared to healthy controls[41]. In contrast, they showed differences in the phasic volume events in the proximal stomach, which were abolished in vagotomized patients [104]. Others have also documented a normal gastric relaxation in response to a meal in longstanding vagotomized patients [77]. Moreover, in a prospective study in which the gastric relaxation response was assessed preoperatively and six weeks and one year after proximal gastric vagotomy, the accommodation reflex was impaired early after surgery but recovered partially after one year [30]. The diabetic patients participating in our study had a normal gastric volume response to a meal, despite evidence of vagal neuropathy. This suggests the existence of a non-vagal control of the gastric volume that allows the postprandial volume increase in the presence of vagal dysfunction. This is consistent with other reports showing similar gastric accommodation to distension in vagotomized subjects compared to healthy controls [104] and preserved gastric accommodation in response to a meal in vagotomized animals [28, 78] and after surgical vagotomy in humans [30, 77]. These data from several studies are consistent, and yet, at first glance they appear to contradict conventional wisdom. Thus, several studies in the literature have suggested gastric compliance and accommodation are reduced in patients with diabetic vagal neuropathy [50, 51, 79]. Samson et al. reported [51] decreased gastric compliance in diabetic patients with autonomic dysfunction plus dyspeptic symptoms but

accommodation to a meal was not tested. In a later study [50], the same group showed that a group of diabetic patients had decreased postprandial gastric accommodation response; however, careful scrutiny of individual data shows that 6 out of 9 diabetic patients with vagal neuropathy presented a normal accommodation response. Undeland et al.[79] also reported decreased gastric volume response to feeding in diabetic patients with decreased vagal tone. However, they compared tertiary referral diabetic patients to non-matched healthy medical students in contrast to our study that recruited community diabetes without dyspepsia. In all these reports, the accommodation responses of diabetic and healthy subjects overlap considerably, and the inclusion of referred diabetic patients with symptoms may confound assessment of the effects of diabetic neuropathy on gastric accommodation. Dyspeptic symptoms in diabetic patients may be related to factors other than vagal neuropathy and, symptoms may conceivably induce changes in gastric motor function.

To avoid this potential confounder, we studied diabetic patients from the community with evidence of vagal impairment and excluded patients with gastrointestinal symptoms. We considered these selection criteria were essential to answer the question whether the gastric volume response to a meal is impaired in the presence of chronic vagal neuropathy. Our studies lead to the hypothesis that there is adaptation of the gastric accommodation response to feeding, and this needs to be assessed in a larger study in which the mechanisms potentially involved in this adaptation can be explored. However, it is important to point out that our observation is backed by a large volume of data from experimental animal and human studies [28, 30, 77, 78, 104].

Given the evidence that hyperglycemia may alter gastric tone [105-107], we performed all the studies under euglycemic conditions to avoid this potential confounder. Hence, we believe that the normal gastric volume response to the meal in vagotomized diabetic patients is not the result of differences in postprandial glycemia. We postulate that, in the presence of vagal dysfunction, the postprandial gastric volume response occurs through a non-vagal entero-enteric reflex. Our studies do not determine whether this is a peripheral reflex arc via prevertebral ganglia or a local enteric reflex. Moreover, we cannot exclude a potential contribution of a hormonal mechanism in the adaptation of the stomach volume increase after the meal.

In summary, by evaluating its effects in patients with vagal neuropathy, we have shown that the GLP-1-induced increase in gastric volume in humans is likely to be mediated through the vagus. Further studies will be needed to explore whether GLP-1 increases gastric volume by inhibition of vagal cholinergic innervation or by activating vagal NANC or adrenergic inhibitory pathways. We have also shown that chronic vagal denervation secondary to diabetes does not necessarily impair gastric accommodation, as had been suggested by some studies [50, 79, 108]. Our data confirm prior experimental animal studies [28, 78] that suggested the potential of non-vagal pathways to induce gastric reflex responses to feeding, including the accommodation response.
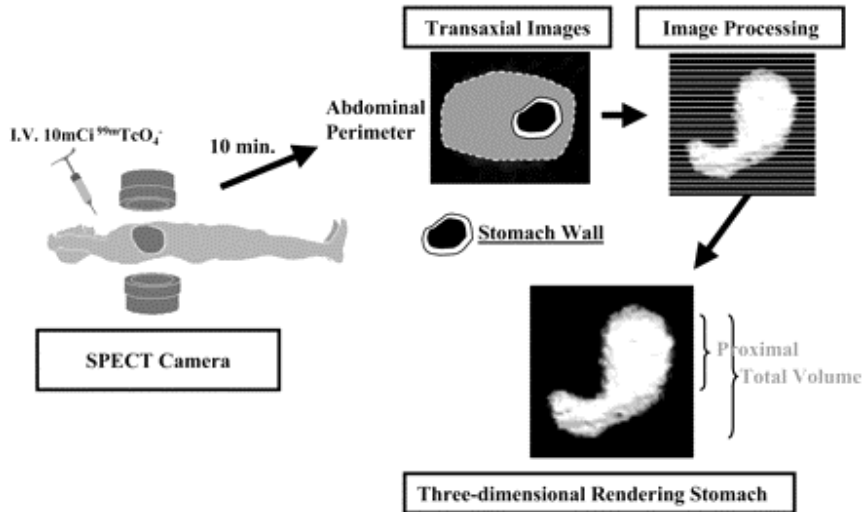
3. Figures

Fig. 1. ^{99m}Tc-SPECT technique to measure gastric volume. Ten minutes after intravenous injection of ^{99m}Tc-sodium pertechnetate to allow gastric mucosal uptake of the isotope, dynamic tomographic acquisition was performed with the single photon emission computed tomography (SPECT) camera. Tomographic images were processed to obtain a 3-dimensional stomach and its volume.
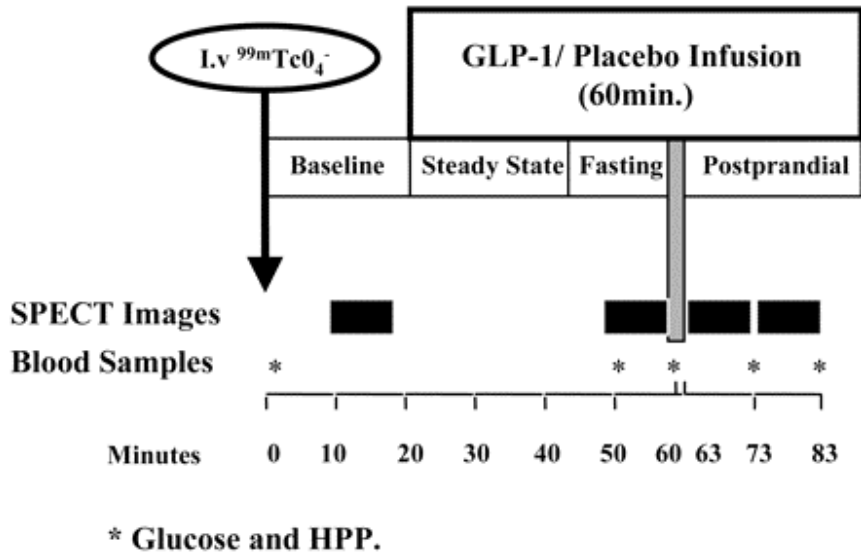
Fig. 2. *Protocol 1*. Gastric volumes and plasma levels of glucose and human pancreatic polypeptide (HPP) were obtained at baseline, before glucagon-like peptide-1 (GLP-1) or placebo infusion, and during fasting and the postprandial period, while GLP-1 or placebo infusion was ongoing.
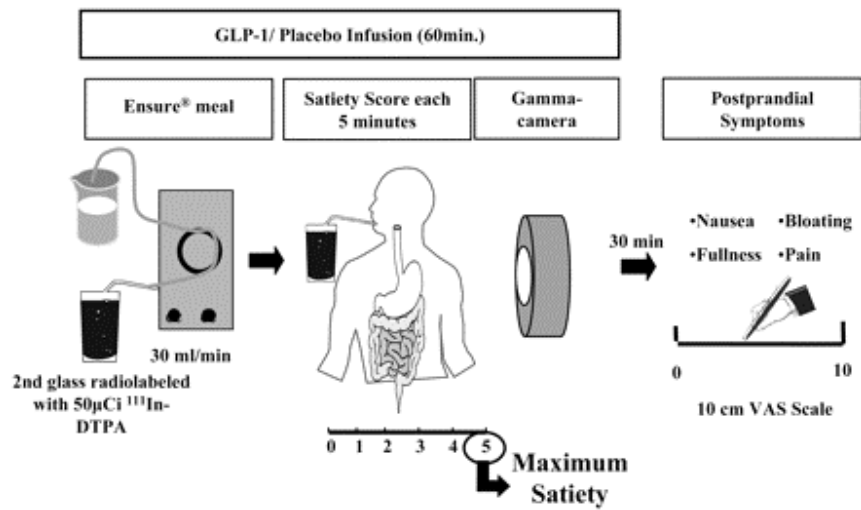

Fig. 3. *Protocol 2*. Measurement of maximum tolerable volume (MTV), gastric emptying, and postprandial symptoms. Participants drank Ensure at a constant rate until maximum satiety was reached (i.e., MTV). The second glass of Ensure ingested was radiolabeled with ^{111}In-diethylenetriaminepentaacetic acid (DTPA) to measure gastric emptying. Thirty minutes after the drink test was finished, postprandial symptoms were assessed using a visual analog scale (VAS).
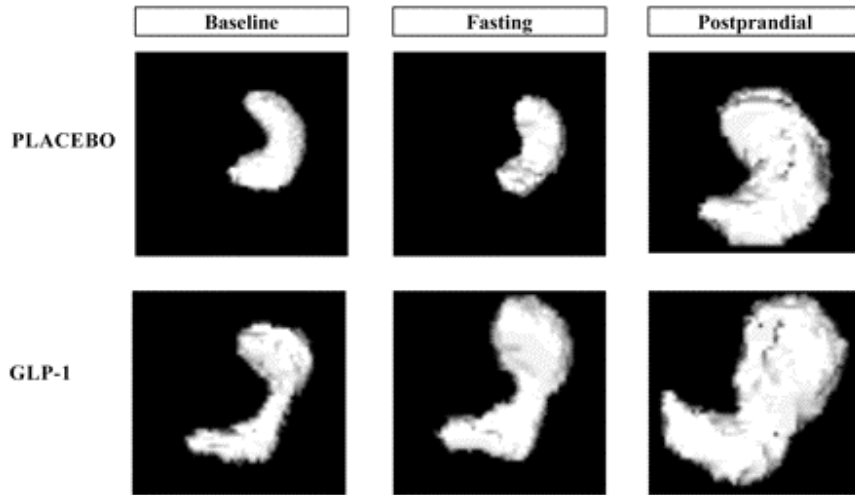
	Baseline	Fasting	Postprandial
PLACEBO			
GLP-1			

Fig. 4. Examples of gastric volumes obtained at baseline, during fasting, and during postprandial periods from 2 participants in the study, 1 treated with placebo and the other with GLP-1. Note the visibly larger volume of the stomach with GLP-1 infusion.
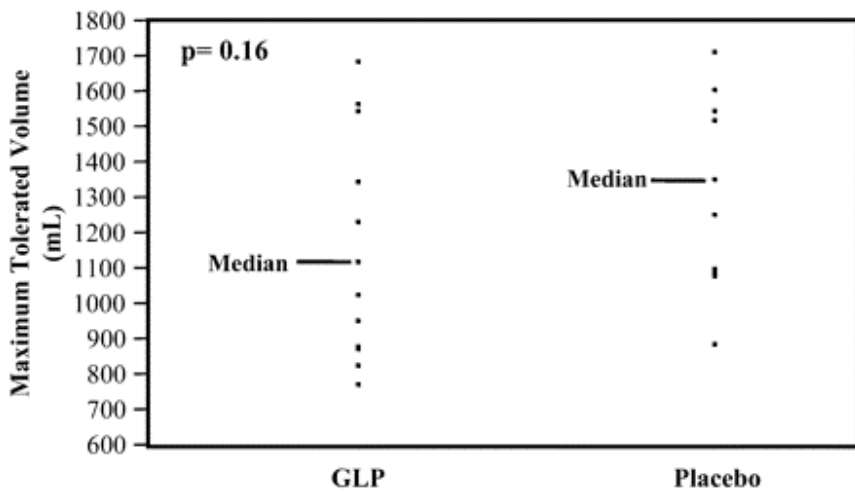

Fig. 5. Individual values for the MTV ingested in each of the 2 groups.
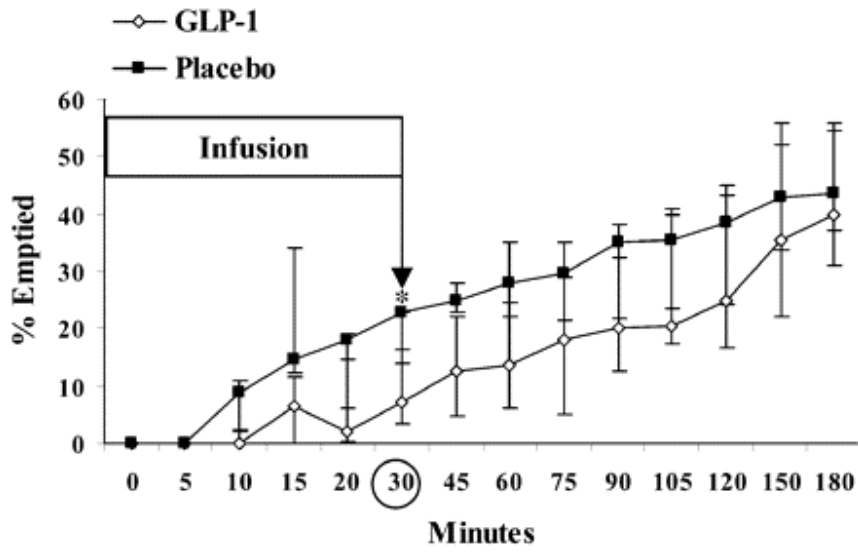
Fig. 6. Gastric emptying of a nutrient liquid meal. Gastric emptying curves observed following radiolabeled Ensure ingestion are shown. The primary end point for assessing the gastric emptying effects of GLP-1 and placebo was the proportion emptied at the time the infusion ended, at 30 min. Median proportion emptied in the GLP-1 group was 7% and in the placebo group was 23%. Data shown are median and interquartile ranges. *$P < 0.05$.
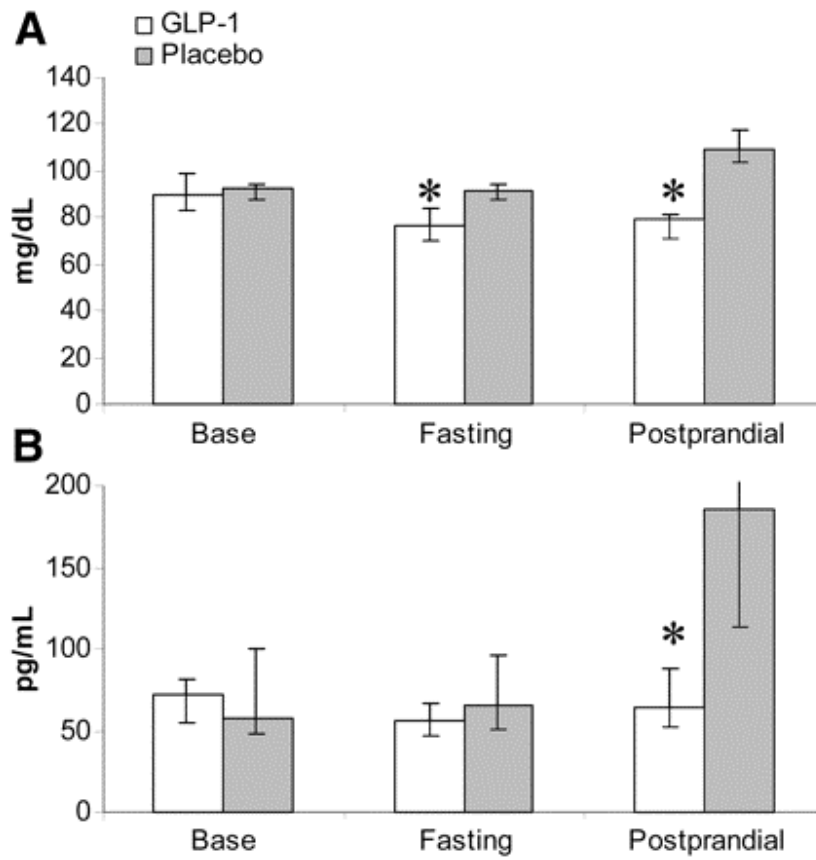
Fig. 7. Plasma levels of glucose (*A*) and HPP (*B*). GLP-1 decreased plasma levels of glucose and inhibited the normal postprandial increase in HPP. Bars are medians; error bars show interquartile ranges. *$P < 0.05$.
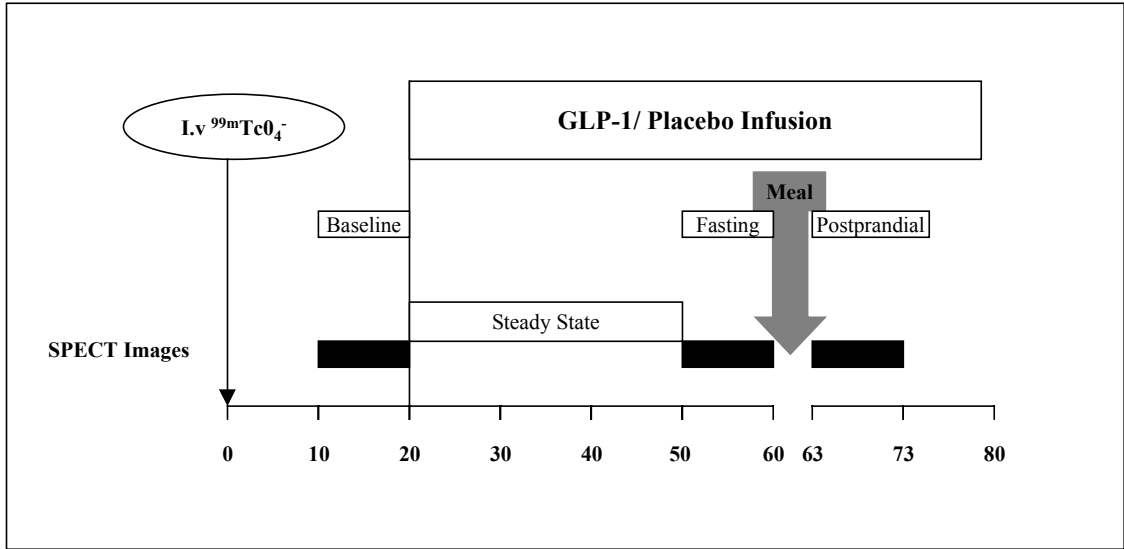
Fig. 8. Gastric volumes were obtained at baseline, prior to GLP-1 or placebo infusion, and during fasting and the postprandial period, while GLP-1 or placebo infusion was ongoing.
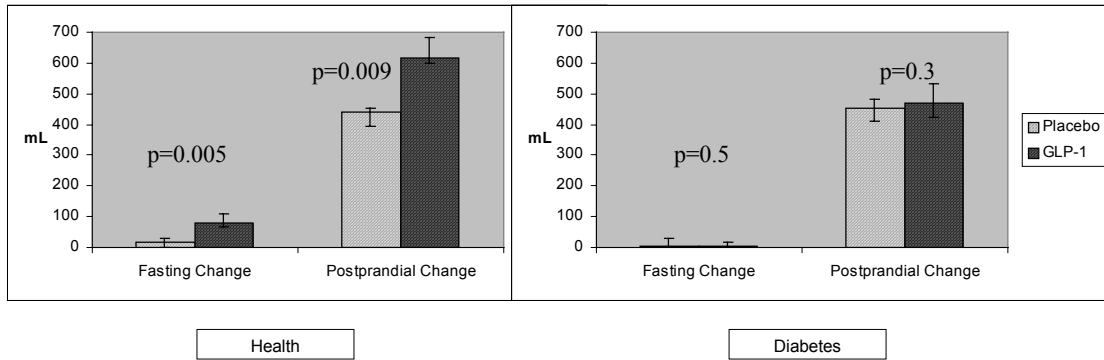
Fig. 9. Gastric volume change in response to placebo or GLP-1, during fasting and postprandially, in healthy and diabetic subjects. GLP-1 only increased gastric volumes in healthy individuals. Data shown are median (IQR).

(A)

(B)

Fig. 10. Examples of stomach reconstructions before meal and postprandially under the effect of placebo in healthy (A) and diabetic patients (B). Note the normal increase in gastric volume after the meal in diabetic patients.

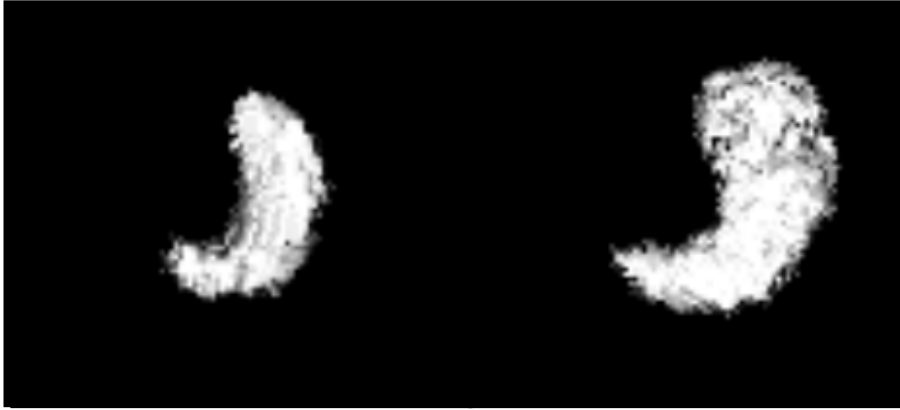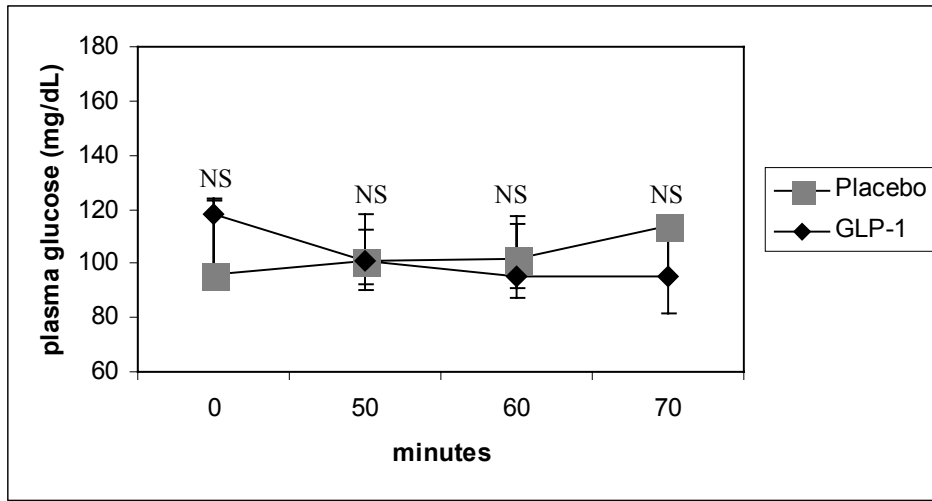**Fig. 11.** Plasma levels of glucose during both study days in diabetic patients. There were no significant differences at any time point. Data shown are median and interquartile ranges. NS: $p>0.05$

4. Tables

Table 1. Demographic and Baseline Variables.

	GLP-1 n=13	Placebo n=11	P value
Gender (m/f)	5/8	5/6	0.7
Age (years)	33 (26-39.5)	33 (28-43)	0.5
BMI (Kg/m^2)	25 (21-26)	28 (24-29)	0.4
Baseline HPP (pg/mL)	77 (60-104)	99 (55-134)	0.8
Baseline Glucose. (mg/dL)	90 (82-99)	92 (88-95)	0.6
Baseline Gastric Volume proximal 2/3 (mL)	115 (80-180)	112 (82-164)	0.8
total (mL)	239 (169-346)	205 (176-262)	0.6

Values are median (interquartile range). n= number of subjects in each group. NS: $p > 0.05$.

Table 2. Total Gastric Volumes (mL) and

	GLP-1 n=13	Placebo n=11	P value
Baseline Vol.	239 (169-346)	205 (176-262)	0.6
Fasting Vol.	312 (253-365)	225 (185-239)	0.002
Fasting Difference	80 (61-128)	17 (-21-25)	0.005
Fasting Ratio	1.48 (1.26-1.60)	1.08 (0.90-1.12)	0.003
Postprandial Vol.	848 (789-899)	651 (602-801)	0.004
Postprand. Difference	608 (532-671)	435 (401-549)	0.008
Postprandial Ratio	3.53 (3.19-4.39)	3.14 (2.79-3.61)	0.15

Values are median (interquartile range). n= number of subjects in each group. NS: p value > 0.05.

Table 3. Proximal Gastric Volumes (mL)

	GLP-1 n=13	Placebo n=11	P value
Baseline Vol.	115 (80-180)	112 (82-164)	0.8
Fasting Vol.	147 (112-243)	137 (97-156)	0.2
Fasting Difference	43 (8-85)	5 (-23-24)	0.3
Fasting Ratio	1.34 (1.04-1.90)	1.07 (0.86-1.21)	0.5
Postprandial Vol.	629 (581-647)	455 (338-485)	<0.0001
Postprand. Difference	461 (400-553)	302 (223-389)	0.0003
Postprandial Ratio	5.77 (3.42-7.45)	3.72 (2.84-4.81)	0.08

Values are median (interquartile range). n= number of subjects in each group. NS: p value > 0.05.

Table 4. Maximum Tolerable Volume and Postprandial Symptoms.

	GLP-1 n=13	Placebo n=11	P value
MTV (mL)	1119 (874-1546)	1350 (1082-1606)	0.16
Aggregate Satiety Score (end GLP-1 infusion)	12.5 (6.3-15.8)	10 (8-17)	0.7
Aggregate Symptoms Score(30 min.Postprandial)	185 (121-250)	169 (121-199)	0.5
Nausea Score	40 (8.5-60.5)	35 (4-51)	0.4
Bloating Score	69 (37-79.5)	47 (34-69)	0.4
Fullness Score	77 (65-86.5)	70 (67-79)	0.4
Pain Score	4 (0-34)	10 (4-17)	0.6

Values are median (interquartile range). n= number of subjects in each group. NS: p value > 0.05.

Table 5. Demographic Variables

	Healthy Placebo	Healthy GLP-1	Diabetic
Age (y)	33 (28-43)	33 (26-39)	65 (56-74)
Gender (M/F)	5/6	5/8	5/2
Body Mass Index (Kg/m^2)	28 (24-29)	25 (21-26)	26 (24-35)
Diabetes Mellitus Type 1/2	NA	NA	5/2

Values are median (interquartile range).

Table 6. Effect of GLP-1 on Gastric Volume (mL)

DIABETES WITH VAGAL NEUROPATHY

	Placebo (n=7)	GLP-1 (n=7)	P
Baseline Volume	157(139-231)	193(166-258)	0.4
Fasting Volume	208(145-232)	202(158-255)	0.3
Fasting Difference	4(-14 - 50)	5(-3 - 30)	0.5
Fasting Ratio	1.02(0.92-1.32)	1.03(0.99-1.09)	0.5
Postprandial Volume	631(582-814)	677(605-855)	0.1
Postprandial Difference	452(400-493)	469(383-563)	0.3
Postprandial Ratio	3.99(2.73-4.26)	3.18(2.72-4.43)	0.5

HEALTHY CONTROLS

	Placebo (n=11)	GLP-1 (n=13)	P
Baseline Volume	205(176-262)	239(169-346)	0.6
Fasting Volume	225(185-239)	312(253-365)	0.002
Fasting Difference	17(-21 - 25)	80(61-128)	0.005
Fasting Ratio	1.08(0.9-1.12)	1.48(1.26-1.6)	0.003
Postprandial Volume	699(586-774)	855(761-886)	0.01
Postprandial Difference	442(381-533)	615(487-649)	0.009
Postprandial Ratio	3.03(2.72-3.49)	3.42(3.08-4.31)	0.1

Values are median (interquartile range).   n = number of subjects in each group.

Table 7.   Effect of GLP-1 on Proximal Gastric Volume (mL)

DIABETES WITH VAGAL NEUROPATHY

	Placebo (n=7)	GLP-1 (n=7)	P
Baseline Volume	71(61-173)	125(105-164)	0.2
Fasting Volume	106(72-177)	125(104-157)	0.3
Fasting Difference	10(-9 - 54)	3(-7 - 26)	0.9
Fasting Ratio	1.15(0.93-1.33)	1.03(0.95-1.12)	0.8
Postprandial Volume	454(406-537)	446(398-565)	0.2
Postprandial Difference	366(294-402)	355(293-409)	0.5
Postprandial Ratio	3.54(2.96-6.69)	3.56(2.84-4.62)	0.7

HEALTHY CONTROLS

	Placebo (n=11)	GLP-1 (n=13)	P
Baseline Volume	112(82-164)	115(80-180)	0.8
Fasting Volume	137(97-156)	147(112-243)	0.3
Fasting Difference	5(-23 - 24)	43(8-85)	0.1
Fasting Ratio	1.07(0.86-1.21)	1.34(1.04-1.9)	0.2
Postprandial Volume	457(330-526)	623(554-640)	0.003
Postprandial Difference	328(184-414)	447(379-546)	0.01
Postprandial Ratio	4.14(3.0-5.15)	5.81(3.19-7.33)	0.2

Values are median (interquartile range).   n = number of subjects in each group.

Table 8. Gastric Volumes (mL) in Healthy Controls and Diabetes with Vagal Neuropathy

	Diabetes Placebo (n=7)	Healthy Placebo (n=11)	P
TOTAL GASTRIC VOLUME			
Fasting Volume	208(145-232)	225(185-239)	0.7
Postprandial Volume	631(582-814)	699(586-774)	0.7
Postprandial Difference	452(400-493)	442(381-533)	0.9
Postprandial Ratio	3.99(2.73-4.26)	3.03(2.72-3.49)	0.7
PROXIMAL GASTRIC VOLUME			
Fasting Volume	106(72-177)	137(97-156)	0.6
Postprandial Volume	454(406-537)	457(330-526)	0.9
Postprandial Difference	366(294-402)	328(184-414)	0.8
Postprandial Ratio	3.54(2.96-6.69)	4.14(3.0-5.15)	0.9

Values are median (interquartile range).   n = number of subjects in each group.

5. Final Remarks

GLP-1 increases gastric volume in healthy humans. Potential mechanism of action

In these studies we have shown a previously unknown effect of GLP-1 on gastric motor function, that is an inhibition of gastric wall tone, as expressed by the increase in gastric volume measured by the SPECT technique. Moreover, we have shown that this effect is absent in the presence of cardio-vagal neuropathy associated with diabetes. This suggests that GLP-1 inhibits gastric tone through a mechanism that involves the vagus nerve.

Based on our data and previous reports, the inhibition of such a wide spectrum of gastrointestinal functions by GLP-1 would suggest that this peptide is acting through inhibition of vagal reflexes in response to different stimulus in the gut, most likely at the level of the afferent pathways, as suggested by others previously [24].

Nevertheless, positive immunoreactivity for GLP-1 and binding of radiolabeled GLP-1 have been demonstrated at different levels of the brainstem, such as at the NTS, area postrema, DMVN, the thalamus or hypothalamus [109-112]. This suggests that GLP-1 may act as a modulator or regulator in the gut-brain axis at different levels.

Moreover, our data are consistent with an inhibitory effect of GLP-1 on vagal cholinergic efferents. In our first study we observed a decrease in pancreatic polypeptide secretion after meal ingestion in the group receiving GLP-1. The secretion of pancreatic polypeptide is under vagal cholinergic control during fasting and during the cephalic phase of the postprandial period [66-69]. During the gastrointestinal phase of the postprandial period, the secretion of this hormone is not only under vagal extrinsic control, but also influenced by enteropancreatic neural, mostly vagal-cholinergic, and hormonal reflexes [69, 113, 114]. In our study, we observed a median decrease of fasting pancreatic polypeptide plasma levels of 12mg/dL in the group that received GLP-1, whereas there was a median decrease of 0.25 pg/mL in the placebo group. This difference did not reach statistical significance. After the meal, the median increase in pancreatic polypeptide plasmatic levels in the GLP-1 group was 6.5 pg/mL compared to 119.8 pg/mL. Since only the gastric phase of pancreatic polypeptide secretion was measured after the meal, and GLP-1 significantly delayed the rate nutrients entered the small

intestine, we cannot exclude that the difference in the postprandial plasma levels of pancreatic polypeptide could be due to the difference in the enteric stimuli of pancreatic polypeptide secretion. Nevertheless, other studies have shown that exogenous GLP-1 decreases pancreatic polypeptide secretion independently of the gastric emptying rate [44]. GLP-1 also inhibits centrally-induced pancreatic and gastric acid secretions [42], effects that are lost after abdominal vagotomy in animals [23] and humans [22]. These data concur with the hypothesis of an inhibitory effect of GLP-1 on vagal cholinergic efferents.

Inhibition of vagal cholinergic input into the stomach by GLP-1 would induce an increase in gastric volume (relaxation) in the interdigestive state. This would also add to the physiological postprandial gastric relaxation through vagal nitrergic activation, and explain the enhanced volumes observed after the meal during GLP-1 infusion compared to placebo. (See Introductory Schema)

It is also conceivable that in the NTS, a region where afferent information from the gut and other organs is integrated, or at the level of DMVN, locally synthesized GLP-1 induces activation of gastric relaxatory reflex by activation of post-synaptic neurons involved in vagal nitrergic or adrenergic inhibitory pathways. This would induce an increase in gastric volume (relaxation) during the interdigestive state and enhance postprandial gastric relaxation, as observed in our studies.

Further studies will be needed to explore whether GLP-1 increases gastric volume through inhibition of vagal cholinergic post-synaptic neurons or by stimulating post-synaptic nitrergic or adrenergic inhibitory pathways.

GLP-1 effects on gastric volumes are lost in patients with vagal neuropathy associated with diabetes

In a group of seven diabetic patients affected by cardiovagal neuropathy, we did not observe the effect of GLP-1 on gastric volume, which had been documented in healthy volunteers. The patient group differed from the healthy control group, not only in the presence of vagal neuropathy, but also in age and the presence of the diabetes itself with its potential for associated clinical complications. Therefore, one may argue that the lack of effect of GLP-1 on gastric volume in diabetic patients with vagal neuropathy, may have been related to differences between the two groups other than the vagal neuropathy. For instance, the incretin effect of GLP-1 is known to be impaired in diabetic patients,

presumably due to a reduced responsiveness of the islet β cells to incretins in these patients [115]. Therefore, it is conceivable that in diabetic patients other effects of GLP-1 may be also impaired due to a reduced responsiveness of the GLP-1 receptors, irrespective of the status of vagal innervation. However, if this is true, we would expect that effects of GLP-1 on other functions would not be present in diabetic patients with normal vagal function. On the contrary, GLP-1 effects on gastrointestinal responses, such as, gastric emptying, appetite or pancreatic secretion, have been shown to be preserved in diabetic patients with non-insulin dependent diabetes mellitus (NIDDM) [7, 10, 14, 41, 116]. This is strong evidence against the hypothesis that there was a diminished response to GLP-1 due to the diabetes per se, independent of vagal function.

The diabetic participants in our study were clearly older than the healthy controls. Aging could have also contributed to the observed differences in the effect of GLP-1 on gastric volume in the diabetic patients compared to the healthy controls, independently of the vagal function. Against this argument, it has been shown that effectiveness of GLP-1 is not significantly affected by age [117]. Moreover, our diabetic participants are similar in age to the diabetic samples included in studies mentioned above [7, 10, 14, 116]. These studies showed the preserved effects of GLP-1 on gastric emptying, appetite, or pancreatic secretion in elderly diabetic patients.

Therefore, we believe our results are more consistent with a lack of response of the stomach volume to GLP-1 infusion due to vagal impairment. This is also consistent with the observed lost of GLP-1 effects on gastric acid secretion after vagotomy in non-diabetic humans [22].

<u>Normal gastric volume response to a meal in patients with vagal neuropathy associated with diabetes</u>

When testing the effects of GLP-1 on gastric volumes in patients with vagal neuropathy associated with diabetes, we observed an unexpected finding, that is, that the gastric volume response after the meal was comparable to that of a healthy control group. We have already thoroughly discussed the evidence of the literature against and in favor of this observation (Discussion p31.)

Vagal participation in the control of gastric tone, and hence in gastric relaxation responses, has been acknowledged for a long time [27, 118-121]. However, it is also

recognized that splanchnic nerves and intrinsic neuronal and humoral mechanisms are also involved in the control of the tone of the stomach [122, 123]. Furthermore, there is increasing evidence that gastric tone may be adequately controlled in the absence of extrinsic vagal innervation [28, 104]. Studies in animals and humans have shown that gastric relaxation responses recover with the passage of time after vagal denervation [28, 30, 77, 78].

Others have suggested that the postprandial gastric relaxation response is impaired in patients after vagotomy [72]. In this study from Troncon et al. [72] the gastric volume responses to imposed pressures and to a standard meal were studied by means of an intragastric balloon connected to a barostat device. They compared the results in seven dyspeptic patients that had undergone bilateral truncal vagotomy and pyloroplasty for peptic ulcer with the results in eleven healthy volunteers. The gastric volume response to distention was similar in both groups. Overall, they reported decreased gastric volume response after meal ingestion in the vagotomized dyspeptic patients. Nevertheless, a careful review of the data in this study shows a normal postprandial gastric relaxation response in three of the seven vagotomized patients, and an unusual absence of gastric accommodation after meal ingestion in two of the eleven healthy volunteers. These unusual observations may have led to erroneous conclusions, and it is relevant that almost 45% (3/7) of vagotomized patients presented normal accommodation.

Previous studies have reported impaired gastric relaxation after meal ingestion in diabetic patients diagnosed withcardio-vagal neuropathy [79]. We have also discussed some of the concerns regarding the validity of those studies based on variability of gastric volume responses, inappropriate controls and the presence of concomitant dyspeptic symptoms in diabetic patients (Discussion p30.).

Since we used an indirect measurement of vagal dysfunction, one could argue that the normal gastric volume response to a meal observed in our diabetic participants could be explained by the lack of a real vagal neuropathy in these patients. Certainly, since these were *in vivo* studies, rather than pathological studies of the vagus nerve, we cannot exclude this possibility. Nonetheless, heart rate variability in response to deep breathing is the gold standard test currently used to diagnose cardio-vagal neuropathy [89-91]. It is considered that gastric vagal function is affected when cardio-vagal impairment is present, since the vagus is a long nerve and metabolic neuropathies start in distal fields

before progressing proximally. Thus, demonstration of functional impairment in the territory innervated by more proximal vagal branches, such as the cardiac nerves, implies more distal impairment of the nerve (e.g., gastric branches). This has been shown in several studies performed by pathologists [81-83]. When trying to develop a test for an early diagnosis of vagal neuropathy in diabetic patients, it was found that impaired abdominal vagal responses (ie pancreatic polypeptide and gastrin responses to sham feeding) preceded the development of cardio-vagal impairment, and it was always present in patients with positive cardiovagal neuropathy [84-87]. Therefore, a positive test of cardio-vagal dysfunction implies abdominal vagal dysfunction. Thus, we believe that our diabetic participants present the features of diabetes autonomic vagal neuropathy, and that the normal gastric volume response to a meal observed in the presence of vagal neuropathy adds to the growing evidence of the existence of an adaptive response preserving postprandial gastric relaxation when the main vago-vagal reflex is impaired.

6. Summary and Future Studies

The presented data provides new information on the effects of GLP-1 on upper gastrointestinal functions and increases the knowledge of its mechanism of action in humans. These data also suggests that the gastric accommodation response, a long-recognized vago-vagal reflex present in animals and humans, is maintained in the presence of diabetes associated with vagal neuropathy, suggesting that adaptation of other extrinsic or intrinsic neural pathways compensate for vagal denervation. This is consistent with previously observed adaptation of other visceral vago-vagal reflexes in animals and humans.

Further studies are required to evaluate the mechanism of action of GLP-1 on inhibition of gastric tone. Several plausible hypotheses may be proposed from an appraisal of the presented results and the literature. Experimental data suggest that radiolabeled GLP-1 can bind to DMVN neurons. It is possible that GLP-1 could bind and inhibit cholinergic motor neurons in the DMVN. Inhibition of this pathway would inhibit gastric tone consistent with an inhibitory effect of GLP-1 on vagal cholinergic efferents. Secondly, it is also possible that GLP-1 binds to cholinergic motor neurons in the DMVN that activate nitrergic neurons in the stomach [124], or GLP-1 may bind directly to nitrergic motor neurons in the DMNV [125, 126]. Activation of the last two pathways would also lead to inhibition of gastric tone [126]. Concomitant studies with the nitric oxide synthase inhibitor L-NMMA and GLP-1 would allow us to test the hypothesis that nitrergic pathways participate in the inhibition of gastric tone by GLP-1.

A further series of studies in the future should explore whether endogenous GLP-1, released in the small intestine and colon after meal ingestion, plays a role in the control of gastric accommodation to a meal. Thus, (9-39)-exendin, an antagonist of the GLP-1 receptor, could be used to test this interesting hypothesis.

7. Acknowledgments

8. References

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