Acceleration of Nutrient Emptying from the Stomach to the Small Bowel. A New Potential Mechanism to Decrease Caloric Intake in Humans

Tesi Doctoral

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GENERAL INTRODUCTION

Obesity: Current Therapeutic Options

Obesity has reached epidemic proportions worldwide [1]. Analyses of data from the National Health Surveys (2006) show that more than 50% of the spanish population over 18 years of age are overweigth or obese [2]. Obesity, as defined by a body mass index (BMI) equal or superior to 30 Kg/m^2, has shown a great impact on quality of life, morbidity and mortality [3, 4].

Longstanding modification of dietary habits, lifestyle and increasing of physical exercise are the cornestone of obesity interventions. However, current lifestyle in developed countries favors the ingestion of high energy density foods and low energy expenditure [5], and thus, these objectives are difficult to reach in most cases. Hence, adjuvant therapies that might help to attain the desired energy balance in patients with high metabolic risc are of interest and have been the foccus of several research studies [6]. Currently, there are only two drugs approved for the treatment of obesity, sibutramine and orlistat. Orlistat, an inhibitor of the intestinal lipase, reduces fat absorption and induces a statistically significant, albeït clinically moderated, weigth loss [7, 8]. Notwithstanding, after initial weight loss subjects ussually return to their initial weight even if the treatment is maintained, and diarrea and severe bloating leads patients to drop treatment in some instances [9] . Sibutramine is a serotonin and adrenaline central uptake inhibitor that induces the sensation of fullness. Weight loss associated to sibutramine treatment is moderate and the side effects profile of the drug limits its generalized use [6].

When medical treatment fails, the only currently available therapeutic alternative is bariatric surgery. This option is currently accepted for class III or class IV obesity (BMI ≥

40) subjects or for class II (IMC=35-39,9) obesity patients with associated comorbidities [10, 11]. Surgical bariatric techniques in use include derivative and/or restrictive techniques that aim to induce intestinal malabsorption and/or reduce gastric capacity, respectively [11]. Bariatric surgery has shown to be effective to induce weight loss and to improvecomorbidities. However, the short-term mortality and long-standing associated comorbidities recommends the restrictions of its use inside multidisciplinary groups with long lasting experience. Moreover, bariatric surgery does not exempt the patient to adhere to a healthy lifestyle in order to ensure that weight loss will be maintained on the long-term follow up [12].

Thus, it is of great public health interest to develop new medical treatments that, associated with cognitive interventions, might help the patient to control energy intake, as surgery does, but with less risk for the patient and that could be more easily generalized to the population at risk.

This thesis is framed within this broad objective and studies hypotheses are based on our current knowledge on gastrointestinal motor physiology and its role in the regulation of energy intake in humans.

Acute Caloric Intake Regulation: The Gastrointestinal Tract

The interruption of a meal depends on a basic stratta on the balance between hunger levels before starting eating and the progresive perception of satiation during feeding, which, ultimately, will lead to stop meal intake [13, 14]. There is scientific evidence that mechansims that control satiation might be altered in obese subjects [15-21]. We have shown that overweight and obese subjects have delayed perception of satiation when drinking a nutrient drink at a constant rate and decreased postprandial fullness after a

fully satiating meal [22]. The potential mechansims underlying this diminished satiation responses have yet to be clarified.

From the gastrointestinal tract arise a variety of signals that convey the sensation of satiation to the central nervous system (CNS). These gastrointestinal signals participate in the regulation of energy intake and are known as satiation factors [23-28]. These include:

- Neuropeptides, citokines and hormones that inhibit energy intake (such as amilin, apolipoprotein A-IV, bombesin, colecistoquinin (CCK), enterostatin, glucagon, glucagon-like peptide-1 (GLP-1), peptide YY-(3-36), pancreatic polipeptide (PP) or somatostatin), which are secreted in response to the arrival of nutrients into the small bowel. These satiation factors estimulate specific receptors sited in the nerve endings of the small bowel wall, which start firing reaching the CNS contributing to the fullness sensation that is building up during the meal ingestion period and that contributes to meal termination [29-34].
- Other factors, such as ghrelin, an orexigenic signal that participate in the regulation of energy balance, are secreted at the gastric level [35]. Ghrelin is secreted by cels in the gastric mucosa in fasting conditions and is absent or significantly reduced in gastrectomized patients or after gastric derivative surgery with exclusion of the gastric fundus [36-38]. Ghrelin is considered a meal initiating hormone. Plasma ghrelin rises to its maximum level in fasted humans and other mammals prior starting feeding and returns to basal levels at the end of the feeding period [36].

Vagal and splanchnic afferent fibers in the gastric wall also signal satiation in response to chemical and mechanical stimuli during ingestion [24, 27, 28]. Thus, the rate of emptying of gastric contents determines, at least partly, the time that nutrients are stimulating chemo- and mechanic neural receptors in the stomach [28, 39, 40]. Slow gastric emptying has been associated with increased fullness levels and decreased caloric intake [39, 41-43]

and has been proposed as a mechanism to reduce food intake in humans. On the other hand, as mentioned above, when nutrients enter into the small bowel also estimulate chemo- and mechano-receptors in the intestine wall and estimulate secretion of satiation factors mentioned above [44, 45]. Moreover, postprandial decrease on ghrelin plasmatic levels has been shown to depend on nutrients emptying from the stomach. Williams et al. compared ghrelin supression after gastric or duodenal nutrient infusions in rats and controlled gastric emptying using a pyloric cuff preparation [46]. Thus, they showed that the presence of nutrients in the stomach was not sufficient to supress ghrelin secretion and that must require feedback from intestinal or postabsorptive sites. Furthermore, they showed, in a different study, that ghrelin postprandial supression could be induced from the jejunum in the absence of duodenal contact with nutrients. Finally, it has been shown that increased gastric emptying of solid food is associated with lower intake capacity and higher postprandial fullness in patients with dyspepsia syndrome[47]. All these data suggested as, that acceleration of emptying of nutrients from the stomach to the small bowel might be a potential mechanism to reduce meal size by precociously stimulating the neuro-hormonal satiation cascade.

Measurement of *Ad Libitum* Caloric Intake or Satiation

Currently, acute caloric intake, usually referred as satiation, the final signal during meal ingestion that leads to meal termination [14], is measured by computing the energy intake during an ad libitum free buffet-type meal. Buffet meals try to mimic a real-world eating situation. However, they are expensive, cumbersome and have not been standardized. Thus, different laboratories use different food options and perform the test at different day times. All these caveats make it difficult to use buffet meals in large-scale studies where satiation is an outcome of interest.

Recently, a laboratory-based and easy to perform nutrient drink test has been developed to measure acute intake capacity and gastrointestinal symptoms in response to a meal challenge [48]. It uses a commercialized nutrient drink, which is cheap and internationally available in a variety of flavors. During this test the subject is asked to consume the nutrient drink at a constant rate until reaching the maximum level of satiation. The nutrient drink test has shown good reproducibility and sensitivity to change in healthy and dyspeptic subjects [49-52]. Thus, the nutrient drink test might be a good candidate to measure ad libitum energy intake in human studies.

STUDIES

In the studies included in this thesis we propose 1) to compare *ad libitum* energy intake, as measured by a free -buffet meal and by the nutrient drink test, to assess if both tests are comparable and correlate in healthy subjects and 2) to assess the effect of pharmacological acceleration of gastric emptying of nutrients on ad libitum intake capacity in humans.

Study 1. Comparison of ad-libitum Energy Intake and the Neuro-Endocrine Satiation Response as Measured during a Buffet-Type Meal and a Standardized Nutrient Drink Test

Introduction

Whole day energy intake is controlled by two different neuroendocrine circuits. One that is on control while we are feeding and that finalizes when we feel satiated. And the second, which is on control during fasting and which, eventually, finishes when hunger sensation reaches a threshold level that leads us to start feeding again. The first is known as satiation and is expressed as the total amount of energy ingested until satiated, as opposed to the second, known as satiety, which is expressed as the time between feeding periods[14].

Current gold standard to measure satiation is a free buffet-type meal where subjects are offered to eat *ad libitum* from a buffet with food in excess [53, 54]. Several research groups working in this field use different type of buffets, with different meal items and variable macronutrient composition, depending on regional food preferences and availability [55, 56]. Some laboratories offer a breakfast buffet meal in the morning after an overnight fast

and others offer lunch buffets [57]. Some limit the time subjects are feeding, usually to 30 minutes, while others let the subjects eat freely until they feel satiated. Buffet meals offer the opportunity of assessing satiation in a setting that, although somehow controlled, tries to mimic a real-world eating situation. However, its lack of standardization across different labs does not permit to make results comparable [55, 57]. Moreover, preparing the buffet meal, weighting food items before and after the subject eats to calculate the energy and macronutrient contents of the food consumed is cumbersome and costly. All these caveats prevent buffet meal to be used in large-scale population studies or big Phase III clinical trials.

Recently, a laboratory-based and easy to perform *ad libitum* nutrient drink test was developed by gastroenterologists working in the field of dyspepsia to measure acute intake capacity and gastrointestinal symptoms in response to a meal challenge[49]. It uses a commercialized nutrient drink, with known energy content and macronutrient composition, which is internationally available and cheap. During test subjects are asked to drink at a fixed rate until the satiation sate. The subject is allowed to choose among available flavors (usually strawberry, chocolate or vanilla) and is excluded from the study if he or she expresses a dislike for the drink. The nutrient drink test has allowed standardization of assessment of acute caloric intake capacity and gastrointestinal symptoms after feeding in a controlled setting. It has been tested in health and disease populations and has shown good reproducibility and sensitivity to change [49-52, 58]. The dynamics of the test allow simultaneous measurement of gastric emptying rate, gastric wall accommodation and small bowel transit plus the neuro-endocrine response to arrival of nutrients to the gut. This may allow establishing the partial roles of the different players in the regulation of human food intake and symptoms response to feeding. With this test we and others have been able to demonstrate that caloric intake capacity depends partly on gastric volume before meal as well as the rate the nutrients reach the small intestine

and the levels of plasmatic satiation peptides like cholecystokinin [47, 59]. The nutrient drink test has allowed establishing previously unknown relationships between several genetic variants in genes controlling gastrointestinal function, obesity and satiation [60]. A variant of the FTO Gene, specifically the A allele of rs9939609 has been linked to increased caloric intake with the buffet meal and the nutrient drink test [60, 61]. Thus, the nutrient drink test may be a good candidate to substitute buffet meals in future human satiation studies.

However, the nutrient drink test may have several limitations to assess real satiation. Since palatability and central reward systems influence food intake, it is conceivable that during a buffet meal, where foods with different palatability are freely offered and is the subjects' choice to take one or the other, food might be consumed beyond the point of strict satiation. On the contrary, during the nutrient drink test, where subjects are offered a limited choice of tastes, subjects might finalize ingestion prior to the "real" point of satiation. Moreover, in one test subjects drink and eat while in the second only a liquid is ingested and furthermore, during the buffet subjects ingest at their own pace whereas they drink at a fixed rate during the drink test. Therefore, it seems rather plausible that energy intake will not be the same under these two rather different circumstances. However, due to the great advantages of the nutrient drink test regarding standardization and application use in large population trials we wished to test within subject correlation of both measurements under the hypothesis that, even though not the "real" satiation, the nutrient drink test could provide us with a good surrogate of satiation to be used in studies to advance our knowledge on the mechanisms of satiation control. Moreover, apart from correlation of satiation measurements between both tests we also aimed to test if the two tests, under the same circumstances, would provide the same information regarding the factors that influence satiation.

Materials and Methods

Study Population

Participants were recruited by public advertisement, which was previously approved by the hospital ethical committee. All of them signed the informed consent after receiving detailed explanation of the study procedures before enrollment.

We included healthy subjects from the community, 18-65 years old, with no gastrointestinal disease or symptoms. Exclusion criteria included: pregnant or breastfeeding females, prior gastrointestinal surgery (including fundoplication, appendectomy and laparoscopic cholecystectomy), organic or functional diseases or conditions that may affect the gastrointestinal tract, positive symptoms on an abridged bowel disease questionnaire [62], diabetes, neurologic, kidney or psiquiatric disease, any form of neuropathy, unable to withdrawn medications 48 hours prior to the study other than contraconceptive pills, thyroid hormone and estrogen replacement and vitamin supplementation and unable to give valid informed consent due to physical or mental condition. Eating disorders were excluded with the validated Eating Attitude Test [63, 64] and anxiety and depression with the validated Hospital and Anxiety Depression Scale (HADS) [65, 66].

Study Design and Protocol

All subjects underwent the two tests, the free-buffet meal and the nutrient drink test, on two different days, one week apart and in random order. On both days, tests were performed at the same time in the morning after an overnight fast. Plasma levels of satiation factors were also measured before and during feeding for both tests.

Ad libitum Caloric Intake by Standardized Nutrient Drink Test

Briefly, participants drank a nutrient liquid meal (Ensure®: 1Kcal/mL, 11% fat, 73% carbohydrate and 16% protein) at a constant rate (30mL/min.). Participants were allowed to choose among three different Ensure® flavors (chocolate, vanilla and strawberry) and were excluded from enrolment if they expressed a dislike for the taste of the test meal during the consent process.

At baseline and every five minutes, participants were requested to score their satiation state, using a 6-grade scale that combines verbal descriptors and numbers (0 = no satiation sensation, 1= 1st sensation of fullness, 2= Mild fullness, 3= Moderate fullness, 4= Full/ No hunger and 5 = Max. Satiation, I can't eat more). Participants were asked to stop meal intake when they reached the score of 5.

We recorded time (minutes), total number of calories (Kcal.) ingested to reach maximum satiation state and the macronutrient distribution (in % and grams of energy).

Ad libitum Caloric Intake by a Buffet-Style Meal

Participants were offered a buffet meal with food in excess after an overnight fast, which included whole meal bread, white bread, toasted bread, cereals, muffins, lettuce, cabbage, corn, cookies, tomato, olive oil, butter, marmalade, quince, sugar, saccharine, pork sausage, Serrano ham, ham, cheese, plain and fruit flavored yogurt, a variety of fruit pieces, orange and pineapple juice, coffee, chocolate drink, infusions, water and whole milk. Participants were allowed to eat freely until they felt fully satiated. At baseline and every ten minutes participants were requested to score their satiation levels as performed during the drink test.

The amount of food consumed from the buffet meal was assessed by S.R, who weighed the food items before and after eating, as usually done when using this test [67]. Energy and

macronutrient composition were obtained from the manufacturer information and standard nutrition tables as available.

We recorded the time (minutes), the total number of calories (Kcals) ingested to reach maximum satiation and the macronutrient distribution (in % and grams of energy).

Neuro-Endocrine Control of Satiation

Entero-insular and satiation related neuropeptides and hormones were measured at baseline and during feeding. We measured plasma levels of glucose, glucagon, insulin, cholecystokinin (CCK), active glucagon-like peptide-1 (GLP-1), pancreatic polypeptide (PP), peptide YY (PYY), active ghrelin and leptin.

Blood draws were taken during fasting (Time 0) and during feeding (at 10, 20, 30 minutes and at the time participants finished feeding [Final]).

Plasma concentrations of glucose were measured in serum using an automated hexoquinase method on a Roche's Modular TM instrument (Roche Diagnostics GmbH, Mannheim). Insulin was measured in serum by a solid-phase chemiluminiscent immunometric assay using different anti-insulin antibodies on an IMMULITE 2000 TM analyzer (Siemens Healthcare Diagnostics Inc. Llanberis, UK) with a lowest detectable concentration of 2 μIU/mL and coefficients of variation ranging between 4.1% and 7.3% (7.7 – 291 μIU/mL) for total imprecision. Glucagon was analyzed in plasma collected in EDTA-Aprotinin tubes using a competitive radioimmunoassay kit with an anti-glucagon antibody plus 125I labeled glucagon (Siemens Healthcare Diagnostics Inc. Llanberis, UK) and showing the following performance data: 13 pg./mL (analytical sensitivity) and 6% to 11.9 % (interassay precision for samples ranging 59 -351 pg./mL).

Plasma levels of CCK were measured, as in previous studies [68], by a competitive radioimmunoassay using an antiserum raised against CCK-8 sulphate N-terminally

conjugated to bovine albumin (Euro-Diagnostica, Malmö, Sweden). Plasma was obtained from blood collected in tubes containing EDTA and Trasylol (500 KIU/ml), and extracted with 96% ethanol previous to RIA.

The lowest detectable concentration was 0, 3 pmol. /l, being the intra and inter assay variation <10% and <15%, respectively.

Hormone measurements of active GLP-1, PP, PYY, active ghrelin and leptin were performed on a Luminex 200 TM system using a commercially available kit and according to the manufacturer's directions. Calibration and validation beads as well as sheat fluid were purchased from Bio-Rad Laboratories (Hercules, CA) and fluorescence data were analyzed by the Bio-Rad's Bioplex 6.0 software. Multianalyte profiling were analyzed with the Milliplex-Map-Kit TM Human Gut Hormone 5-plex panel (Millipore #HGT-68K) assaying duplicates of all plasma samples in several batches. Plasma samples were collected using EDTA tubes adding several protease inhibitors too for the active hormone's measurements (Millipore's DPPIV inhibitor, Sigma's protease inhibitor cocktail and Roches's Pefabloc SC). The minimum detectable concentrations (pg./mL) were 5.2 (active GLP-1), 2.4 (PP), 8.4 (PYY), 1.8 (active ghrelin) and 157.2 (leptin) respectively, reporting intra and inter-assay variation (%CV) <11% and <19% across two different concentrations of all hormones.

Data Analysis

Satiation was summarized as the number of calories (Kcal.) ingested until the point of maximum satiation was reached. Neuroendocrine satiation response was summarized, as usual, by computing the ratio of the highest feeding plasmatic level over the fasting plasmatic level (Fed/ Fast Ratio) for each satiation factor.

Sample Size and Statistical Analysis

The main variables to compare the two tests under study were: total energy intake (Kcal.) and time (minutes) until fully satiated. We used matched-pairs analyses with sign-rank test if data were not normally distributed. Means and their 95% confidence intervals (95% CI) are reported. However, we were interested in the "correlation" of the variables measured by the two different tests, rather than a pure statistical comparison of means. Thus, we calculated Pearson or Spearman correlation coefficients, depending on the normality of the variables' distribution. Mean energy intake, as measured by the nutrient drink test is about 1330 Kcal. (95%CI 1250; 1400) [59]. Thus, a sample n= 24 gave us 95, 90, 85 and 80% power, to detect correlation coefficients of 0.62, 0.56 and 0.4, respectively, with a significance level of 5%.

A secondary analyses' aim was to compare if factors influencing or associated with satiation would be the same for both tests. Hence, we used multiple (lineal) regression analyses to build models of energy intake during the buffet meal and the nutrient drink test. We used stepwise regression to fit models using forward, backward and mixed methods for selecting effects. For the construction of the models we included as independent variables: plasma levels of the satiation factors measured during fasting, feeding, and the feeding/ fasting ratios, gender, body mass index, HADS scores and EAT-26 scores The same potential regressors were explored for both models. Best fitted regression models were selected based on adjusted R^2 with the least number of independent variables.

Results

Study Population

We evaluated 24 healthy subjects (16 females and 8 males). Mean age was 37 years (31; 41) and mean BMI was 24.5 Kg/m^2 (23; 26). None had eating disorders, nor anxiety or depression. Baseline characteristics of the study population are shown in Table 1.

Time to Satiation State

The progression of the feeling of fullness throughout time during feeding was similar during the two tests (Figure 1). On average, subjects reached the satiation state 4.8 (0.3; 9.2) minutes later during the free-buffet meal as compared with the drink test (p= 0.04 paired t test). However, time to reach maximum satiation on each test was significantly correlated within subjects (Pearson correlation: 0.57; p= 0.004).

We explored whether age, gender and BMI, variables that have been shown in previous studies to influence time to satiation, as measured by the nutrient drink test [59], might also show the same effect in the current study. We report least square means (LSM) and their standard errors (SE) from ANCOVA models. During the drink test, overall mean time to satiation was 34minutes (29; 40) and was significantly and independently affected by age (ANCOVA p=0.01) and gender (ANCOVA p=0.002), but not BMI (R^2: 58%; p=0.0001). Thus, during the drink test, the satiation state was reached, on average, after 30 (2.14) minutes, by females, compared to 46 (3.09) minutes by male subjects; and 4.4 (1.57) minutes earlier per each 10 year increase in age. On the other hand, during the buffet meal, overall mean time to satiation was 39minutes (35; 43) and it was significantly influenced by age (ANCOVA p= 0.03), but not by gender (p=0.10) or BMI (p=0.14). Thus,

satiation state during the buffet-like meal was reached, on average, 3.7 (1.58) minutes earlier per each 10 years increase in age.

Total Energy Intake and Macronutrient Composition

Regarding calories ingested until the satiation state; on average, subjects ingested 1191Kcal. (995; 1388) and 1017Kcal. (855; 1179) during the buffet and the drink tests, respectively (p= 0.03 paired t-test). Still, there was a significant within subject correlation between calories ingested on both days (Pearson correlation: 0.62; p= 0.001). Figure 2A

Subjects consumed similar grams of proteins and carbohydrates on both days (p= 0.4 and 0.2, respectively; paired t-tests) and there were correlated within subjects (Pearson correlations: 0.6 and 0.7; p= 0.004 and 0.0003, respectively). However, there was a greater ingestion of fat during the buffet meal compared to the nutrient drink test (57grs. [45; 68] vs. 34grs. [29; 40], respectively; p=0.0001 paired t-test). Nevertheless, fat intake on both days was, again, significantly correlated within subjects (Pearson correlation: 0.50; p=0.02).

Plasmatic Neuroendocrine Satiation Responses

Figure 3 shows the plasmatic levels of the different glucose-homeostasis and satiation-related factors measured during the fasting and feeding periods.

Glucose homeostasis was similar in both days with the only difference of an earlier increase of glucose and insulin during the feeding period of the drink test (Figure 3A).

Ghrelin descended during feeding similarly during the buffet and the drink test. However, during the buffet meal there was a 10 minute lag period before ghrelin started to descend compared to the drink test, during which ghrelin was observed to decrease right after

initiating drinking (Figure 3B). Nevertheless, overall satiation response of ghrelin, as measured by the feeding/ fasting ratios, was almost identical between tests (0.6 and 0.6, for buffet and drink test respectively; p=0.97 paired t-test).

As expected, GLP-1 and PYY both increased during feeding. The plasmatic levels of both peptides increased in response to feeding similarly during the buffet meal and the drink test (Figure 3B). Overall feeding response, as measured by the fed/ fasting plasmatic ratios, were highly comparable, for both peptides, during the buffet and drink test. Thus, GLP-1 ratios were 4.97 (4.11; 5.83) and 5.46 (3.84; 7.08) for the buffet and drink test, respectively (p=0.5 paired t-test) and PYY ratios were 1.60 (1.41; 1.78) and 1.64 (1.37; 1.91), for the buffet and drink test, respectively (p= 0.72 paired t-test).

Similar feeding responses were observed as well for PP during the buffet and drink test (Figure 3C). During the buffet there was an immediate rise in plasmatic levels of PP and then a steady descend after 20 minutes of feeding. The same was observed during the drink test, though the rise on plasmatic PP was less pronounced. However, there were no significant differences on PP feeding/ fasting ratios on both days (9.41 [7.0; 11.82] and 7.63 [5.30; 9.95] for the buffet and drink test, respectively; p=0,15 paired t-test).

We observed a biphasic CCK feeding response on both study days. Plasmatic levels of CCK rose during the first 10 minutes of feeding and again, yet much more powerfully, on the second half of the feeding period (Figure 3C). Even so, CCK feeding response was significantly more intense during the drink test compared to the buffet meal (7.52 [4.12; 10.92] vs. 3.15 [2.13; 4.17], respectively; p= 0,01 paired t-test). However, within subjects CCK ratios were highly correlated (Pearson correlation: 0,66; p= 0,01. Figure 2B).

Satiation Explanatory Models

As explained, we compared factors' effects on satiation for each meal test using stepwise regression modeling. Final models reported are the best fitted least square models, that is, those that explained the highest variance (R^2) of energy intake with the less number of independent variables.

Both meal tests yielded the exact same stepped models. Table 2 shows the regressors explored, with their unadjusted effect on energy intake (univariate analysis) and the final stepped model with the variables that, independently, influenced energy intake after adjusting for the other variables. Thus, in both cases, gender and the GLP-1 fed/ fasting ratios were the factors that were found independently associated with energy intake (Table 2).

Discussion

Current gold standard to measure satiation is a free buffet meal, which is difficult to standardize, hazardous and expensive and thus, does not seem suitable to be used in large-population studies. Recently, a simple nutrient drink test that measures energy and volume intake until satiation has been developed, as a tool to evaluate symptoms' response to feeding. In this study we aimed to test if the nutrient drink test, as described by Camilleri et al. [48], could reliably measure satiation.

The presented results show that, as expected, ad libitum energy intake is greater during a free-buffet meal than during the nutrient drink test. However, calories ingested by a subject during a buffet meal seem to correlate well with calories ingested by the same subject during the drink test. Moreover, even though during the buffet meal subjects could make their own choices regarding type of food item consumed, the overall ingestion of

carbohydrates and proteins were very similar both days and correlated highly within subjects. Only fat ingestion was clearly superior during the buffet. We believe this might be explained by the low proportion of fat in the composition of the commercialized nutrient used in the study, 32grs for 2000Kcals, which is half the average recommended daily fat intake in a healthy diet and is, probably, below average fat content of participants' usual meals. Thus, it would not be unconceivable that these differences might fade away in the alleged use of a nutrient drink with greater fat content, similar to the contents of a usual diet. Not withstander, though average group lipid intake differed from one day to another, it was highly correlated within subjects. Hence, we believe this adds support to our proposal of the potential use of the nutrient drink test to measure satiation in a more simple and standardized manner that may be easily reproduced across studies from different labs.

Albeit subjects ate at their own pace during the buffet meal while had to drink at a fixed rate during the drink test, we were surprised that subjects reached the satiation state only 5 minutes earlier, on average, during the drink test compared to the buffet meal. Moreover, the progression of fullness or satiation levels was very similar across time in both tests. One potential explanation could be that the intake rate we elected for the drink test might highly resemble the physiologic feeding rate. This is actually a matter that might be of interest to discuss further. The nutrient drink test was proposed with the aim of standardizing assessment of dyspepsia, as an in-lab provocative test. Dyspepsia refers to a wide array of noxious sensations that arise from the proximal gastrointestinal tract usually during and after feeding. Different groups have used different nutrient liquids, some groups have even used a water load test [69], and different ingestion rates. Thus, some investigators [70] have evaluated performance characteristics of a nutrient drink test using Nutridrink ®, which contains 1.5Kcal/mL with 39% fat at 100mL/min and have shown a mean ingestion of 1962Kcal (1,308mL) to achieve satiation. Others have shown, using the same Nutridrink® but with a much slower ingestion rate, 15mL/min, an average

ingestion of 1507Kcal (1,005mL) until full satiation. With the nutrient drink test used in the present study, which contains less fat and 1Kcal/mL, and an ingestion rate of 30mL/min, average caloric ingestion until the satiation state has been shown in a previous large study on 133 healthy volunteers to go from 1000Kcal to 1400Kcals, depending on gender and BMI[59]. Thus, extremely higher rates of ingestion are associated with greater caloric intake, whereas caloric intake seems more stable when using rates of ingestion of 15 to 30mL/min. Higher ingestion rates might be useful when the aim is to assess dyspepsia, but probably not suitable to assess physiologic satiation.

In this study we aimed to assess the potential application of the nutrient drink test in human satiation studies. Therefore, a valid estimate of *ad libitum* energy intake from the test would not suffice for this. In order for the nutrient drink test to be useful it would need to induce comparable neuro-hormonal satiation responses if we were to use it to get insight on human satiation control mechanisms. Thus, we measured plasmatic levels of factors that are related to food intake control. Our data show a precocious plasmatic response to feeding of glucose, insulin and ghrelin during the drink test. This might be conceivable explained, at least partly, due to the liquid nature of the test meal. Liquids reach the stomach and small bowel before solid that needs chewing and trituration in the stomach prior stimulating the secretion of these gastrointestinal factors. Contrarily, pancreatic polypeptide plasmatic levels increased more powerfully, initially, during the buffet meal, probably due to an enhanced cephalic phase during solid, and more palatable, food ingestion [71]. However, overall feeding plasmatic responses of these factors, as assessed by the ratio between the maximum plasmatic levels during the feeding period over the plasma level during fasting, were highly comparable, except for CCK. Cholecystokinin increased significantly more during the drink test than during the buffet-type meal. Cholecystokinin is secreted by I cells in the duodenum and jejunum in response to, mostly, fat and proteins. However, fat ingestion was lower during the drink test than during the buffet. Thus, this heightened CCK response during the drink test might as well

be explained by the earlier arrival of the liquid nutrient, compared to the solid food, into the small bowel. Moreover, fatty acid composition of the nutrient drink used in this study may also explain this difference. The release of CCK needs the triglycerides to be hydrolyzed into fatty acids, and unsaturated long-chain fatty acids are known to induce a much more powerful CCK response, whereas short (<12C) saturated fatty acids do not generate any CCK release[53, 72, 73]. Ensure® fat content comes from canola, safflower and corn oils, all rich in monounsaturated long-chain fatty acids as opposed to fat content of food items offered in the buffet meal, which were mostly saturated. This difference between the two tests might be relevant since CCK is one of the factors that have deemed to be relevant in the regulation of food intake [74, 75]. However, our results show that, although different, CCK responses were correlated within subjects and that, in the conditions of this study, both testing conditions yield the same satiation explanatory models. In a group of rather homogeneous healthy people, with no psychological distress or eating disorders, and taking into account that other variables that are thought to be important for the control of food intake have not been measured, like gastric emptying or gastric volume, gender and the postprandial response of GLP-1 explained most of the variance of measured energy intake. GLP-1 is released from L cells of the jejunum 5 to 30 minutes after feeding in proportion to energy intake. This increase in plasma levels of GLP-1 is known to induce satiety and, thus, inhibit further caloric intake as well as inhibition of gastric acid secretion and motility. Thus our observation is simply adding evidence to the known relationship between energy intake and postprandial increase in GLP-1 plasmatic levels. We could not show a significant relationship between energy intake and other factors known to influence satiation such as CCK [74]. The present study was designed to compare the nutrient drink test with the buffet meal. Thus, it was not powered to explore factors that explain satiation. Hence, a type II error may explain that we could not demonstrate for example a relationship between CCK fed/fast ratio and energy intake. Another conceivable explanation might be that plasma measurements of

CCK may not fully reflect all nutrient-induced CCK release. CCK effects on energy intake are thought to be neurally mediated through activation of CCK A receptors in the enteric wall. And there exists evidence showing that locally released CCK can activate enteric neural afferents in the absence of significant plasma CCK elevations [76].

In summary, the data from this study suggest that the estimates of *ad libitum* energy intake obtained by the nutrient drink test correlate well with those obtained with the gold standard free-buffet. Moreover, plasmatic responses of hormones and peptides that participate in the regulation of satiation are also comparable and correlate well between the two meal tests. In the controlled setting of our study and with the limitations of not having assessed gastric functions that are known to be relevant in satiation, we have been able to show that both tests provide comparable satiation explanatory models. Thus, we believe these data further support the potential of the use of a simple nutrient drink test to translate important data on satiation/obesity physiology obtained on experimental studies into large population human studies.

Study 2. Meal Size can be decreased in Obese Subjects through Pharmacological Acceleration of Gastric Emptying. (The OBERYTH Trial)

Introduction

The devastating effects of the obesity epidemic worldwide on the population health and its enormous costs for our society has stimulated concerted action to prevent excessive weight gain [5]. Most healthy individuals increase weight unless energy intake and energy

expenditure are adequately balanced. Hence, increasing energy expenditure through exercise and decreasing caloric intake remains the cornerstone of obesity management and prevention. However, this goal is increasingly difficult to attain in modern society that has evolved towards comfort and where processed food with high energetic content is conspicuous and much cheaper than non-processed, healthier food [5]. Thus, therapeutic options that aim at diminishing food intake trough either decreasing hunger or increasing satiation would seem useful adjunctive therapies to achieve adequate energy balance in obese subjects.

Arrival of nutrients to the small intestine normally stimulates the secretion of several peptides from cells of the intestinal mucosa. These peptides are part of the so called satiety or satiation factors,[34] since they induce a sensation of fullness, satiety or satiation, leading to meal termination, and thus decreased caloric intake, through neuro-hormonal mechanisms.[34, 77]

Our hypothesis was that pharmacological acceleration of gastric emptying of nutrients towards the small intestine would induce precocious activation of intestinal satiation factors resulting in precocious meal termination and decreased caloric intake in overweight or obese human subjects. The present study was designed to test this hypothesis.

Materials and Methods

Study Population

Participants were recruited by public advertisement, which was previously approved by the hospital ethical committee. All of them signed the informed consent after receiving detailed explanation of the study procedures and before randomization.

We included overweight and obese (BMI ≥ 25 Kg/m^2) healthy subjects from the community, 18-65 years old, with no gastrointestinal disease or symptoms. Exclusion criteria included: pregnant or breastfeeding females, prior gastrointestinal surgery (including fundoplication) other than appendectomy, laparoscopic cholecystectomy or tubal ligation, positive symptoms on an abridged bowel disease questionnaire, diabetes, any form of neuropathy, unable to withdrawn medications 48 hours prior to the study other than contraconceptive pills, thyroid hormone and estrogen replacement and vitamin supplementation and unable to give valid informed consent due to physical or mental condition.

Study Design and Protocol

To evaluate the effects of accelerating gastric emptying on satiation, we studied patients in two different days, at baseline (Day1), without the effect of any drug, and, on Day 2, 1 week apart, under the effect of 60 minutes infusion of intravenous saline (placebo) or erythromycin (3mg/Kg), as randomly assigned in a double blind fashion. The dose of erythromycin used in this study was chosen on the basis of a previous validation study [78] that showed it accelerates gastric emptying of an identical test meal. The randomization, blocked by gender, was performed by personnel of the hospital pharmacy and the code was not unsealed to the study investigators until all participants completed the studies.

The study protocol was the same on both study days. Subjects underwent simultaneous assessment of ad libitum caloric intake and postprandial gastrointestinal symptoms using a standardized and validated nutrient drink test, during which gastric emptying was measured using scintigraphy. Plasma levels of satiation factors were also measured during the drink (Figure 1).

Caloric Intake and Postprandial Symptoms by Standardized Nutrient Drink Test

To measure satiation (*ad libitum* caloric intake)[79] and postprandial gastrointestinal symptoms, we used a modified nutrient drink test as described previously.[51]

Participants were allowed to choose among three different Ensure® flavors (chocolate, vanilla and strawberry) and were excluded from enrolment if they expressed a dislike for the taste of the test meal during the consent process.

Briefly, participants drank a nutrient liquid meal (Ensure Plus®: 1'5Kcal/mL, 11% fat, 73% carbohydrate and 16% protein) at a constant rate (30mL/min.). Every five minutes, participants scored their perception of fullness using a 6-grade scale that combines verbal descriptors and numbers (0 = no sensation, 5 = Max. Fullness, I can't eat more). Participants were asked to stop meal intake when they reached the score of 5.

At baseline, every five minutes and thirty minutes after completing ingestion of the Ensure®, participants were requested to score their symptoms (hunger, fullness, nausea, bloating, pain) using a 100mm visual analog scale (VAS) anchored with the words unnoticeable and unbearable at the left and right ends of the lines.

We recorded the total number of calories (Kcal.) ingested to reach maximum satiation, and the scores (mm) for each symptom at baseline (pre-drug) and every 5 minutes during ingestion and thirty minutes after completing ingestion of Ensure®.

Liquid Gastric Nutrient Emptying by Scintigraphy

We measured gastric emptying of Ensure Plus® using scintigraphy as in previous studies.[10] The first 300 mL of Ensure Plus® ingested were radiolabelled with 18.5 MBq (0.5 mCi) of ^{99m}Tc-DTPA, allowing gastric content tracing with a dual head gamma-camera (E.Cam Siemens, Erlangen, Germany). Simultaneous anterior and posterior abdominal

images of 1-minute duration were obtained at baseline (Time 0), at 15, 30, 45 and 60 minutes post-initiation of the meal while subjects were completing the nutrient drink test as in previous studies (Figure 4).[51, 78]

Neuro-Endocrine Control of Satiation

Entero-insular and satiation related neuropeptides and hormones were measured at baseline and during the satiation and gastric emptying assessments. We measured plasma levels of glucose, insulin, cholecystokinin (CCK), pancreatic polypeptide (PP), peptide YY (PYY), and leptin.

Blood draws were taken during fasting (on two occasions) and postprandially (at 10, 25, and 40 minutes after initiation of the Ensure Plus® meal). (Figure 4)

Plasma concentrations of glucose and insulin were measured as in prior studies by our group.[16] Glucose was measured using a glucose oxidase method (Yellow Springs Instrument, Yellow Springs, OH). Insulin was measured by a two-site immunoenzymatic assay performed on an Access automated immunoassay system (Beckman Instruments, Chaska, MN 55318).[16] Insulin intra-assay coefficients of variation (C.V.'s) are 2% at 6.75 uU/mL and 2.6% at 116 uU/mL. Insulin inter-assay C.V.'s are 3.9% at 12.7 uU/mL , 3.9% at 48.8 uU/mL, and 4.6% at 121 uU/mL.

Plasma levels of CCK were measured by a competitive radioimmunoassay using an antiserum raised against CCK-8 sulphate N-terminally conjugated to bovine albumin (Euro-Diagnostica, Malmö, Sweden). Plasma was obtained from blood collected in tubes containing EDTA and Trasylol (500 KIU/ml), and extracted with 96% ethanol previous to RIA.

The lowest detectable concentration was 0,3 pmol./l, being the intra and inter assay variation <10% and <15%, respectively.

Hormone measurements of PP, Leptin and PYY were performed on the Luminex 100 ™ system using a commercially available kit and according to the manufacturer's directions. Calibration microspheres as well as sheat fluid were purchased from Luminex Corp. and fluorescence data were analyzed by the Luminex 100 IS 2.3 software.

Multianalyte profiling were analyzed by the Linco Milliplex ™ Human Gut Hormone 3-plex panel kit (Linco Research Inc. #HGT-68K) assaying duplicates of all plasma samples in several batches. The lower limit of detection (pg./mL) for the assay was 2.4 (PP), 157.2 (Leptin) and 8.4 (PYY). The coefficients of variation (%) were: 11.7 (PP), 9.3 (Leptin) and 4.9 (PYY) within assay and 6.8 (PP), 8.9 (Leptin) and 13.1 (PYY) between assays.

Eating Attitude Test (EAT-26)

The Eating Attitudes Test (EAT) is a widely used self-report instrument to screen for eating disorders in the general population.[80] This questionnaire has long and short versions with 40 and 26 items (EAT-26), respectively.[63] Both have shown an excellent criterion validity for diagnosing the presence of DSM-IV eating disorders (i.e. anorexia, bulimia and Eating Disorder Not Otherwise Specified –EDNOS-) in non-clinical samples.[64] The negative predictive value when the EAT-26 total score is below 20 is 94%.[64]

Since eating behavior influences acute caloric intake,[81-83] we used this tool to control for potentially altered feeding behavior in the study participants to include this variable as a potential confounder when assessing the effects of gastric emptying on satiation.

Hospital Anxiety and Depression Scale (HADS)

Depression and anxiety may influence feeding behavior. Hence, we used the Hospital Anxiety and Depressions Scale (HADS) to measure depression and anxiety scores and

include them in the analysis of the effects of gastric emptying on satiation as potential confounders.

The HADS is 14-item questionnaire split into two subscales (anxiety and depression) that can be combined to create a total score. It has been validated to measure clinically significant anxiety and depression in both outpatients and inpatients. It is an instrument of easy application and has been used extensively in studies with non-psychiatric clinical populations.[65, 66]

Data Analysis

Satiation was summarized as the number of calories (Kcal.) ingested until the point of maximum satiation was reached. Postprandial symptoms were summarized by the sum of individual (fullness, nausea, bloating and pain) symptoms scores 30 minutes after finishing drinking. Acceleration of gastric emptying (GE) was summarized by the difference (Δ) between the proportion (%) of radiolabelled nutrient drink emptied at each time point between Day 2 and Day 1.

As in previous studies, the primary variable to assess the effect of erythromycin on gastric emptying was ΔGE at 15 minutes (ΔGE15), the first acquisition time post initiation of the meal. This was also the primary variable to assess the effect of acceleration of gastric emptying on caloric intake. The rationale was first the fact that the amount ingested at this time point was equal for all study patients and, second, because we were evaluating the effect of accelerating the arrival of nutrients to the small bowel on prospective caloric intake. Thus, the variable of interest is the initial gastric emptying, since gastric emptying beyond the initial phase of ingestion might already be influenced by the arrival of nutrients to the small intestine due to the ileal/duodenal brake.

The primary variable to assess the effect of acceleration of gastric emptying on symptoms reported 30 minutes after finishing the nutrient drink test was ΔGE at 60 minutes (ΔGE60), since the median time for finishing the nutrient drink test was 30 minutes.

Plasma levels of the satiation parameters in fasting conditions prior to the satiation test were summarized by computing the average of the two fasting measurements obtained. Postprandial levels were summarized by the maximum level obtained within 30 minutes of initiating the meal for each parameter. Changes in plasma levels from fasting to postprandial periods were summarized by computing the ratio [postprandial /fasting levels] for each satiation parameter.

Sample Size and Statistical Analysis

Sample size was calculated focused on the primary endpoint, detecting the effect, if any, of ΔGE on *ad libitum* caloric intake. This was based on the power to detect partial correlation coefficients in a ANCOVA analysis. Based on previous data[49, 51, 78] using the same methodology, a sample size of 30 individuals gives us 87% power to detect a 20% change (250Kcal.) on caloric intake with a 20% increase in gastric emptying 15 minutes post-meal with a significance level of 5%.

To evaluate the effect of erythromycin on ΔGE we used analysis of covariance (ANCOVA) and repeated measures ANOVA (MANOVA) for ΔGE at specific time points and ΔGE throughout the study, respectively. Gender, age and BMI were included in the analysis as potential confounding variables based on previous reports.[59]

To evaluate the effect of ΔGE at 15 and 60 minutes post initiation of meal ingestion on caloric intake and postprandial symptoms, respectively, we used multiple (linear) regression modeling. GE parameters were entered in the models as the principal independent variables, and gender, age, BMI, and baseline variables were explored as

covariates. Models were explored using backward, forward and mixed stepwise approaches. Best fitted regression models were selected based on adjusted R^2 with the least number of independent variables. To explore if GE effects on satiation/symptoms might be explained by plasma levels of satiation factors, fasting and postprandial changes of each were incorporated in the above mentioned models. Least square mean estimates ± SEM from best fitted regression models are reported.

Results

Study Population

We evaluated 30 overweight or obese (BMI ≥25 Kg/m^2) otherwise healthy subjects (24 females and 6 males). Median age was 36 years (IQR: 30-42, range: 22-56) and median BMI was 30 Kg/m^2 (IQR: 27-36, range: 25-42). Baseline characteristics were equally distributed between erythromycin (n=15) and placebo (n=15) groups (Table 3).

The median EAT-26 score was 12 (IQR: 8-23, range: 4-57). Six female subjects presented EAT-26 scores between 20-30, 4 were overweight and 2 were obese class II. Three subjects, 2 females and 1 male, all of them overweight, presented scores above 30 in the EAT-26.

Baseline ad libitum Caloric Intake, Postprandial Symptoms and Plasma Levels of Glucose, Insulin, Leptin and Satiation Factors

At baseline (Day 1), calories ingested to reach maximum satiation and symptoms scores (VAS-mm) 30 minutes after finishing the satiating meal were similar in both study groups (Table 3).

Plasma concentrations of glucose, insulin, leptin, CCK, PP and PYY were also similar in the two treatment groups at baseline (Day 1) during both fasting and postprandial conditions (Table 4).

Acceleration of Gastric Emptying by Erythromycin

Gastric emptying at baseline (Day 1) was similar in both treatment groups (Figure 5A). As expected, erythromycin significantly increased gastric emptying on Day 2 throughout the study period (Repeated measures MANOVA-p=0.0002, unadjusted and p= 0.0008, adjusted by gender, age and BMI). (Figure 5B). The mean difference in the percentage emptied from stomach at 15 minutes post-meal (ΔGE15) was 11±2.8 % and −1.2±2.7% in the erythromycin and placebo groups respectively (ANCOVA p=0.007, unadjusted and p=0.02 adjusted by gender, age, and BMI (Figure 5C).

Effects of Gastric Emptying Acceleration on ad libitum Caloric Intake

Acceleration of initial gastric emptying (ΔGE15) had a significant effect on ad libitum caloric intake. From the best fitted regression model (R^2=81%, p<0.0001), a 10% increase in gastric emptying in the first 15 minutes post-initiation of the meal induced an average decrease of 135±43.5Kcal on caloric intake (F Ratio: 10, p=0.004, adjusted by gender and baseline caloric intake) (Figure 6). We tested if intra-meal symptoms (during meal, before having finished ingestion) might explain the effects of gastric emptying acceleration on caloric intake. Only fullness scores 10 minutes after initiating eating had a significant effect on prospective caloric ingestion (p=0, 02). However, fullness did not fully account for the effects of gastric emptying acceleration on caloric intake, since ΔGE15 remained significant (F Ratio: 10, p=0.004) after including fullness in the above mentioned model.

The scores in the eating attitude test and anxiety-depression scale did not have a significant effect on ad libitum caloric intake in our sample (both $p>0.05$).

Effects of Gastric Emptying Acceleration on Postprandial Symptoms

Acceleration of gastric emptying at 60 minutes post-initiation of meal (ΔGE60) had a significant effect on postprandial symptoms. From the best fitted regression model (R^2= 50%, p=0.003) a 10% increase in gastric emptying 60 minutes post-initiation of the meal induced an average increase of 22±7.5mm on the sum of the VAS for individual symptoms assessed 30 minutes post-meal termination (F Ratio: 8.5, p=0.0095, adjusted by age) (Figure 7).

Again, in this sample, the scores in the eating attitude test and anxiety-depression scale had no significant effect on postprandial symptoms (both $p>0.05$).

Effects of Gastric Emptying Acceleration on Satiation Factors

As expected, there was a significant increase in plasma levels of glucose and insulin as well as satiation factors such as PP, PYY and CCK during the postprandial period (Table 2).

However, we did not observe any significant effect of acceleration of gastric emptying on postprandial, absolute or relative, changes in plasma levels of hormones and neuropeptides measured (all $p>0.05$).

Effects of Plasma Satiation Factors on ad libitum Caloric Intake and Postprandial Symptoms

Even though we were not able to find any significant effect of increased gastric emptying on plasma levels of satiation factors, we assessed if plasma levels of such factors might further explain caloric intake and postprandial symptoms beyond gastric emptying rate.

Regarding *ad libitum* caloric intake, beyond ΔGE15, gender and baseline intake capacity, postprandial increase in plasma CCK ($p=0.003$) and insulin ($p=0.02$) were both significantly and independently associated with decreased maximum caloric intake capacity, increasing the R^2 of the prediction model developed above up to 89%. There was an average decrease of -385±114 Kcal. and of -112 ±60Kcal for each 10 points increase in the postprandial/prepandrial CCK and Insulin ratios, respectively (Figure 8).

Once the above variables were introduced in the model, other hormones and neuropeptides did no further explain caloric intake variability.

Regarding symptoms 30 minutes after meal; beyond ΔGE60, age and nutrient intake fasting levels of glucose (F Ratio: 18, $p=0.0009$) and CCK (F Ratio: 5, $p=0.04$) and postprandial increase in plasma levels of CCK (F Ratio: 15, $p=0.002$) and PP (F Ratio: 7, $p=0.02$) were all significantly associated with postprandial symptoms scores. Increased fasting levels of glucose and CCK and increased postprandial change in PP were associated with increased symptoms scores. Decreased postprandial change (ratio) in CCK was associated with decreased symptoms. These factors increased the R^2 of the prediction model developed above up to 88% ($p<0.0001$) and their effect was not explained by volume or caloric intake ($p>0.05$).

DISCUSSION

Our results suggest that by pharmacologically accelerating gastric emptying of nutrients to the small intestine we may induce individuals to reduce spontaneously their acute caloric intake or meal size.

The perception of satiation, a major determinant of meal termination, is believed to be the final result of different neuro-hormonal signals that arrive mostly from the gastrointestinal tract.[34] The stomach has been one of the sites that have been more exhaustively investigated in this regard. Afferent neurons at the gastric level signal satiety in response to gastric wall distention (mechanoreceptors) as well as to nutrients, hormones and neuropeptides (chemoreceptors).[26-28, 84, 85]

The small intestine is also a key segment of the gastrointestinal tract involved in the control of food intake. As in the stomach, neurons from the small bowel wall signal satiety in response to distention and nutrients. Moreover, intestinal mucosa cells respond to the arrival of nutrients by secreting several peptides and hormones that regulate food intake through neuronal and endocrine mechanisms.[86] Thus, the small bowel is the main source of satiation signals arising from the gut. Our hypothesis was that pharmacologically accelerating the time of arrival of nutrients to the small intestine would significantly accelerate satiation perception and induce meal termination prematurely.

We used erythromycin at a dose tested previously that was known to significantly accelerate gastric emptying [47]. Erythromycin is known to cause digestive symptoms through different mechanisms (i.e. increased gastric tone). Hence, we aimed, not to test erythromycin global effects on satiation or symptoms but the particular effect of the acceleration of gastric emptying, which may be variable among subjects receiving erythromycin.

The results of the study appear to corroborate our hypothesis.

Precocious stimulation of mechano and chemoreceptors of the small bowel as well as precocious release of intestinal satiety signals participating in the control of nutrient intake would add to satiety signaling from the already distended gastric wall.

We explored if the observed reduced caloric intake was in partly due to precocious induction of symptoms due to accelerated emptying of nutrients towards de small intestine. We observed that precocious perception of fullness after initiating eating influenced significantly prospective caloric intake. However, initial fullness after initiating eating did not fully explain the effects of gastric emptying acceleration on caloric intake.

However, we did not observe higher plasma levels of the satiation factors measured following acceleration of gastric emptying. In the current study we planned an exploratory analysis of the role of such factors on caloric intake and postprandial symptoms. However, this was not the primary endpoint and thus, the study was not formally powered to find an effect of gastric emptying on plasma levels of satiation factors. Hence, we cannot exclude a type II error as a potential explanation for this lack of significance. It might also be argued that changes in satiation factors released from the small intestine might not be adequately reflected in plasma concentrations. Plasma concentrations will depend not only on the amount of substance released to the blood stream but also on its plasma clearance. Moreover, satiation factors released from the intestinal mucosa act not only through direct activation of central nervous system and brain stem neuronal receptors reached from the blood stream.[87, 88] More efficiently, several of gut satiation peptides also act locally, activating neuronal terminals located at the same gastrointestinal mucosa, in the brain-gut axis.[89-93] Hence, local changes in the release of gut satiation neuropeptides might not be adequately captured by variations in plasma concentrations.

However, in our exploratory analyses we have been able to reproduce well known relationships. We observed for instance that increased CCK response within 30 minutes of

starting a standard meal was associated with faster perception of satiation and thus, decreased caloric intake. Physiological postprandial levels of CCK have now long been known to induce satiation and influence food intake [74] [75] through, as mentioned above, both hormonal and neuronal mechanisms [94]. The same association was observed with insulin response to the meal. Increased postprandial insulin was associated with decreased meal size. Brain neurons involved in energy intake express insulin receptors [95]. From circulation, insulin enters the brain and stimulates a net catabolic response in part through inhibition of food intake [96]. As weight increases, insulin secretion increases in both the basal state and postprandially to compensate for insulin resistance [97]. Increased insulin secretion as obesity progresses is thus hypothesized to increase delivery of insulin to the brain to prevent further weight gain, partly through reducing food intake. Our results showing an inverse relationship between postprandial increase of endogenous insulin and meal size would support that hypothesis. This independent (additive) effects of CCK and insulin levels on caloric intake observed in our study are also in concordance with animal data showing a synergistic effect of CCK and insulin administered centrally on food intake [98].

Intensity of fullness, nausea, bloating and abdominal pain was also significantly linked to baseline glucose and CCK values. CCK is known to increase perception of mechanical and biochemical both physiological and non-physiological stimuli in the gut.[99, 100] Hence, it is not surprising that higher levels of CCK might be associated with heightened perception after a fully satiating meal. Postprandial levels of PP were also associated with increased intensity of symptoms. PP polypeptide is a 36-amino-acid peptide and a member of the PP fold peptide family (also including NPY and PYY). It is released in response to food intake from endocrine cells of the pancreas and has also been proposed as a satiation factor. In rodents and in healthy humans, peripheral administration of PP reduced food intake [101, 102].

However, postprandial endogenous levels of PP have never previously been associated with heightened perception of nutrient gut stimuli and we have no good biological explanation for this observation. Hence, we believe these results should be interpreted with caution, as the effects of plasma hormones were not primary endpoints of the study, there were no specific hypotheses to test and a type I error is possible due to multiple testing.

In conclusion, the results of the study support our hypothesis that meal size can be reduced in humans through pharmacological acceleration of gastric emptying and provide us with a new potential approach to reduce excessive acute caloric intake. Specifically, we may speculate that administration of a prokinetic drug before meals might help to prevent weight gain. Unfortunately, the practicality of such an approach at present is questionable because of current unavailability of suitable agents. Moreover, agents accelerating gastric emptying of nutrients might also induce faster gut transit of nutrients and thus might conceivably decrease the time interval until the subjects feel hungry again. This could lead the individual to eat more frequently and hence, total caloric intake in a day would not be diminished as pretended.

With all these caveats, our findings should at least stimulate further research on the mechanism of early satiation induced by nutrient dumping into the upper intestine and to evaluate whether this novel principle can be successfully translated into clinical practice.

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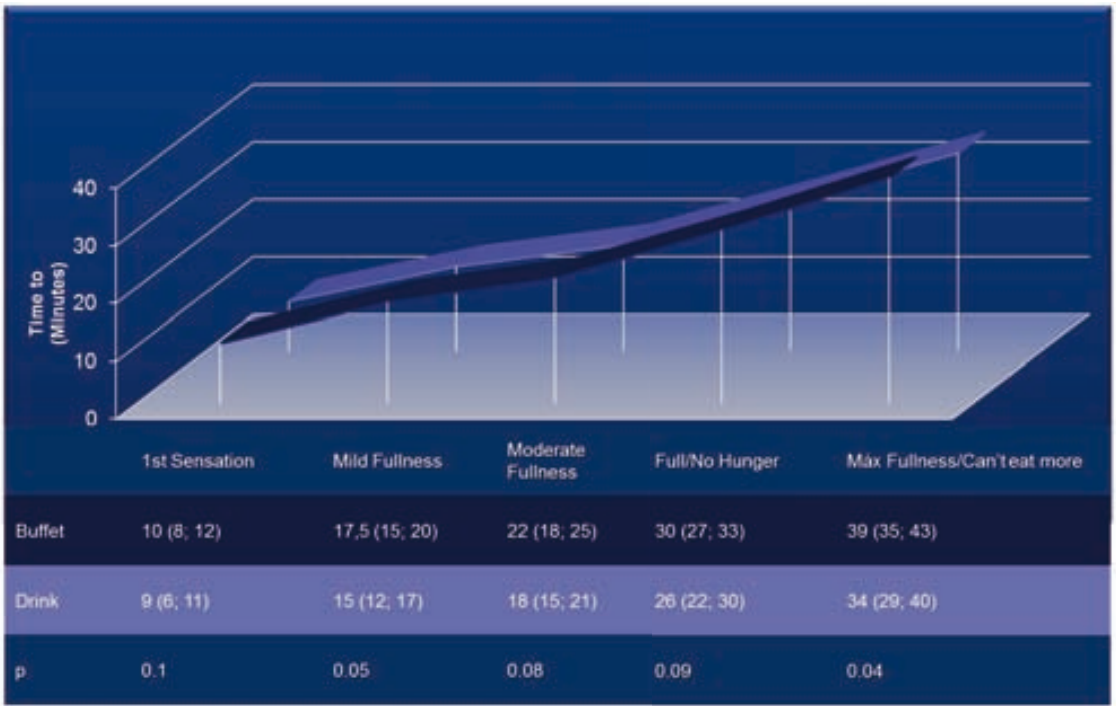

	1st Sensation	Mild Fullness	Moderate Fullness	Full/No Hunger	Máx Fullness/Can't eat more
Buffet	10 (8; 12)	17,5 (15; 20)	22 (18; 25)	30 (27; 33)	39 (35; 43)
Drink	9 (6; 11)	15 (12; 17)	18 (15; 21)	26 (22; 30)	34 (29; 40)
p	0.1	0.05	0.08	0.09	0.04

[illegible]

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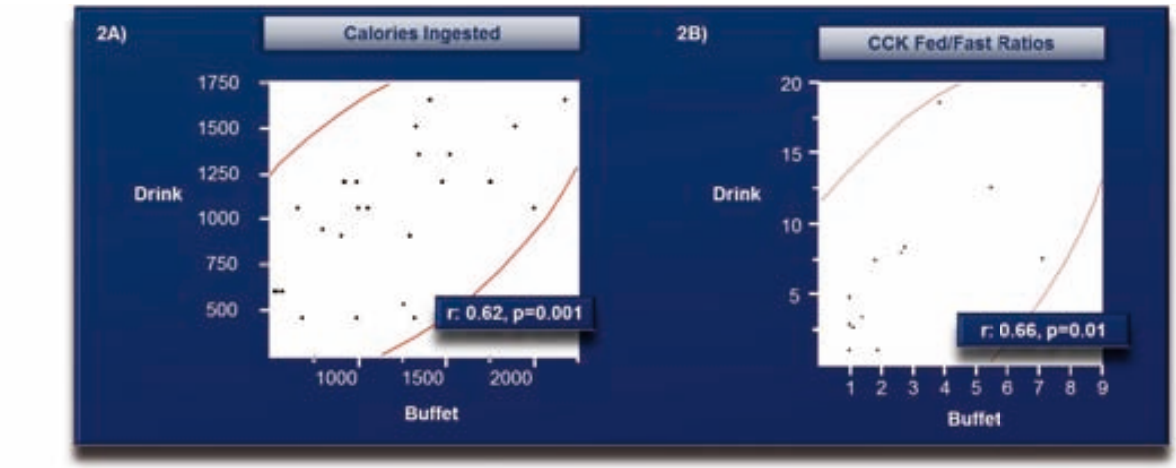

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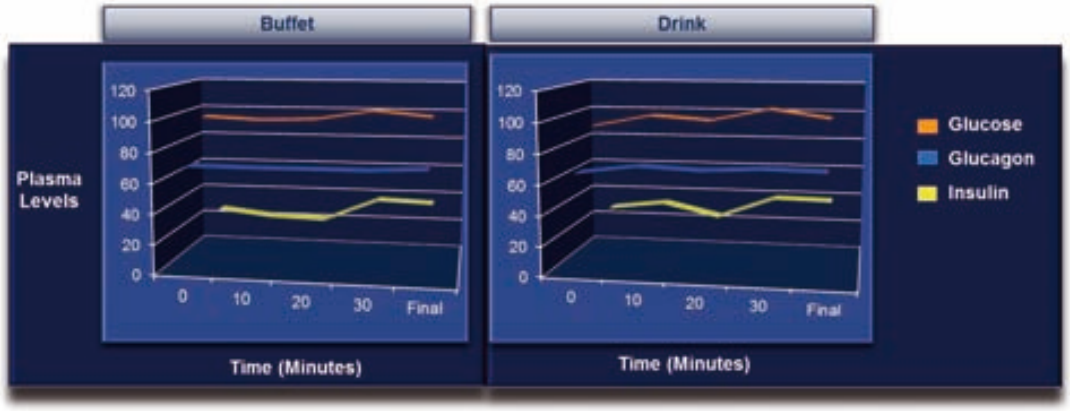
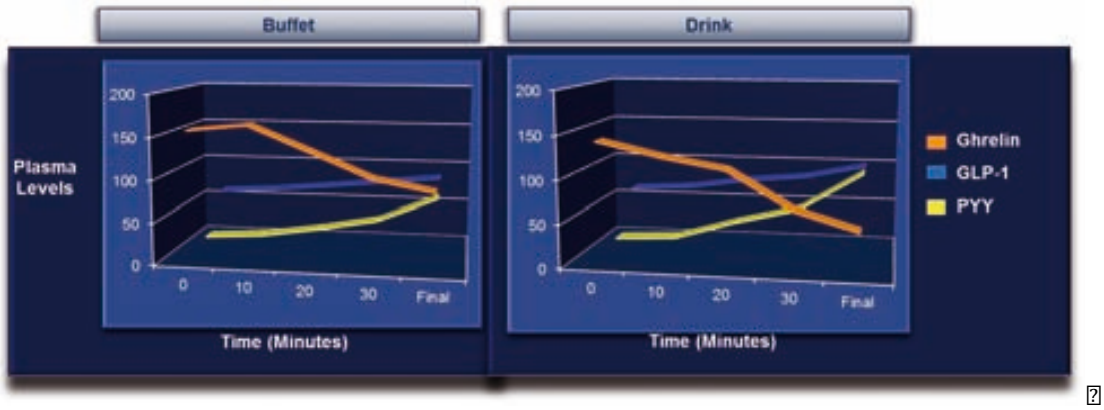
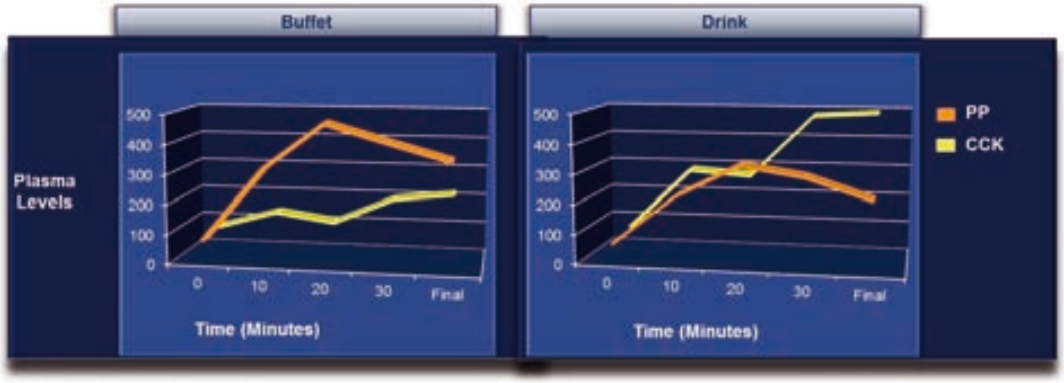
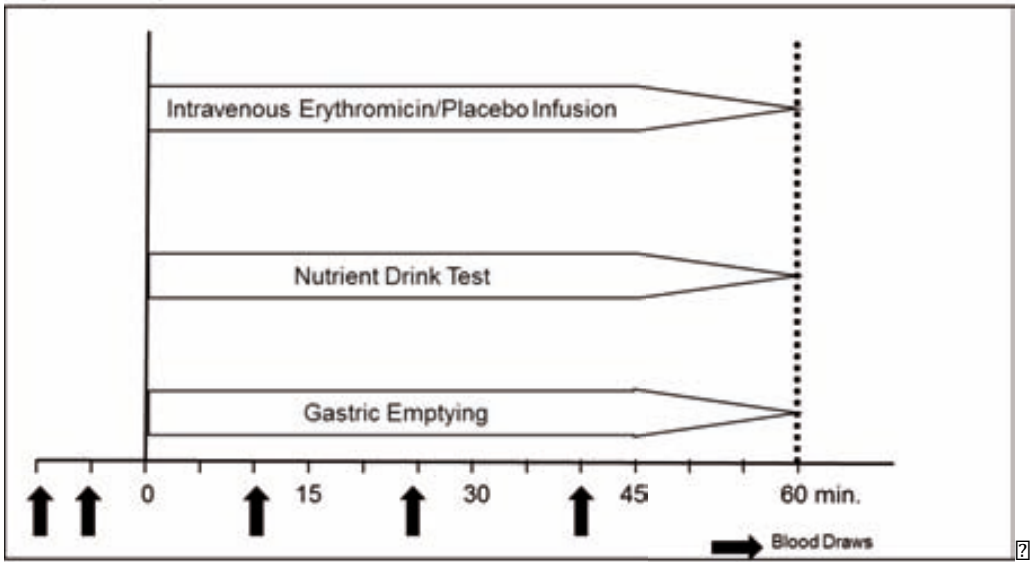

Buffet
Drink
Plasma Levels
120
100
80
60
40
20
0
0
10
20
30
Final
Time (Minutes)
Glucose
Glucagon
Insulin


Buffet
Drink
Plasma Levels
200
150
100
50
0
0
10
20
30
Final
Time (Minutes)
Ghrelin
GLP-1
PYY


Buffet
Drink
Plasma Levels
500
400
300
200
100
0
0
10
20
30
Final
Time (Minutes)
PP
CCK

oi p □f□Er□□Efct □A□□□fct l □□Nuff□□□□or□Na □uc□□□m□rN□□cNor□g□□□p i voA□N□ff□Noi t N□□t □□i uA□□NuN□□
uAi E A□i f□□tu□t N□□□Efct □A□□□fct l □□Nuf□

Figure 4. Study Protocol



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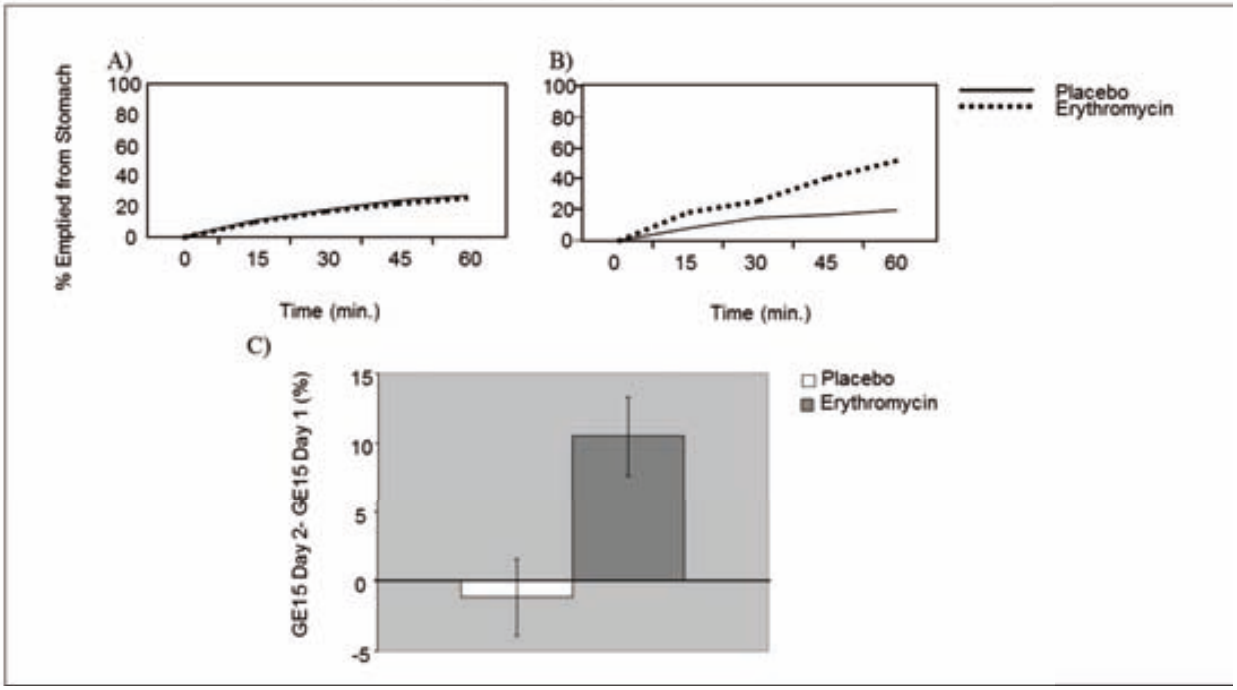
a □□Nf□□□rE□i N□□□t □□t NErct □or□Na □□□m□nNf□

Figure 5. Acceleration of Gastric Emptying by Erythromycin (adjusted Least Square Means) □



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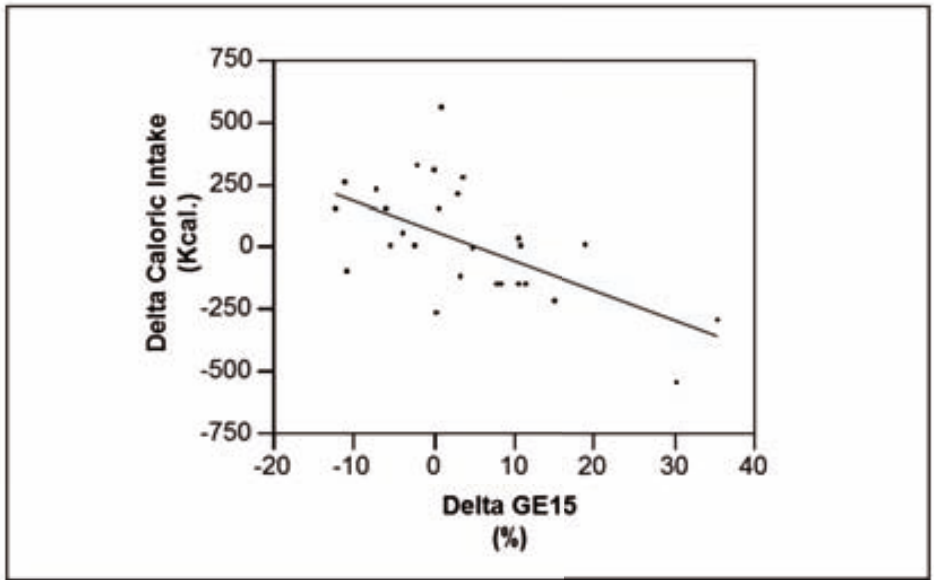
[illegible]

Figure 6. Effects of Gastric Emptying Acceleration on *ad libitum* Caloric Intake (from best fitted regression model excluding satiation factors R^2=81%)



[illegible]

Figure 7. Effects of Gastric Emptying Acceleration on Postprandial Symptoms (from best fitted regression model excluding satiation factors R^2=50%)

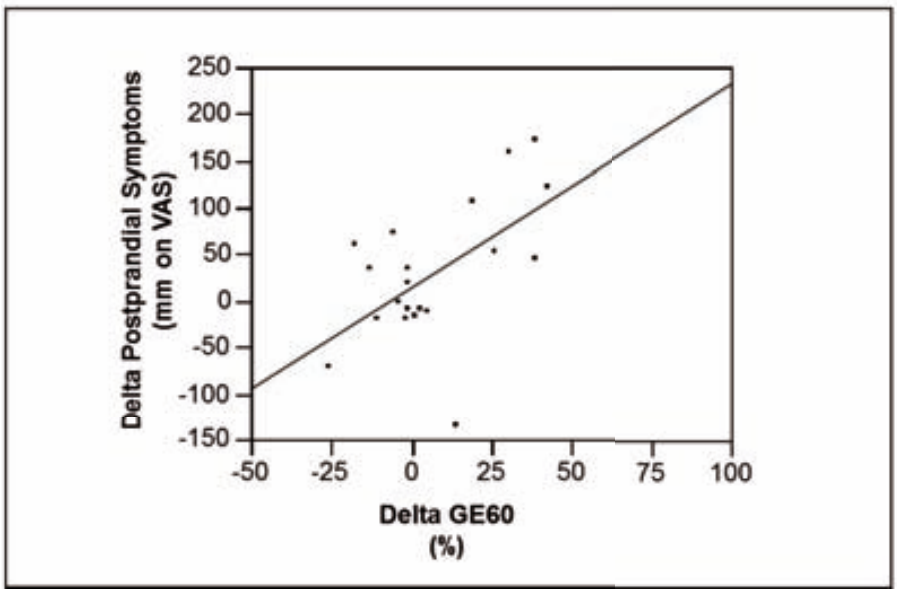


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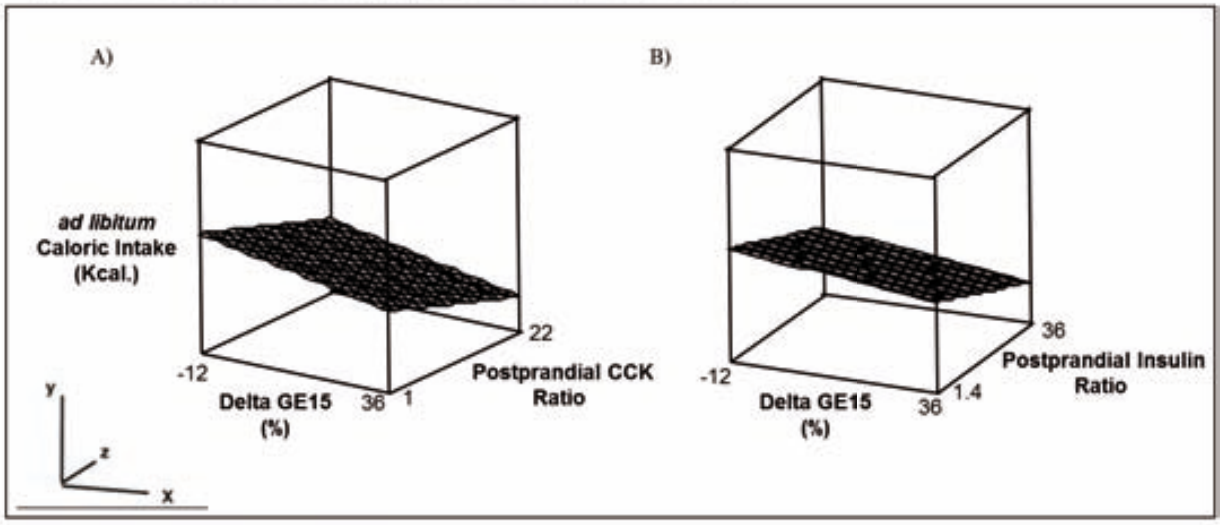
Figure 8. Effects of Postprandial CCK and Insulin Changes on Caloric Intake Independently of Gastric Emptying Acceleration *
(from best fitted regression model includying ΔGE15 and satiation factors R^2=89%)



* The model also included, sex and baseline caloric intake capacity

Tables

Table 1. Study population demographic and baseline characteristics. Mean (95%CI) are reported

	N=24
Females /Males	16/8
Age (years)	37 (31; 41)
Weight (Kg)	67 (62; 71)
BMI (Kg/m²)	24.5 (23; 26)
Leptin (pg./mL)	8462 (5802; 11122)
EAT-26 (Normal score < 20)	16 (9, 22)
Anxiety (Normal score < 7)	7 (5, 10)
Depression (Normal score < 7)	4 (2, 7)

Table 2. Effect of Variables on Energy Intake on both Test days and final Standard Least Squares satiation models.

Variables	Buffet			Nutrient Drink		
	Univariate	Stepped Model		Univariate	Stepped Model	
		E (SE)	p		E (SE)	p
Gender (F vs. M)	0,0003	-341 (51)	<0,0001	0,0009	-242 (52)	<0,0001
BMI	0,9072			0,0619		
Glucose Fed/ Fast	0,1151			0,0717		
Insulin Fed/ Fast	0,2883			0,0346		
Ghrelin Fed/ Fast	0,0133			0,3452		
GLP-1 Fed/ Fast	0,0037	129 (48)	<0,0001	0,0304	46 (13)	0,0021
PYY Fed/ Fast	0,0918			0,5431		
PP Fed/ Fast	0,0611			0,6066		
CCK Fed/ Fast	0,1751			0,2320		
		R^2 : 79%, p<0.0001			**R^2 :** 65%, p<0.0001	

Fed/Fast: ratio of the maximum plasmatic level during feeding over the fasting plasmatic level

E (SE): Estimate of the effect (Standard Error)

Table 3. Study population demographic and baseline characteristics by treatment group. Mean (95%CI) are reported

	Erythromycin N=15	Placebo N=15	p
Females /Males	12/3	12/3	1.00
Age (years)	37 (32, 42)	36 (31, 41)	0.73
BMI (Kg/m²)	33 (30, 36)	30 (28, 32)	0.07
EAT-26 score	16 (9, 22)	17 (9, 24)	0.79
Anxiety Score	7 (5, 10)	8 (6, 10)	0.58
Depression Score	4 (2, 7)	5 (4, 7)	0.50
***ad lib* Caloric Intake (Kcal.)**	1576 (1245, 1906)	1546 (1309, 1783)	0.88
PP Symptoms (VAS mm)	182 (150, 214)	187 (154, 220)	0.81

PP: Postprandial; 30 minutes after finishing a satiating meal
VAS: Visual analog scale

Table 4. Baseline (Day 1) plasmatic levels of satiation signals by treatment group. Mean (95%CI) are reported

	Erythromycin N=15	Placebo N=15	p
Leptin Fast(pg./mL)	15.603 (10308, 20898)	14.104 (9656, 18552)	0.65
Leptin PP(pg./mL)	15.859 (10064, 20192)	14.956 (10167, 19746)	0.79
Glucose Fast (mg/dL)	83 (79, 87)	83 (79, 86)	0.91
Glucose PP (mg/dL)	139 (123, 155)	130 (119, 140)	0.31
Insulin Fast (mcUI/mL)	72 (31, 113)	61 (8, 114)	0.74
Insulin PP (mcUI/mL)	546 (374, 718)	370 (244, 496)	0.08
CCK Fast (pmol/L)	1.2 (0.6, 1.8)	1.1 (0.7, 1.6)	0.84
CCK PP(pmol/L)	7.2 (4.7, 9.6)	6.8 (4.9, 8.7)	0.80
HPP Fast (pg./mL)	74 (35, 112)	54 (31, 76)	0.35
HPP PP (pg./mL)	354 (208, 500)	247 (172, 321)	0.18
PYY Fast (pg./mL)	64 (21, 107)	38 (28, 48)	0.21
PYY PP (pg./mL)	114 (70, 158)	71 (55, 86)	0.06

Fast: After 8h fast, before satiation test
PP: Postprandial; max levels within 30 minutes of initiating the meal

Final Remarks

ESTIMATES OF AD LIBITUM CALORIC INTAKE AND TIME TO SATIATION, AS MEASURED BY A STANDARDIZED NUTRIENT DRINK TEST, ARE COMPARABLE TO THOSE OBTAINED WITH A FREE-BUFFET MEAL

In the first study of this thesis we have shown for the first time that a nutrient drink can be used to evaluate ad libitum caloric intake, that is, satiation, in human subjects. The data from our study show that the ingestion of a commercialized nutrient drink at a controlled physiological rate of 30mL/min can provide estimates of satiation that correlate well with those obtained during a free-buffet meal, the current gold standard. Overall and on average, it took subjects five minutes and 143Kcals less to reach the satiation state during the drink test as compared to the buffet meal. During the drink test, subjects were drinking at 30mL/min and were asked about their satiation level every five minutes instead of every ten, as done during the buffet meal. Hence, there it seems that if asked 5 minutes later, as done during the buffet meal, there would not have been any detectable differences between the two tests.

These were somehow unexpected findings. The nutrient drink test does not allow the subject to freely choose from a variety of liquid and solid food items, but involves ingesting a high density energy liquid of the same taste and at a fixed rate until satiated. Thus, a much greater difference was expected on times and caloric intake to reach the satiation state [103]. We have already discussed previously in this thesis (p15) some potential explanatory hypothesis, pointing out that the physiological ingestion rate chosen for the drink test might partly be responsible of the similarities between the two tests. Another conceivable explanation might be that subjects in our study were led to finish intake until they could not eat more, independently of their hunger or actual desire to eat, potentially forcing them beyond the physiological satiation point. However, time to reach

the previous level of satiation in the scale used during feeding, score 4: Full/No hunger was equally comparable between the two tests: 30mins.(27; 33) and 26mins (22; 30) for the buffet and drink test, respectively (Figure 1); thus, suggesting that leading subjects to eat only until comfortably full would have not significantly impact the results of our study.

Gender-based differences in satiation responses to food have been recognized now for a long time. Studies have shown higher satiety ratings after a fully satiating meal and, as shown in our study, lower ad libitum energy intake in women compared to their male counterparts [104]. Other studies have shown greater dietary restraint and different neuronal responses to food cues in females compared to males [16, 104-106]. These data are generally interpreted as probably showing gender differences in social and psychological mechanisms that influence food behavior. Hence, females would be more conscient of food energy content and self-excess weight what would lead them to have greater dietary restrain [104].

The results of our study would add to the evidence of gender differences in food intake. Thus, our data show that ad libitum energy intake, measured by the nutrient drink or the buffet meal, is significantly influenced by gender. But then, we have also tested time to satiation, a measure that is not usually recorded in satiation studies, which has provided us with a new and interesting observation. Thus, data presented in this thesis show that females reach the satiation state earlier during the drink test compared to male subjects as previously published [47]. However, this was not observed during the buffet meal. Females did not reach satiation earlier than male subjects during the buffet meal, albeit they ingested significantly less calories than males. This might be explained by, as speculated by others, feminine behavioral traits. Thus, females might make healthier food choices, for instance food items with less fat and carbohydrate content. We tested this post-hoc hypothesis by comparing macronutrient ingestion during the free-buffet meal in males and females. While females during the buffet meal ingested, in absolute terms, less

grams of all nutrients, the percentage distributions of carbohydrates, proteins and lipids ingested were the same as those consumed by males. Another potential explanation for gender-related differences on energy intake while time of consumption was unaltered by gender might be differences in the rate of ingestion in males and females. If women were eating more slowly during the buffet compared to males, this would, at least partly, explain why they reached satiation at the same time consuming less calories. The fact that females reached satiation earlier than males when performing the nutrient drink test, during which females were forced to maintain the same ingestion rate than males would support the hypothesis that rate of ingestion might be the key difference to make women ingest less energy during the free buffet.

One of the main objectives of the first study of this thesis was to test if, within the controlled setting of our study, performed in the same subjects with exact conditions, both satiation tests would unveil the same relevant influences on caloric intake. And we have been able to provide valid data that supports, for the first time, this hypothesis. In both tests, ad libitum caloric intake was significantly associated to gender and the GLP-1 feeding response. In this study we aimed to show significant correlations among main satiation measures by the two different tests. Hence, we did not power it to perform an in-depth analysis of all factors that might be influencing satiation. Thus, we acknowledge that the satiation models obtained in this nutrient drink test validation study are lacking many variables that are known to be important in the regulation of satiation. We have already discussed in this thesis potential explanations for the absence of CCK feeding response as a significant factor associated with satiation in our study (p16). However, potential model pitfalls apart, we believe that obtaining the exact same variables when modeling satiation for both tests is reassuring us that both test have indeed a good convergent validity. Therefore, we firmly believe this data supports and provides confidence for the use of this test as a surrogate measure of satiation in studies where the current gold standard might not be applied.

ACCELERATION OF NUTRIENT DUMPING INTO THE SMALL BOWEL ACCELERATES THE SATIATION STATE AND REDUCES ACUTE AD LIBITUM ENERGY INTAKE

The second study presented in this thesis is the proof of concept of a novel mechanism to induce postprandial fullness and decrease acute ad libitum caloric intake in humans. Our data show, for the first time, that accelerating emptying of nutrients from the stomach into the small bowel during the early phases of ingestion (within the first 15 minutes of eating) we can induce increased fullness and decrease prospective nutrient intake, at least in the short-time.

These new data actually may appear to be in controversy with current knowledge. Thus, current mainstream accepts that sensations after feeding, such as fullness or others like nausea, or pain, included in the dyspepsia syndrome, are mainly arising from the stomach. There are many data demonstrating the pivotal role of stomach on the generation of all this sensations. When nutrients enter the stomach neural receptors in the gastric wall generate impulses that signal the sensation of fullness through vagal and splanchnic afferents [26-28, 84, 85]. Many studies have shown relationships between gastric retention and postprandial fullness, appetite and the constellation of the dyspepsia syndrome [107-109]. However, prior studies performed to induce a decrease in food intake through pharmacologically delaying gastric emptying have failed to show this effect [44, 51, 110]. These studies failure to show that delaying gastric emptying might decerase meal size might be explained due to an insufficient effect on gastric emptying by agents tested. Likewise, agents used in these trials also have effects on sensation pathways that might counteract the effect of delayed gastric emptying on satiation. It would also be conceivable that even though we might increase the sensation of "fullness" by delaying emptying of the stomach, this sensation will actually most probably appear once the subject has already finished eating. Physiological emptying of a mixed meal from the

stomach starts on average 30 to 60 minutes after initiating meal [111, 112], a time point when most subjects have already finished their meal. Therefore, delaying gastric emptying might not be the most suitable mechanism to prevent further eating.

Moreover, during feeding a physiological reflex occurs, the gastric accommodation reflex, the result of which is the relaxation of the gastric wall [113, 114]. This relaxation of the stomach during feeding prevents the increase of intra-gastric pressure and participates in the regulation of gastric emptying, mostly of liquids, through progressive recovery of the gastric wall tone [42]. In this regard, we consider necessary to highlight that the maximum relaxation of the gastric wall is achieved very rapidly at the beginning of the meal (< 30 minutes) and gastric tone is recovered progressively until it reaches the baseline levels 60 to 120 minutes after initiating the meal [115, 116]. Hence, it is conceivable that decreasing gastric emptying, which physiologically starts around 30 minutes after initiating a mixed meal, when the distention of the stomach is its maximum point, might probably not enhance signaling from mechanoreceptors in the gastric wall, and most probably won't influence food intake.

On the other hand, and as commented previously in this thesis, the small intestine has also been shown to be relevant in the control of food intake. Neurons from the small bowel wall also signal satiety in response to distention and nutrients. Moreover, cells of the small intestine mucosa respond to the arrival of nutrients by secreting several peptides and hormones that also signal satiety through neuronal and endocrine mechanisms [86]. Thus, as hypothesized in this thesis, it might be conceivable that precocious stimulation of mechano and chemoreceptors of the small bowel as well as precocious release of intestinal satiation signals would add to satiation signaling from the already distended gastric wall. The results of our study seem to corroborate such hypothesis and give us a new potential target in the development of successful strategies in the battle against obesity.

From the presented results one might be led to think that taking a prokinetic drug before meals might be a good solution to avoid excessive energy intake and thus unwanted weight increase. However, there are several potential pitfalls for this strategy that we would like to discuss. First, agents with a prokinetic activity strong enough to achieve the aim of a 10% increase during the first 15 minutes of ingestion are scarce. Moreover, most of currently available agents do not have a pure gastric prokinetic effect [117]. Many of them share their prokinetic properties with actions on different receptors in afferent sensitive fibers arising from the gastrointestinal tract or directly on the central nervous system (CNS). For instance, part of the anti-emetic effect of dopamine antagonists is mediated through dopamine type 2 receptors in the CNS. Metoclopramide, which has shown to improve gastroparesis of different causes, is a dopamine antagonist but also a partial agonist of serotonin type 4 receptors (5-HT4-r) [118-120] and a partial antagonist of serotonin type 3 receptors (5-HT3-r), which are present in sensory visceral afferents [121]. The effect of metoclopramide inhibiting sensory nerves might counteract its potential effect on satiation through its prokinetic effect. 5-HT4-r agonists such as cisapride, tegaserod or prucalopride, have also prokinetic activity, presumably through acetylcholine liberation from motor neurons in the enteric nervous system [122]. However, the first two also act as 5-HT3-r antagonists, and thus inhibit sensory information that arises from the gastrointestinal tract. Prucalopride is the only agent currently available that binds specifically to 5-HT4-r. However, there are scarce data on its effect on human gastric emptying.

Another potential issue that might rise with the use of this strategy to prevent weight gain in humans is related to security. Even if we were to have a pure, strong and safe prokinetic agent, we should keep in mind that no drug can replace an adequate lifestyle. Thus, we as doctors should always provide our patients with the necessary tools to achieve a healthy lifestyle. And limit the use of these drugs in selected patients that cannot achieve adequate energy balance in any other physiological way.

Summary and Future Studies

In summary, data from studies included in this thesis show that a nutrient drink test is a reliable measure of satiation in humans. We believe our data supports the use of this tool to assess energy intake in research studies. The nutrient drink test is cheap, universally available, and easy to perform and thus, we believe it can foster the investigation of satiation physiological control, as well as clinical studies to develop new mechanisms that may help patients to achieve an adequate energy intake to maintain a healthy weight.

In the second study presented in this thesis we have actually tested a new mechanism to decrease energy intake using this tool, the nutrient drink test. It has allowed us to proof a new principle, that accelerating the dumping on nutrients into the small bowel reduces liquid nutrient intake. The confirmation of our hypothesis leads to new research questions that will need to be addressed in order to translate this principle to the clinical setting. Thus, it is not clear if the acceleration of emptying of a solid or mixed (solid and liquid) meal into the small bowel might actually be feasible and lead to similar results. Our data show that in order to inhibit further food consumption in a significant way, it is important to induce around a 10% increase in the gastric emptying during the first 15 minutes of the meal. Solids need time to be triturated before being emptied from the stomach. The time form the start of the ingestion and the beginning of the emptying of solids from the stomach is known as the Tlag. Tlag has been estimated to be, on average around 20 minutes, as measured by the current gold standard, the gastric scintigraphy [123]. Thus, the mechanism we propose to decrease food intake might not work with a normal daily meal, where people consume mixed solid and liquid foods. However, there are several studies that have suggested that delivery of solid particles from the stomach may start before 10 minutes of eating [124, 125] or even, right after the start of the meal[126]. Thus we believe that future studies that confirm our hypothesis with solid or mixed meals are warranted.

Another question that was raised after analyzing the data from our second study was if increasing gastric emptying during a specific meal might also induce a faster transit of nutrients through the small bowel. If that would be confirmed, it might conceivably lead to a more rapidly return to a hunger state, that is, the satiety period might be shortened. On the other hand, it would not be unconceivable either that faster small bowel transit might cause a decrease in absorption of nutrients and thus provide an extra-benefit to an hypothetical drug for weight control. We tested fullness and other postprandial symptoms only until 30 minutes post-meal and, therefore, cannot answer these questions.

Hence, we believe future studies are needed to respond these questions in order to evaluate if the novel principle proven in this thesis can be successfully translated into clinical practice.

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