Comparison of the mixed-meal stimulated gut hormone secretion profile after classical gastric bypass and the long biliopancreatic limb variant.

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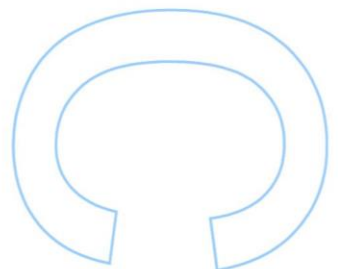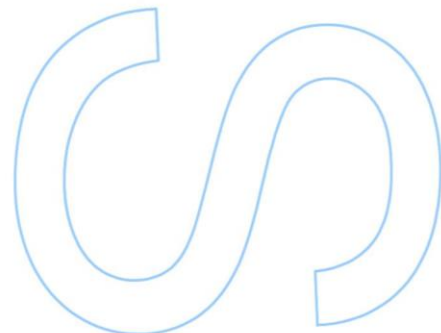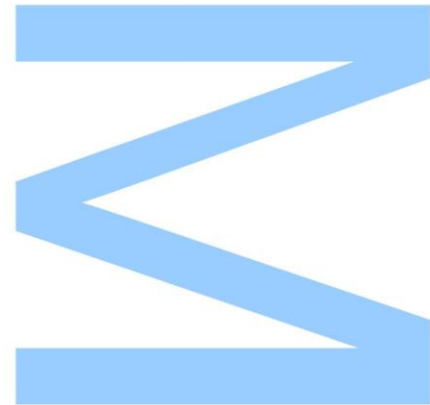
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O Presidente do Júri,
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Resumo

Introdução: O Bypass Gástrico em Y-de-Roux (BGYR), um dos procedimentos bariátricos mais usados, demonstrou ser eficaz na indução de perda de peso sustentada e remissão prolongada da diabetes tipo 2. O perfil de secreção de hormonas gastrointestinais (GI) desencadeado pelo rearranjo anatómico do tubo digestivo tem sido apontado como um importante mecanismo para a melhoria metabólica da diabetes após a cirurgia bariátrica. O BGYR de ansa biliopancreática (BP) longa foi associado a melhores resultados metabólicos quando comparado com o procedimento clássico. No entanto, a influência do comprimento da ansa BP do BGYR na resposta hormonal pós-prandial era desconhecida e por esse motivo foi o objetivo deste estudo.

Objetivo: Comparar o perfil hormonal GI em resposta a uma refeição mista de indivíduos submetidos a BGYR com dois comprimentos de ansa BP diferentes.

Métodos: Indivíduos não-diabéticos previamente submetidos a BGYR com ansa BP clássica (n=9; BP: 87.8±6.4cm) ou longa (n=11; BP: 200cm) foram submetidos a um teste de tolerância à refeição mista. Procedeu-se à colheira de sangue em jejum e novamente aos 15, 30, 45, 60, 90 e 120 minutos após a refeição para medição dos níveis de glucose, hormonas pancreáticas e GI, e ácidos biliares totais.

Resultados: Após a refeição mista, não foram encontradas diferenças entre os grupos relativamente às curvas de glucose, glucagon, PP, PYY e ácidos biliares totais. Os indivíduos submetidos ao BGYR com ansa BLP longa apresentaram níveis de GLP-1 (t=45 min, $p<0.05$; T-AUC: 11205±3399 *vs* 7800±1817 pmol/L x min, $p=0.01$) e neurotensina (t=45 min, $p<0.05$ and t=60 min, $p<0.01$; T-AUC: 11205±3399 *vs* 7800±1817 pmol/L x min, $p=0.02$) significativamente mais elevados, enquanto os níveis de GIP (t=15 min, $p<0.01$), insulina e péptido-c (t=30 min, $p <0.001$) foram significativamente menores quando comparados com o grupo BGYR com ansa BLP clássica.

Conclusão: A resposta hormonal pós-prandial observada em indivíduos submetidos a BGYR com ansa BLP longa, com aumento dos níveis de GLP-1 e neurotensina, sugere que o procedimento do BGYR pode ser personalizado de modo a otimizar os efeitos antidiabéticos da cirurgia.

Palavras-chave: Obesidade, cirurgia bariátrica, bypass gástrico em Y-de-Roux, hormonas gastrointestinais.

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Abstract

Introduction: Roux-en-Y gastric bypass (RYGB), one of the most widely performed bariatric surgery procedures, results in long-term weight-loss and improved glycaemic control in obese type-2 diabetes mellitus (T2DM) patients', often with prolonged disease remission. Changes in gastro-intestinal (GI) hormone secretion profile after surgery in result of the anatomical rearrangement of the digestive tract has long been hypothesized to play an important role in the metabolic effects of bariatric surgery. Long biliopancreatic limb (BPL) RYGB improves the metabolic outcomes of the surgery as compared to the classical procedure. However, the influence of the RYGB limb length on the hormonal response to food intake was unknown.

Aim: The purpose of this study was to compare the GI hormone profile in response to a mixed-meal in subjects submitted to RYGB with two different BPL lengths.

Methods: Non-diabetic weight stable subjects previously submitted to classical RYGB (n=9; BPL length: 87.8±6.4cm) or long BPL RYGB (n=11; BPL length: 200cm) underwent a mixed-meal tolerance test. Blood was sampled at fasting before the meal and again at 15, 30, 45, 60, 90 and 120 minutes after the meal for measurements of plasma glucose, GI and pancreatic hormones, and total bile acids (TBA).

Results: There were no differences in plasma glucose, glucagon, PP, PYY and TBA response between the groups. Comparing both experimental groups, the long BPL RYGB group displayed significantly higher GLP-1 (t=45 min, $p<0.05$; T-AUC: 11205±3399 vs 7800±1817 pmol/L x min, p=0.01) and neurotensin (t=45 min, $p<0.05$ and t=60 min, $p<0.01$; T-AUC: 11205±3399 vs 7800±1817 pmol/L x min, p=0.02) levels, while exhibited significantly lower levels of GIP (t=15 min, $p<0.01$), insulin and c-peptide (t=30 min, $p<0.001$) as compared to classical RYGB.

Conclusion: The distinct postprandial hormone response with increased GLP-1 and neurotensin observed after the long BPL RYGB, suggests that RYGB technique could be customized to achieve optimized metabolic outcomes in T2DM patients.

Key-words: Obesity, bariatric surgery, Roux-en-Y gastric bypass, gut hormones.

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Abbreviations

%EBMIL   Percentage of Excess BMI Loss

5-HT   Serotonin

5-HT2C   5-Hydroxytryptamine 2C Receptor

AL   Alimentary Limb

BMI   Body Mass Index

BPD   Biliopancreatic Diversion

BPL   Biliopancreatic Limb

CCK   Cholecystokinin

CHEDV   Centro Hospitalar de Entre o Douro e Vouga

CL   Common Limb

CNS   Central Nervous System

DBP   Diastolic Blood Pressure

DPP-4   Dipeptidyl Peptidase-4

ECLIA   Electrochemiluminescence Sandwich Immunoassay

EEC   Enteroendocrine Cells

EMA   European Medicines Agency

ER   Extended Release

FDA   US Food and Drug Administration

GI   Gastrointestinal

GIP   Glucose-dependent Insulinotropic Polypeptide

GLP-1   Glucagon Like Peptide-1

GLP-2   Glucagon Like Peptide-2

HOMA-IR   Homeostatic Model Assessment - Insulin Resistance

HOMA-β   Homeostatic Model Assessment – β-cell function

HR	Heart Rate
MMTT	Mixed-Meal Tolerance Test
NT	Neurotensin
OGTT	Oral Glucose Tolerance Test
OXM	Oxyntomodulin
PC	Prohormone Convertase
PG	Proglucagon
POMC	Pro-opiomelanocortin
PP	Pancreatic Polypeptide
PYY	Peptide YY
RIA	Radioimmunoassay
RYGB	Roux-en-Y Gastric Bypass
SBP	Systolic Blood Pressure
SD	Standard Deviation
SEM	Standard Error of the Mean
SR	Sustained Release
T2DM	Type-2 Diabetes Mellitus
TBA	Total Bile Acids
VSG	Vertical Sleeve Gastrectomy
WHO	World Health Organization

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INTRODUCTION

1. Obesity and energy imbalance

1.1. Defining obesity

Obesity is a metabolic disease characterized by abnormal or excessive fat accumulation in adipose tissue and other organs, to the extent that health may be impaired [1]. In 2014, according to the World Health Organization (WHO) it was estimated that 13% of the world's adult population (11% of men and 15% of woman) was obese (Figure 1). The tendency points to an increase in the prevalence of this disease by 2030, when 60% of the world's population is likely be overweight or obese [2].
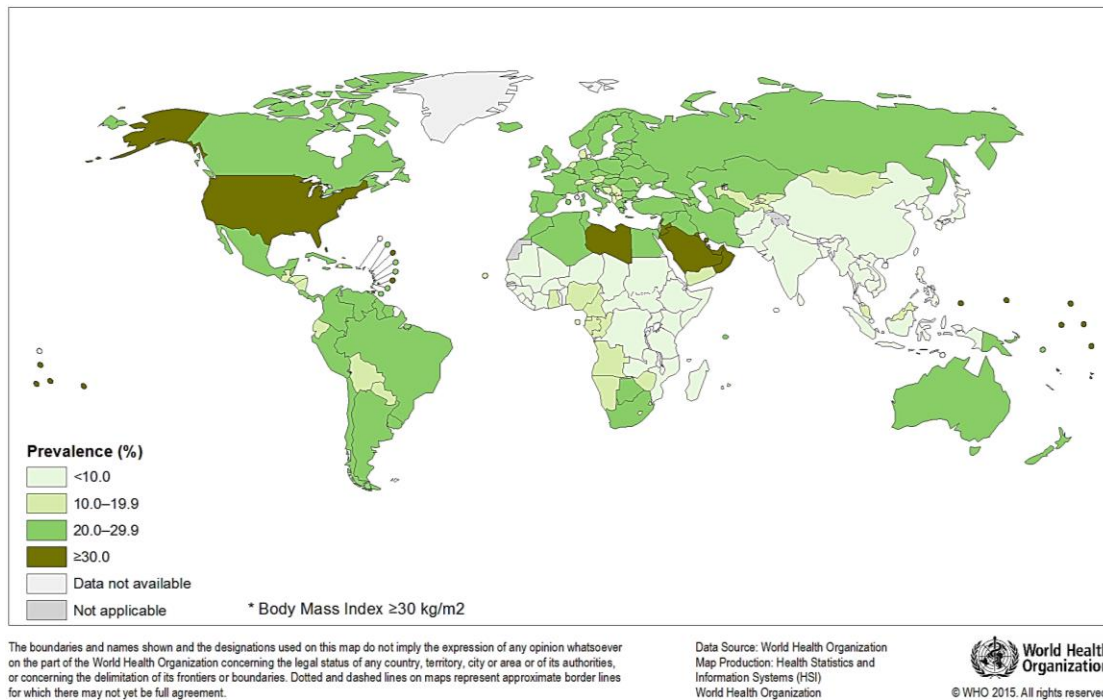
Figure 1. Age standardized estimate of the prevalence of adult obesity worldwide in 2014 *(adapted from the WHO Global Health Observatory[1]).*

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[1] http://gamapserver.who.int/mapLibrary/, last access in October 2016.

1.2.   Associated co-morbidities

Obesity has long been associated with a wide variety of other health-related complications especially due to augmented body fat, coupled with other genetic, endocrine and metabolic defects. Obese related co-morbidities include increased risk of developing type-2 diabetes mellitus (T2DM), lipid metabolism abnormalities (increased serum cholesterol, low-density lipoproteins, very low-density lipoproteins and triglycerides, as well as a reduction in serum high-density lipoproteins) and cardiovascular disorders (hypertension, stoke and coronary heart disease) [3, 4].

Obesity has also been associated with increased prevalence of several types of cancer (oesophagus, colon and rectum, liver, gallbladder, pancreas, kidney, stomach, prostate, breast, uterus, cervix and ovary), osteoarthritis, gout and pulmonary diseases, such as obstructive sleep apnoea [3, 5]. The numerous obesity related health complications lead to increased morbidity and risk of all-cause mortality, and may have a significant impact on quality-adjusted life years [6].

1.3.   Defining obesity

The body mass index (BMI), despite harbouring some limitations, is the most widely used surrogate measure of body fat in clinical and epidemiologic studies. Formerly known as Quetelet index as a measure of the nutritional status in adults, is defined as a person's weight in kilograms divided by the square of the person's height in metres (kg/m^2) [7]. According to the WHO guidelines, obesity can be defined by a BMI equal to or greater than 30 kg/m^2. This definition is further subdivided into obesity classes 1 (30–34.9 kg/m^2), 2 (35–39.9 kg/m^2), and 3 (>40 kg/m^2), as shown in Table 1. These classifications allow the identification of individuals at increased risk of obesity-associated complications and specific disorders [8].

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Table 1. BMI classification in adults *(adapted from WHO Regional Office for Europe[2])*

BMI (kg/m^2)	Weight category
< 18.5	Underweight
18.5 – 24.9	Normal weight
25.0 – 29.9	Overweight
30.0 – 34.9	Obesity class I
35.0 – 39.9	Obesity class II
> 40	Obesity class III

1.4. Management of obesity

As a chronic disease, obesity management requires long term interventions, which depending on the severity of the disease and co-morbid conditions may include pharmacological or surgical treatment in association to lifestyle modifications [9].

1.4.1. Lifestyle changes

Lifestyle and dietary modification constitute the cornerstone of every intervention for obesity treatment [10]. Nevertheless, in severe obesity lifestyle interventions alone have limited long-term efficacy due to complex and persistent hormonal, metabolic and neurochemical adaptations that defend the body against weight loss and promote weight regain [11].

1.4.2. Pharmacotherapy

Obese patients with a BMI ≥30 or ≥27 with comorbid conditions who have failed to achieve weight loss through diet and exercise alone can be endorsed for a pharmacological approach, to be used in combination with caloric restriction, increased physical activity and behaviour modification. However, there are few pharmacological treatments available for obesity management that aim to downturn the biological responses of weight gain and enable patients to achieve sustained weight loss [12]. Currently approved obesity medications and their actions are listed in Table 2.

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[2] http://www.euro.who.int/en/health-topics/disease-prevention/nutrition/a-healthy-lifestyle/body-mass-index-bmi, last access in April 2017.

Table 2. Anti-obesity drugs currently approved by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) [13, 14].

Drug	Dosing	Action	Effects
Phentermine*	15-30mg orally	Sympathomimetic	Increase CNS activity. Decrease appetite and food intake
Orlistat	120mg orally 3x day before meals	Pancreatic lipase inhibitor	Reduces digestion and absorption of fats.
Lorcaserin	10mg orally 2x day	$5\text{-}HT_{2C}$ serotonin agonist	Inhibits the subsequent uptake of 5-HT.
Phentermine/ topiramate ER*	1.5mg/46mg or 15mg/92mg orally	Sympathomimetic anticonvulsant	Glutamate antagonist. Carbonic anhydrase inhibitor. Releases catecholamine in the hypothalamus.
Naltrexone SR/ bupropion SR*	32mg/360mg orally	Opioid receptor antagonist.	Stimulates POMC neurons. Suppresses appetite. Increases energy expenditure. Inhibits reuptake of dopamine and noradrenaline.
Liraglutide	3.0mg injection	GLP-1 receptor agonist	Stimulates POMC neurons and glucose-dependent insulin secretion. Suppresses glucagon secretion.

*Approved by FDA only. Abbreviations - 5-HT: Serotonin; $5\text{-}HT_{2C}$: 5-hydroxytryptamine 2C receptor; CNS: Central Nervous System; POMC: hypothalamic pro-opiomelanocortin; ER: extended release; SR: sustained release.

The major limitations of pharmacological therapies are the need for continued treatment to sustain the weight loss effects, being prone to tolerance and lack of efficacy over time and often associated with considerable and undesirable side effects [8].

1.4.3. Bariatric Surgery

Surgical treatment for obesity, generically designated as bariatric surgery, is becoming increasingly more common as other treatments for obesity fail. Bariatric surgery stands as the most effective treatment for severe obesity by allowing considerable and sustained weight loss, along with improvement of obesity-associated comorbidities, and thereby reducing mortality [15, 16].

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1.4.3.1.   Inclusion criteria

Based upon the European Guidelines on Metabolic and Bariatric Surgery, patients qualify for bariatric surgery if aged from 18 to 60 years with a BMI ≥ 40.0 kg/m^2 or BMI 35.0 - 39.9 kg/m^2 with co-morbidities in which surgically induced weight loss is expected to improve the disorder (such as metabolic disorders, cardiorespiratory disease, severe joint disease, obesity-related severe psychological problems), when less invasive therapeutics have failed to sustain weight loss and the patient is at high risk for obesity-associated morbidity or mortality [17].

1.4.3.2.   Types of bariatric surgery

Bariatric surgery procedures according to the putative mechanism of action can fall under the following categorization: purely restrictive, such as adjustable gastric banding (AGB) and vertical sleeve gastrectomy (VSG), which only decrease the volume capacity of the stomach; malabsorptive procedures that decrease absorption of nutrients by decreasing the length of small intestine that is exposed to food (biliopancreatic diversion (BPD) and duodenal switch); and mixed procedures that combine intestinal rearrangement with gastric restriction, such as, Roux-en-Y gastric bypass (RYGB) (Figure 2).

In AGB (Figure 2A), a band or collar is placed around the proximal portion of the stomach, thereby creating an approximate 30-mL upper gastric pouch, which has a balloon that lines the inside portion of the band. In VSG (Figure 2B), most of the greater curvature of the stomach is resected creating a tubular section along the lesser curvature of the stomach which results in a marked reduction in gastric capacity. BPD (Figure 2C) diverts bile and pancreatic secretions to the distal bowel for mixing with nutrients, much further distal than in RYGB procedure, combined with a 50% to 80% gastrectomy. In BPD with duodenal switch (Figure 2D), the pylorus is retained allowing normal gastric emptying to occur, while a much longer section of intestine is bypassed as compared to RYGB, which also increases the risk of malabsorption [18, 19].
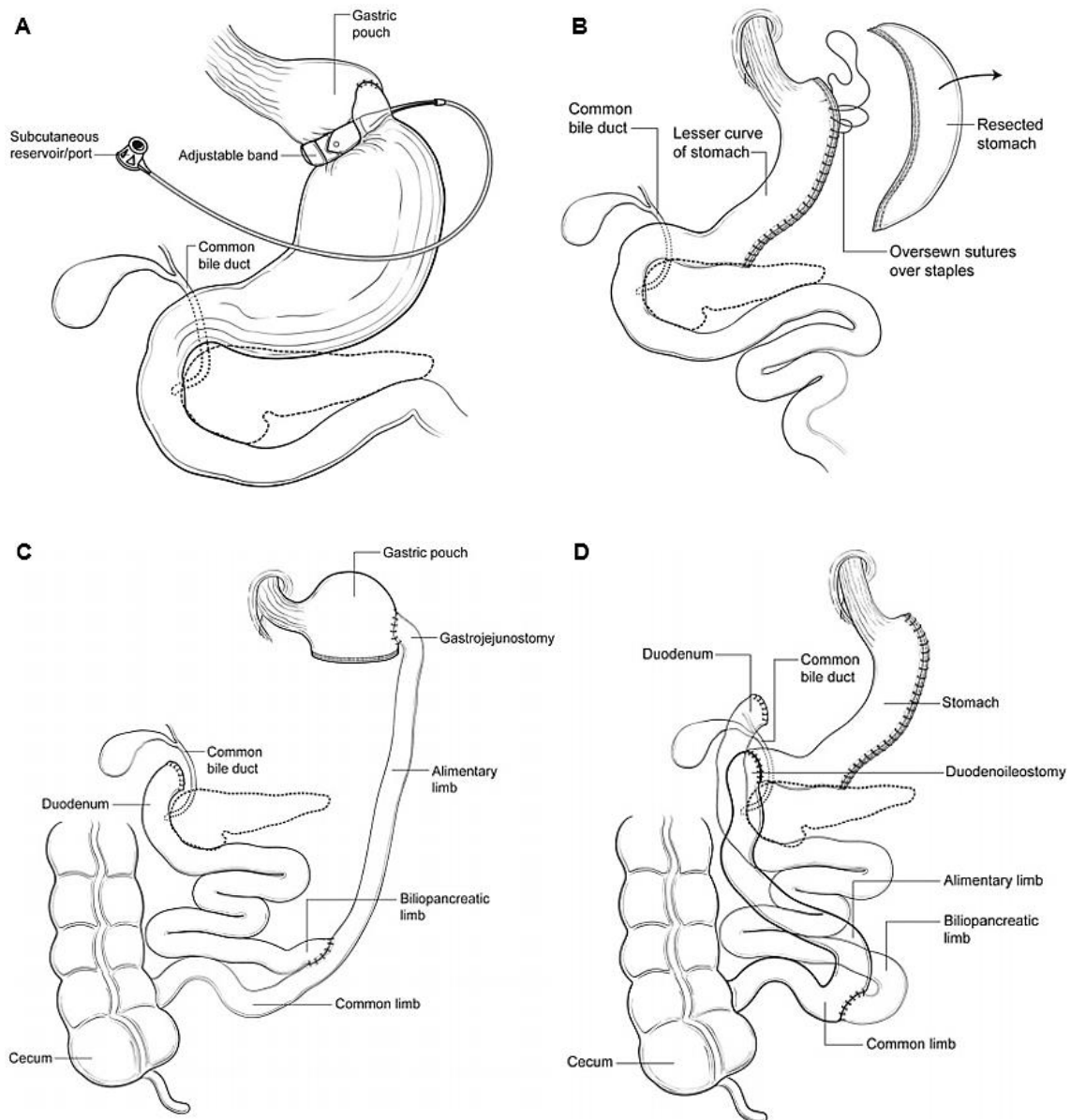
Figure 2. Bariatric surgery procedures: AGB (A), VSG (B), BPD (C), and BPD with duodenal switch (D) *(Adapted from Thompson, C.C. ed., (2013), Bariatric Endoscopy [20]).*

2. Roux-en-Y gastric bypass (RYGB)

RYGB is the most commonly performed bariatric operation worldwide and thus considered by many as the gold standard in bariatric surgery. This procedure is highly effective in reducing excess body weight through combination of malabsorption and gastric restriction and has a substantial efficacy in reverting multiple comorbid conditions associated with obesity, in particular T2DM [21, 22].

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2.1. Anatomical changes

In RYGB, the stomach is divided, creating a small gastric pouch (15-30 mL) and a much larger excluded component, the gastric remnant. The gastric pouch is connected to a distal segment of jejunum through a gastrojejunostomy, which forms the Roux or alimentary limb (AL). The gastric remnant drains into the bypassed portion of bowel, referred to as the biliopancreatic limb (BPL), is then connected to the AL, restoring bowel continuity. The intersection of these limbs forms the "Y" configuration of the operation and the remaining ileum and colon is normally referred as the common limb (CL) [23].

Given the anatomical changes (Figure 3), ingested nutrients proceed rapidly through the stomach pouch and move immediately into the jejunal AL in the absence of bile and pancreatic secretions. Bile and pancreatic secretions drain via the biliopancreatic limb and interact with the nutrients at the point of the jejuno-jejunostomy and afterwards at the CL [24].
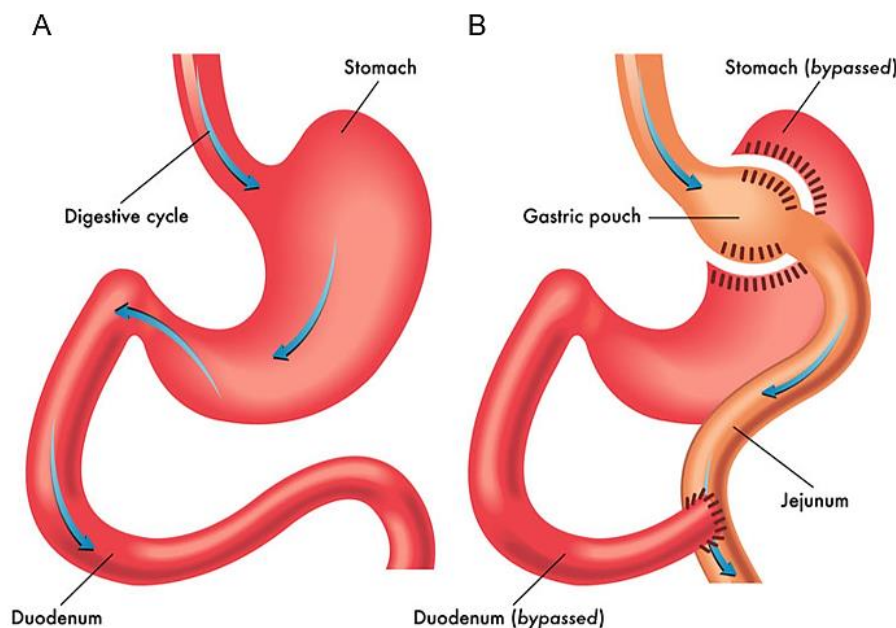
Figure 3. Schematic illustration of the preoperative anatomy (A) and postoperative anatomy after RYGB (B) *(Adapted from http://surgery.ucla.edu/bariatrics-gastric-bypass, last access in May 2017).*

2.2. Complications

Given the technical complexity of the RYGB procedure this can be associated with several surgical and medical complications, such as bleeding, anastomotic leakage, wound infections, pulmonary embolism, micronutrient deficiencies, protein malnutrition, postprandial hypoglycaemia and dumping syndrome [25]. A recent systematic review

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and meta-analysis including 161,756 patients concluded that bariatric surgery results in substantial and sustained effects on body weight and obesity comorbidities and that the risk of complications and death, although exist occur at relatively low rates, averaging 21% (12–33%) for all postoperative complications and 0.72% for mortality (<30 days after surgery) [26].

Dumping syndrome occurs due to the lack of pyloric control over gastric emptying that allows the rapid delivery of a high osmotic load to the small intestine, bypassing the stomach and prompting fluid to enter the gut lumen. This complication has been reported to occur in 12 to 68% of patients after Roux-en-Y gastric bypass, depending on the series and diagnostic criteria that were used [27, 28].

Early dumping symptoms occur within 30 minutes after eating, when food and fluid pass into the small intestine, and may include nausea, vomiting, stomach pain or cramping, diarrhoea, feelings of fullness, bloating or increased heart rate. Late dumping symptoms occur 1 to 3 hours after eating and include flushing, sweating, intense need to lie down, weakness, dizziness, tremor, along with a decrease in blood pressure and reactive hypoglycaemia. The prevalence of early dumping after gastric bypass was estimated to be between 12 and 42% when assessed by patient or changes in the haematocrit. The prevalence of late dumping when assessed by oral glucose tolerance test (OGTT) or mixed-meal tolerance test (MMTT) was estimated to occur in 17 to 68% of patients [27-29].

Dumping syndrome diagnosis relies mostly on clinical features supported by the use of Sigstad's scoring system or by the response to provocative tests such as the OGTT or the MMTT, while late hypoglycaemia (<60mg/dl or 3.33 mmol/l), an increase in haematocrit over 3% or in heart rate over 10 beats per minute within the first hour of a provocative test performed after a 10-hour fasting, is considered positive [30-32].

2.3. Outcomes of the surgery

RYGB produces substantial weight-loss and dramatically ameliorates the comorbidities associated with obesity both at short- and long-term. Moreover, the clinical outcomes of RYGB are better when compared with other bariatric surgery procedures, such as adjustable gastric band [33].

RYGB leads not only to major weight loss and considerable improvements in glycaemic control, but also to an increased incretin effect and GLP-1 levels. Glucose homeostasis is markedly improved in insulin-resistant patients after RYGB surgery. This effect is in

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part independent of weight loss and may involve early absorption of dietary carbohydrates together with enhanced secretion of gut peptides stimulating insulin secretion [34].

3. Glucose homeostasis and energy balance

In healthy adults, blood glucose levels are tightly regulated within a range of 70 and 100 mg/dL (3.9 and 5.5 mmol/L, respectively) to meet metabolic requirements and to ensure a normal body function. During the postprandial period, the processes responsible for glucose regulation may be particularly exacerbated once, under normal physiologic circumstances, glucose levels rarely rises beyond 140 mg/dL (7.7 mmol/L), even after consumption of high-carbohydrate meals [35]. This is accomplished by a highly-sophisticated network of various hormones and neuropeptides released mainly from the brain, pancreas, liver, intestine as well as adipose tissue and muscle [36].

3.1. Pancreatic regulation

The pancreas, located behind the stomach within the left upper abdominal cavity, plays an important role in macronutrient digestion and metabolism homeostasis by releasing various digestive enzymes and pancreatic hormones. This secretory organ is divided in two principal components: one exocrine which consists of acinar and duct cells arranged in a tubule-acinar structure that synthetizes, stores and releases pancreatic juice containing digestive proenzymes and enzymes, such as amylase, pancreatic lipase and trypsinogen; and an endocrine component consisting of neuroendocrine cells organized into clusters known as Langerhans islets where pancreatic hormones are produced and then released directly into the bloodstream [37].

Each islet is typically composed of five different cell subtypes: glucagon-producing α-cells, amylin-, C-peptide and insulin-producing β-cells, pancreatic polypeptide (PP)-producing γ-cells, somatostatin-producing δ-cells, and ghrelin-producing ε-cells, whose produced hormones have distinct functions in glucose homeostasis [38].

3.1.1. Insulin and glucagon

Insulin is a 51-residue peptide with two chains (A and B) linked by disulphide bonds. After cleavage by signal peptidases, the biosynthetic precursor of insulin, proinsulin, yield

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equimolar amounts of insulin and C-peptide, a 31-amino-acid cleavage product of insulin biosynthesis responsible for insulin A- and B-chains linkage and stabilization [39, 40].

Glucagon, a peptide hormone consisting of 29 amino acids, is produced by the action of prohormone convertase (PC) 2 upon pro-glucagon (PG), also releasing glicentin-related PP, and the major PG fragment [41].

Through its various hormones, particularly glucagon and insulin, the pancreas maintains blood glucose levels within a very narrow range. This preservation is accomplished by the opposing and balanced actions of glucagon and insulin, referred to as glucose homeostasis (Figure 4).

During sleep or in between meals, when blood glucose levels are low, glucagon induces a catabolic effect, mainly by activating liver glycogenolysis and gluconeogenesis, which results in the release of glucose to the bloodstream. In contrast, insulin acts mainly on muscle, liver and adipose tissue with an anabolic effect, triggering glucose uptake into these tissues and its accumulation as glycogen and fat [42].
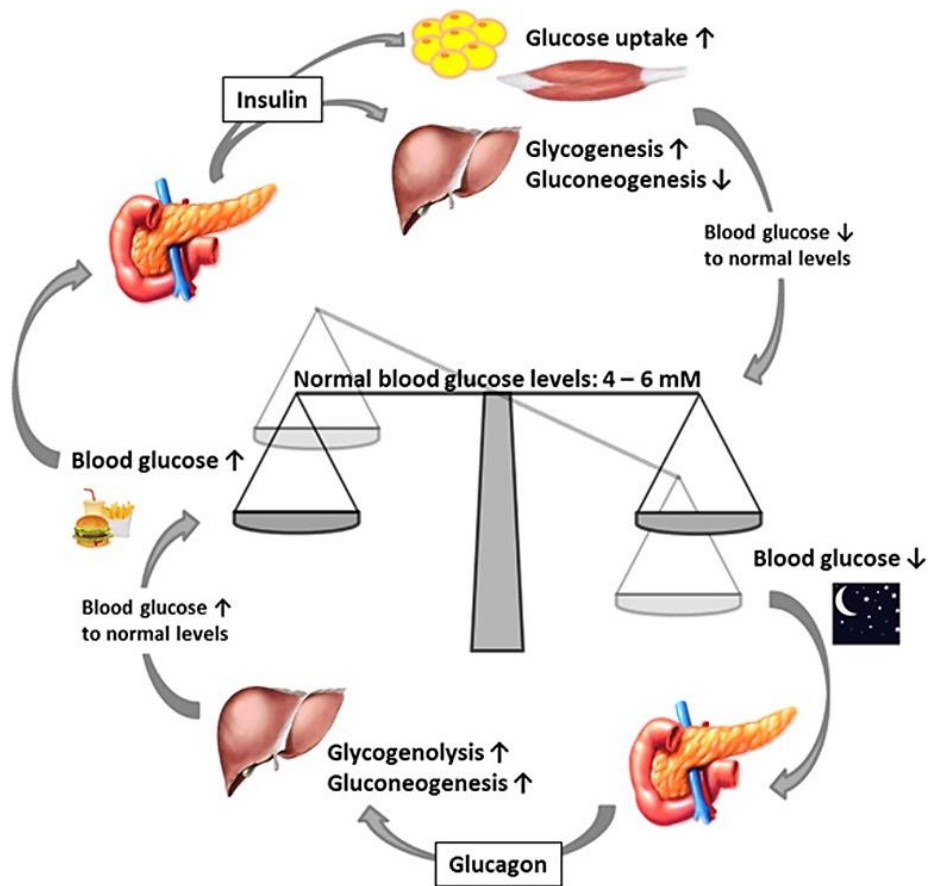
Figure 4. Maintenance of blood glucose levels by pancreatic hormones glucagon and insulin [36].

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3.1.2. Pancreatic Polypeptide

Pancreatic polypeptide (PP) is a 36-amino acid peptide hormone from the PP fold family of peptides and secreted by the PP-cells of the islets of Langerhans in the pancreas following meal consumption, especially meals high in protein. It regulates pancreatic exocrine secretion, as well as modulates gastric acid and GI motility. In addition to reducing food intake, PP also reduces gastric emptying, potentially providing feedback to central satiety centres via the vagus nerve [43, 44].

3.2. Gastrointestinal regulation

The gastrointestinal (GI) tract is responsible for the production of multiple signals which play a critical role in the regulation of glucose homeostasis and energy balance. These signals are synthesized in response to ingested nutrients and include peptides/hormones originated from intestinal epithelial and neuroendocrine cells, bile acids, and molecules produced by the gut microbiota [45].

Enteroendocrine cells (EEC), widely distributed along the entire GI mucosa in the crypts and villi, are capable of sensing luminal content, producing and releasing signalling molecules that modulate a variety of physiological GI and homeostatic functions [46, 47].

Several EEC types have been identified, collectively secreting a wide range of hormones that can act locally, on other cells, nerve endings, or organs at distant sites including pancreatic islets and the central nervous system (CNS) (Table 3) (Figure 5).

Table 3. EEC types, their location, synthetized compounds, and main effects [47, 48].

Cell type	Products	Location	Main effects
A	Ghrelin	Stomach (corpus)	Appetite control Growth hormone release
G	Gastrin	Stomach (antrum)	Stimulation of gastric acid secretion
D	Somatostatin	Stomach (pylorus), small intestine and pancreas	Inhibition of gastric release Modulation of insulin release
I	CCK	Proximal small intestine	Activation of gallbladder contraction Stimulation of pancreatic enzyme secretion

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K	GIP	Proximal small intestine	Stimulation of insulin release
L	GLP-1, GLP-2, PYY, OXM	Distal small intestine, colon	Stimulation of carbohydrate uptake Slow intestinal transit, appetite regulation and insulin release
N	Neurotensin	Small and large intestine	Inhibition of intestinal contractions
P	Leptin	Stomach	Reduction of food intake
S	Secretin	Proximal small intestine	Stimulation of bicarbonate release Reduction of acidity in upper small intestine

CCK, cholecystokinin; GLP-1, glucagon like peptide-1; GLP-2, Glucagon like peptide-2; PYY, peptide YY; GIP, gastric inhibitory peptide.
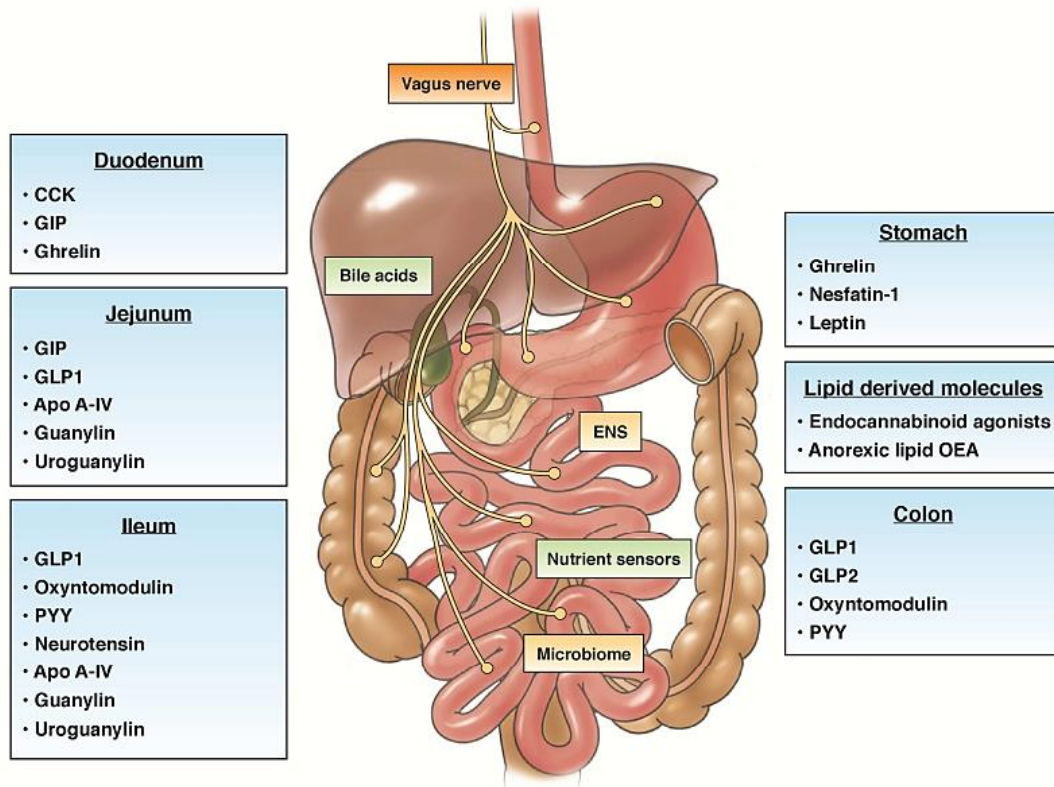
Figure 5. GI signals responsible for the regulation of energy homeostasis [49].

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3.2.1. Incretins and the incretin effect

In healthy individuals with normal glucose tolerance, the incretin effect is a well-defined phenomenon characterized by a greater insulin secretory response from pancreatic β-cells after oral glucose load as compared to intravenous administered glucose that has an important contribution for glycaemic homeostasis, while this effect is reduced or even absent in T2DM patients [50].

The glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), synthesised and secreted from the gut, are the hormones responsible for the incretin effect and interact with the endocrine pancreas, acting via specific G-protein-coupled receptors to potentiate insulin secretion from pancreatic β-cells in a glucose-dependent manner, by increasing the transcription and biosynthesis of insulin, as well as promoting β-cell proliferation and suppressing β-cell death in experimental models [51, 52].

3.2.1.1. Glucagon-like peptide-1

Glucagon-like peptide-1 (GLP-1) is synthetized by L-cells in the distal ileum and colon through the action of prohormone convertase (PC) 1/3 that cleaves proglucagon (PG) to yield not only GLP-1 but also glucagon-like peptide-2 (GLP-2) and the glucagon-containing peptides glicentin and oxyntomodulin (OXM), in a similar way to what happens in pancreatic α-cells [53].

GLP-1 release occurs particularly after fat and carbohydrate intake in proportion to meal size. GLP-1 effects include stimulation of insulin secretion by β-cells, inhibition of glucagon secretion, suppression of gastric emptying and reduction of food intake by inhibiting appetite through CNS-mediated mechanisms [54, 55].

Once secreted, the active forms of GLP-1, GLP-1 $_{7-37}$ and GLP-1 $_{7-36\ amide}$, are converted into the inactive forms GLP-1 $_{9-37}$ and GLP-1 $_{9-36\ amide}$ by dipeptidyl peptidase-4 (DPP-4), and consequently only approximately 25% reaches the hepatic-portal circulation and less than 10% reaches the systemic circulation [56].

3.2.1.2. Glucose-dependent insulinotropic polypeptide

Glucose-dependent insulinotropic polypeptide (GIP) is a 42-amino acid peptide hormone produced by K-cells in the duodenum and jejunum via PC 1/3-dependent posttranslational cleavage of its precursor, proGIP, in response to glucose, lipid or

mixed-meal ingestion in proportional amounts to meal size. At supra-physiologic doses, GIP inhibits gastric acid secretion in the stomach. In the liver, GIP attenuates glucagon-stimulated hepatic glucose production, likely through indirect mechanisms as GIP receptors (GIPRs) have not been detected in the liver [57, 58].

DPP-4 is also responsible for the cleavage of the intact biologically active GIP_{1-42} which has a half-life of less than 7 minutes in healthy individuals, into the inactive metabolite GIP_{3-42} [56].

3.2.2. Other gut-derived signals

3.2.2.1. Peptide YY

Peptide YY (PYY) is a 36-amino acid gastrointestinal peptide hormone synthetized and secreted often together with PG-derived peptides by L-cells in the distal ileum and colon, and released into the circulation in response to food intake. PYY belongs to the pancreatic polypeptide family that also includes PP and NPY and decreases food intake. Circulating PYY levels are low and it is present in plasma in two major molecular forms: PYY_{1-36}, and the main circulating form, a 34-amino acid N-terminally truncated peptide, PYY_{3-36}, which is formed by cleavage by the enzyme DPP-4 [59, 60].

3.2.2.2. Neurotensin

Neurotensin (NT) is a 13-amino acid peptide primarily found in CNS and the GI tract. NT is widely distributed in the CNS and exerts potent effects on appetite and thermoregulation, controlling food reward while interacting with the dopaminergic system and leptin. In the GI tract, NT is produced in the small and large intestine and secreted in response to nutrient-ingestion, and in certain enteroendocrine cells is coexpressed with the anorexigenic hormones GLP-1 and PYY [49].

NT regulates nutrient absorption, GI motility, pancreatic and biliary secretion, fat translocation, insulin secretion [61] and acts together with GLP-1 and PYY in metabolic regulation [62]. However, while it is well recognised that brain NT has anorexigenic effects, the metabolic effects of peripheral NT have been less investigated [63].

3.2.2.3. Bile acids

Bile acids represent the primary pathway for cholesterol catabolism and act as emulsifiers, thus promoting the absorption of lipids. The major primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized in liver and conjugated with taurine or

glycine for secretion into bile, reabsorbed in the ileum and transported back to the liver, mainly via portal blood circulation. Bile acids form mixed micelles with phospholipids and cholesterol and are stored in the gallbladder. After each meal, cholecystokinin (CCK), a GI hormone secreted from I-cells of the duodenum and jejunum, stimulates gallbladder contraction to empty bile acids into the intestinal tract to facilitate digestion and absorption of nutrients [64, 65].

Bile acids mediate control of their own synthesis through activation of farnesoid X receptor in the liver, affecting hepatic metabolism, and activate G-protein-coupled bile acid-activated receptors (TGR5) of the enteroendocrine L-cells, promoting the release of incretin, stimulating energy metabolism and improving insulin sensitivity. Therefore, bile acids also play an important role in glucose homoeostasis [64].

4. Metabolic improvements after RYGB

RYGB was initially designed to promote weight loss by a combination of reduced stomach volume and malabsorption of nutrients. Additionally, RYGB also leads to early-improvements in T2DM glycemic control or even complete clinical remission often before any substantial weight loss has occurred. It is now known that calorie malabsorption alone does not explain these effects, and the outcomes of RYGB are not entirely due to the reduced stomach volume but also to the alteration in the secretion profile of various gut hormones associated with appetite and satiety, as well as energy expenditure [66, 67].

4.1. Altered gut hormone secretion profile

Many of the beneficial metabolic effects observed after bariatric surgery have been attributed to altered peptide hormone profiles, specially involving pancreatic and gut hormones [68]. After RYGB, the anatomy of the proximal part of the intestine is considerably rearranged and most of the stomach is bypassed. Considering that the different EEC are distinctively distributed along the human small intestine and that the secretion of gut hormones is potentiated by the arrival of nutrients to the gut, it is likely that RYGB by changing the intestinal exposure to nutrients leads to a modified secretion of peptide hormones [69-71].

As a bariatric procedure that reduces the nutrient exposure in the gastric antrum and duodenum, RYGB would theoretically produce a decrease in the levels of the upper

intestinal hormones. As so, one would predict that the length of the BPL would be important for postoperative duodenal hormone secretion. As the length of this limb increases, the cells present in the proximal small intestine would not be exposed to nutrients and therefore would be less stimulated, resulting in a lower secretion of duodenal hormones [72], such as gastrin, ghrelin, GIP, CCK, secretin and somatostatin.

Postprandial levels of gastrin fall after RYGB in the first two weeks post-operatively and the same happens for ghrelin and GIP levels. On the contrary, CCK levels increase postprandially after RYGB in response to a mixed-meal. Post-operative changes in somatostatin levels do not seem to occur [73, 74].

The main hormones secreted from the distal small intestine are GLP-1, GLP-2, PYY and NT, and these were shown to be markedly increased after the RYGB when compared to the pre-surgical state [74, 75].

The anatomical changes after RYGB result in bile progressing down the biliopancreatic limb to the distal L-cells without mixing with food. As a result, the availability of undiluted bile acids in the distal intestine may enhance stimulation of TGR5 on L-cells, therefore stimulating GLP-1 secretion [76]. Serum bile acid concentration is raised after RYGB and the post-operative increases in circulating levels have been suggested to contribute to the metabolic benefits of bariatric surgery; however, their role and mechanism of action remain undefined [77].

Regarding the pancreatic hormone changes after RYGB, insulin levels appear to decrease in parallel to the increase in insulin sensitivity [78], as well as postprandial glucagon levels after a liquid meal [79]. Unfortunately, very little is known about the effect of RYGB upon PP since very few studies have assessed its effects.

4.2.   Hindgut and foregut hypothesis

After RYGB along with the significant hormonal changes there is an acute anti-diabetic effect that occurs via a weight-independent mechanism. Moreover, the magnitude of the long-term metabolic control is much greater than would be expected from the degree of weight loss, which might be a clue that the altered hormonal milieu of gut-hormone release could further contribute to explain the metabolic control [80].

Based on this observation, several possible mechanisms were hypothesized to explain the early and long-term glycemic normalization occurring after RYGB surgery. Given the anatomic changes produced by the RYGB procedure, two hypotheses arose to explain

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the mechanisms leading to improved glucose homeostasis and T2DM remission, the foregut and the hindgut hypotheses [81].

The foregut hypothesis proposes that the exclusion of the duodenum and proximal jejunum from the transit of nutrients may prevent the secretion of a putative signal (anti-incretin) originated in the foregut that promotes insulin resistance and T2DM, suggesting that a yet unidentified inhibitory product from the proximal bowel is the cause of metabolic changes [82]. The hindgut hypothesis proposes that T2DM control results from the rapid delivery of nutrients to the distal bowel, thus stimulating L-cells to secrete GLP-1, PYY and NT which results in the enhancement of the incretin effect, insulin secretion and insulin sensitivity [83].

4.3. RYGB limb length variations

Since the first description of the RYGB procedure, introduced by Mason and Ito [84], many variations with the purpose of achieving greater weight loss with a minimum of nutritional deficiencies and a higher resolution of obesity-related comorbidities, were described. Even though there is no consensus on the ideal length of the gastric bypass limbs, bariatric surgeons continue to vary the length of the limbs in order to improve results and find the appropriate balance between benefits and side effects [85].

In a recent survey on the technical preferences of 215 bariatric surgeons' members of the American Society for Metabolic and Bariatric Surgery, the reported lengths for the AL and BPL ranged from 35 to 250cm (average 110cm) and from 10 to 250cm (average 48cm), respectively. Despite most surgeons assumed using a BMI cut-off to vary the length of the limbs, the criteria used also varied significantly [86].

Above all, the lengths of BPL and AL that will produce the maximum effect on hunger and satiety and T2DM remission, remain to be determined, as well as the hormonal pathways that could be useful as a surrogate to estimate these changes.

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AIM OF THE STUDY

The aim of this study was to compare the gastro-intestinal hormone profile after a mixed-meal of non-diabetic patients previously submitted to classic RYGB and the long BPL RYGB variant.

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MATERIALS AND METHODS

1. Patient recruitment

Subjects included in the study were selected among the post-bariatric surgery cohort under routine follow-up at Centro Hospitalar de Entre o Douro e Vouga (CHEDV), Portugal.

Inclusion criteria were having performed a previous laparoscopic RYGB for the primary treatment of obesity and related conditions (BMI>40 or >35 kg/m^2 with comorbidities) and body weight stability for the previous 6 months regardless the time elapsed since surgery.

Exclusion criteria included past medical history of diabetes mellitus or glucose intolerance documented either before or after surgery, dumping syndrome, post-bariatric hypoglycaemia and pregnancy.

Patients selected had been submitted to RYGB (n=20), either to the standard classical RYGB procedure (n=9) or to a RYGB variant procedure consisting of a longer BPL (n=11), 3.8 ± 2.1 or 4.4 ± 1.7 years earlier, respectively.

Subjects meeting the inclusion criteria were heighted, weighted and had their co-morbidities and current medications recorded, both at the time of surgery and at the time of the study visit.

Both the study protocol and the patient information leaflet were approved by the CHEDV Institutional Ethical Review Board and was conducted according to the National Data Protection regulations. Written informed consent was obtained from all participants before enrolment in the study.

2. Surgical procedure

Surgeries were performed at the Department of General Surgery of Centro Hospitalar de Entre-o-Douro e Vouga (CHEDV), Portugal, using a standard laparoscopic RYGB technique and consisted in the creation of a small gastric pouch (15 mL) with a 120 cm long alimentary limb (AL) for both procedures and a biliopancreatic limb (BPL) of 88 ± 21

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cm and 200 cm for the classical RYGB and for the long BPL RYGB variant, respectively, as previously described [23]. Briefly, the small gastric pouch was created by transecting the lesser curvature of the stomach and excluding the gastric fundus. Afterwards the jejunum was divided and anastomosed in an anterocolic position regarding the gastric pouch (AL) and the remaining part of the proximal jejunum (BPL) was anastomosed to the AL 120 cm distally to the gastrojejunostomy.
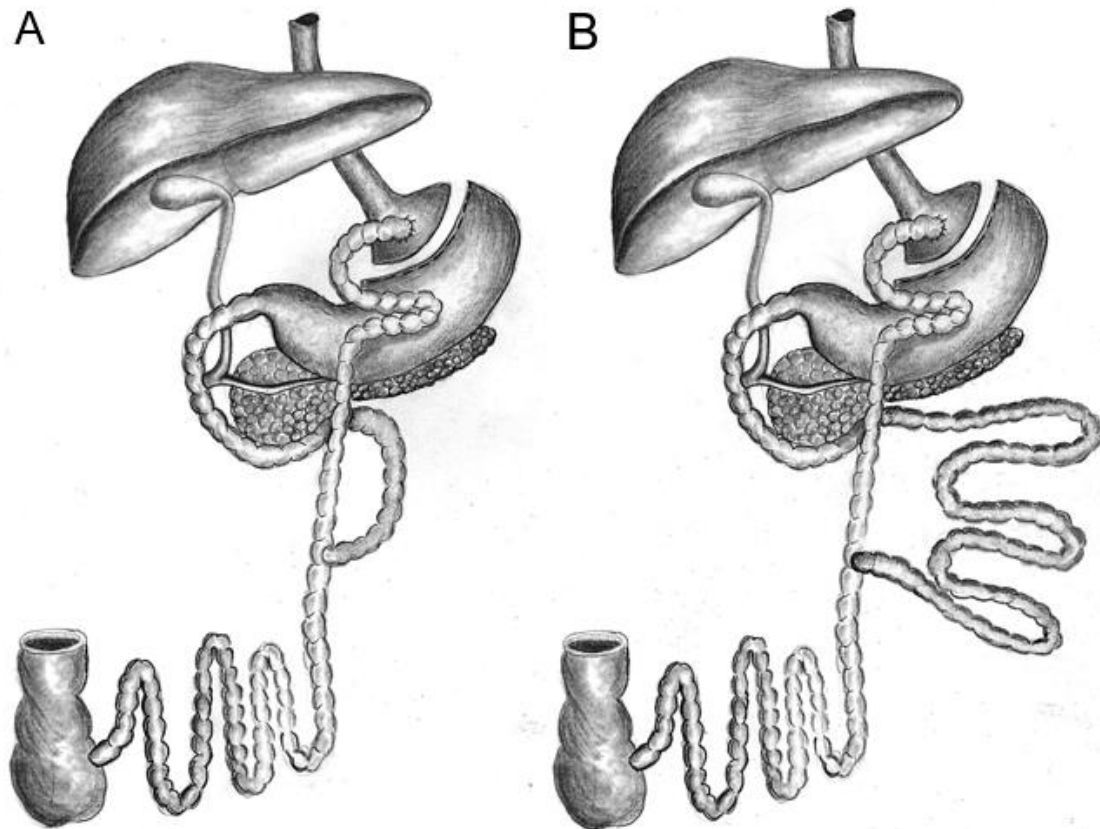
Figure 6. Classic RYGB (BPL= 88 ± 21 cm; AL=120cm) (A) and the long BPL RYGB variant (BPL=200cm; AL=120cm) (B) (kindly drawn by Duarte Monteiro).

3. Mixed-meal test

Subjects were submitted to a mixed-meal tolerance test (MMTT) using a commercially available standardized liquid meal consisting of 300 kcal in 200 mL with 50% energy from carbohydrate, 15% energy from protein, and 35% energy from fat (Fresubin® Energy Drink, Fresenius Kabi Deutschland, Bad Homburg, Germany). Subjects were requested to drink the 200ml meal after a 12-hour overnight fast over a maximum of 15 minutes' time. All patients were able to tolerate the supervised ingestion of the mixed-meal within the requested time frame or less. As dumping syndrome could represent a confounding factor in the interpretation of the hormonal responses, this has been actively

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investigated. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were monitored during the MMTT and the Sigstad's scoring system was used to document the occurrence of dumping syndrome. A Sigstad's score above seven was considered positive for dumping syndrome [87].

3.1. Sample Collection

Venous blood was collected from a catheter placed in the cubital vein into chilled EDTA tubes (S-Monovette® 7.5 ml, K2 EDTA Gel, 1.6 mg/mL, Sarstedt) before (baseline), and again 15, 30, 45, 60, 90 and 120 minutes' after the consumption of the mixed-meal. During the duration of the test, the participants were asked to remain in a reclined seating position and no strenuous activity was allowed during the period of the test.

Blood samples were kept refrigerated until centrifugation at 4ºC and 2500×g (Rotina 380R, Hettich) for plasma separation, which was then frozen (-20ºC) and stored until assayed.

4. Biochemical measures

Plasma glucose was measured by the glucose oxidase method using a glucometer (YSI model 2300 STAT Plus, Yellow Springs Instruments, Yellow Springs, OH). Plasma levels of GLP-1, GIP, glucagon, peptide YY (PYY), pancreatic polypeptide (PP), and neurotensin (NT) concentrations were determined by in-house radioimmunoassays (RIA); insulin and C-peptide concentrations were evaluated by an electrochemiluminescence sandwich immunoassay (ECLIA), and total bile acids (TBA) concentrations were accessed using a commercial assay kit (Total Bile Acid Assay Kit, STA-631, Cell BioLabs, Inc, San Diego, CA, USA).

4.1. Radioimmunoassays

Plasma samples were thawed on ice and immediately extracted with 70% ethanol (volume/volume, final concentration). Preparation of the samples by ethanol extraction was necessary to minimize the presence of non-specific cross-reacting substances in the samples at the expense of some peptide loss in result of the extraction process. After addition of ethanol, samples were vortexed and centrifuged for 35 minutes (3500×g, 4ºC, Sorvall RC-3C Plus, GMI, USA). The supernatant was decanted into new marked tubes and dried under an air stream over-night. The next day samples were reconstituted with assay buffer (80 mmol/L sodium phosphate buffer, pH 7.5, 0.1% (wt/vol) human serum albumin (Calbiochem, USA), 10 mmol/L EDTA, and 0.6 mmol/L thimerosal (Sigma, St.

Louis, USA)) [88]. Peptide concentrations in the reconstituted samples were then analysed using in-house radioimmunoassays (RIA).

The plasma concentrations of GLP-1 were measured against standards of synthetic GLP-1 $_{7-36}$ amide using an in-house polyclonal antibody raised in a rabbit (antiserum code no. 89390), which is specific for the amidated C-terminus of GLP-1 and therefore mainly reacts with GLP-1 of intestinal origin. The assay reacts equally well with intact GLP-1 and its primary metabolite (GLP-1 $_{9-36}$ amide) truncated at the N-terminus [89]. ^{125}I-GLP-1 was used for tracer (a gift from Novo Nordisk, Bagsværd, Denmark).

Total GIP levels were determined using C-terminally directed antiserum no. 867, which cross-reacts fully with human GIP but not with the so-called 8-kDa GIP, whose chemical nature and relationship to GIP secretion is uncertain. The antiserum, which is similar to the previously used R65, reacts equally with intact GIP and GIP$_{3-42}$, the primary N-terminally truncated metabolite. Human GIP and ^{125}I-labeled human GIP (70 MBq/nmol) were used for standards and tracer. Because of the rapid and intravascular conversion of both GLP-1 and GIP to their primary metabolites, to estimate the secretion of these hormones is essential to determine the sum of the intact hormone and the metabolite (total peptide levels) [90].

The glucagon antibody is directed against the intact C-terminus (antiserum code no. 4305) of the molecule and therefore mainly measures glucagon of pancreatic origin. Antiserum 4305 recognizes the COOH-terminus of glucagon and thus cross-reacts with all peptides with the same COOH-terminus as pancreatic glucagon. None of the glucagon antisera cross-reacts with GLP-1 or any other members of the glucagon/secretin family of peptides [91].

RIA for total PYY in plasma was performed using a monoclonal antibody MAB8500 (Abnova, clone RPY-B12), which reacts equally well with PYY $_{1-36}$ and PYY $_{3-36}$. Synthetic human PYY $_{3-36}$ (Bachem, Bubendorf, Switzerland) was used as standard and ^{125}I-PYY $_{1-36}$ (NEX341, Perkin Elmer, Massachusetts, USA) as tracer [92].

PP was assayed using a monoclonal antibody HYB 347-07 (Statens Serum Institut, Copenhagen, Denmark), synthetic human PP (7TM Pharma A/S, Denmark) as standard and ^{125}I-PP (Perkin Elmer, Massachusetts, USA) as tracer.

An in-house developed assay was used to measure plasma concentrations of total NT levels (antibody code no. 3D97, ^{125}I-labeled NT peptide tracer (in-house iodinated tracer)) as previously described [93, 94].

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Plasma samples were handled identically and assayed in duplicate in one single batch to minimise intra-assay variation and avoid inter-assay variation. In all assays, the free and bond moieties were separated with plasma-coated charcoal (E. Merck, Darmstadt, Germany). The experimental detection limits were less than 5 pmol/L and the intra-assay coefficient of variation was below 6% at 20 pmol/L.

4.2. Electrochemiluminescence immunoassay

Insulin and C-peptide levels were determined by an electrochemiluminescence sandwich immunoassay (ECLIA, Cobas 8000, model e602, Roche Diagnostics, USA) and both assays used two liquid human serum based controls (Liquichek™ Immunoassay Plus Control, Level 1 #361 and Level 3 #363, Bio-Rad for insulin; Liquichek™ Specialty Immunoassay Control, Level 1 #364 Level 3 #366, Bio-Rad for c-peptide). The maximum value for the coefficient of variation (CV_{max}) was 5% and 8% for insulin and C-peptide, respectively.

4.3. Total Bile Acid Assay Kit

The total bile acid content was measured using a commercial assay kit (Total Bile Acid Assay Kit, STA-631, Cell BioLabs, Inc, San Diego, CA, USA). The bile acids were measured spectrophotometrically at a primary wavelength of 405 nm and a secondary wavelength of 630 nm using a microplate spectrophotometer. The 630 nm absorbance was subtracted from the 405 nm absorbance to obtain the final absorbance values.

5. Statistical analysis and calculations

Data are presented as mean ± standard error of the mean (SEM) for figures and mean ± standard deviation (SD) for tables unless stated otherwise. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) and was calculated according to the formula: *[fasting glucose (mmol/L) × fasting insulin (mIU/L) / 22.5]* [95]. Pancreatic β-cell function was measured by the HOMA of β-cell function (HOMA-β), which was calculated according to the formula: *[20 × fasting insulin (mIU/L) / fasting glucose (mmol/L) − 3.5]* [95].

The D'Agostino-Pearson test was used to verify the normality of the data. Differences between groups relative to hormone levels were assessed using a two-way ANOVA with Sidak's multiple comparisons test, while comparisons between groups regarding fasting concentrations and area under the curve (AUC) were evaluated with an unpaired two-

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tailed t-test. Total AUC (T-AUC) was calculated using the trapezoidal model. p-values <0.05 were considered significant. All statistical analyses were performed on GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

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RESULTS

1. Patients

Twenty subjects previously submitted to classic RYGB (n=9, 1 men and 8 women) or the long BPL RYGB (n=11, 1 men and 10 women) were included in the study. At the time of the mixed-meal study, there were no significant differences regarding age, body weight, BMI, percentage of excess BMI loss (%EBMIL), HOMA-IR, HOMA-β and time elapsed since surgery, except for the lengths of the BPL that were significantly different between the study groups (p<0.0001) (Table 4).

Table 4. Clinical and anthropometric characteristics of subjects submitted to classic and the long BPL RYGB at the mixed-meal study.

	Classic BPL	Long BPL	p-value
N (% of total)	9 (45%)	11 (55%)	-
Gender (men/women)	1/8 (11%/89%)	1/10 (9%/91%)	-
Age (years)	42 ± 9	47 ± 7	0.14
Body weight (Kg)	74.8 ± 8.5	67.5 ± 8.3	0.07
BMI (Kg/m^2)	28.1 ± 2.3	26.2 ± 2.8	0.12
EBMIL (%)	96.4 ± 2.8	98.5 ± 3.5	0.16
HOMA-IR	1.3 ± 0.6	1.2 ± 0.4	0.55
HOMA-β (%)	107.1 ± 25.6	119.4 ± 73.2	0.63
Post-operative (years)	3.8 ± 2.1	4.4 ± 1.7	0.47
BPL length (cm)	88 ± 21	200	**0.0001**

Data presented as means ± SD or number (%) as appropriate. p-values for the difference between patients submitted to classic RYGB and long BPL RYGB. Statically significant p-values are presented in bold.

At the time of surgery there were no differences in body weight, BMI and HOMA-IR between the two studied groups (Table 5). Both groups presented a significantly lower BMI at the time of the MMTT as compared to before the surgery (p=0.02 and p=0.03 for classic BPL RYGB and the long BPL RYGB, respectively) (Table 6). HOMA-IR was also significantly lower at the time of the MMTT when compared to before the surgery but only in the long BPL RYGB group (p=0.01) (Table 6).

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Table 5. Body weight, BMI, HOMA-IR and HOMA-%B of subjects submitted to classic and the long BPL RYGB at the time of the surgery.

	Classic BPL	Long BPL	p-value
Body weight (Kg)	111.4 ± 15.6	104.9 ± 14.0	0.34
BMI (Kg/m^2)	41.8 ± 3.5	40.6 ± 3.0	0.40
HOMA-IR	3.9 ± 2.1	4.8 ± 2.8	0.51
HOMA-β (%)	281.0 ± 158.4	173.8 ± 68.5	0.10

Data presented as means ± SD. p-values for the difference between patients submitted to classic RYGB and long BPL RYGB.

Table 6. Comparison between BMI, HOMA-IR and HOMA-%B at the time of the surgery (pre-op.) and at the time of the mixed-meal (post-op) in subjects submitted to classic and the long BPL RYGB.

	Classic BPL		p-values	Long BPL		p-values
	Pre-op.	Post-op.		Pre-op.	Post-op.	
BMI (Kg/m^2)	41.8 ± 3.5	28.1 ± 2.3	**0.02**	40.6 ± 3.0	26.2 ± 2.8	**0.03**
HOMA-IR	3.9 ± 2.1	1.3 ± 0.6	0.27	4.8 ± 2.8	1.2 ± 0.4	**0.01**
HOMA-β (%)	281.0 ± 158.4	107.1 ± 25.6	0.29	173.8 ± 68.5	119.4 ± 73.2	0.26

Data presented as means ± SD. p-values for the difference between pre- and post-operative values in patients submitted to classic RYGB and long BPL RYGB. Statically significant p-values are presented in bold.

2. Vital signs

Baseline HR was not significantly different between the groups, however SBP and DBP were significantly higher in the long BPL RYGB group when compared to the classic RYGB group (p=0.04) (Table 7, Figure 7 A, B and C). There was no increase in HR over 10 beats per minute in the first hour after the meal (t=15 minutes) when compared to baseline values [30] and none of the patients included in the study depicted a Sigstad's score compatible with dumping syndrome during the MMTT.

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Table 7. Fasting heart rate, systolic and diastolic blood pressure in subjects previously submitted to classic BPL and the long BPL RYGB.

	Classic BPL	Long BPL	p-value
HR (bpm)	65 ± 13	64 ± 9	0.83
SBP (mmHg)	118.6 ± 15.0	137.3 ± 22.1	**0.04**
DBP (mmHg)	66.9 ± 10.2	80.0 ± 15.2	**0.04**

Data presented as means ± SD. p-values for the difference between subjects previously submitted to classic BPL and long BPL RYGB. Statically significant p-values are presented in bold.
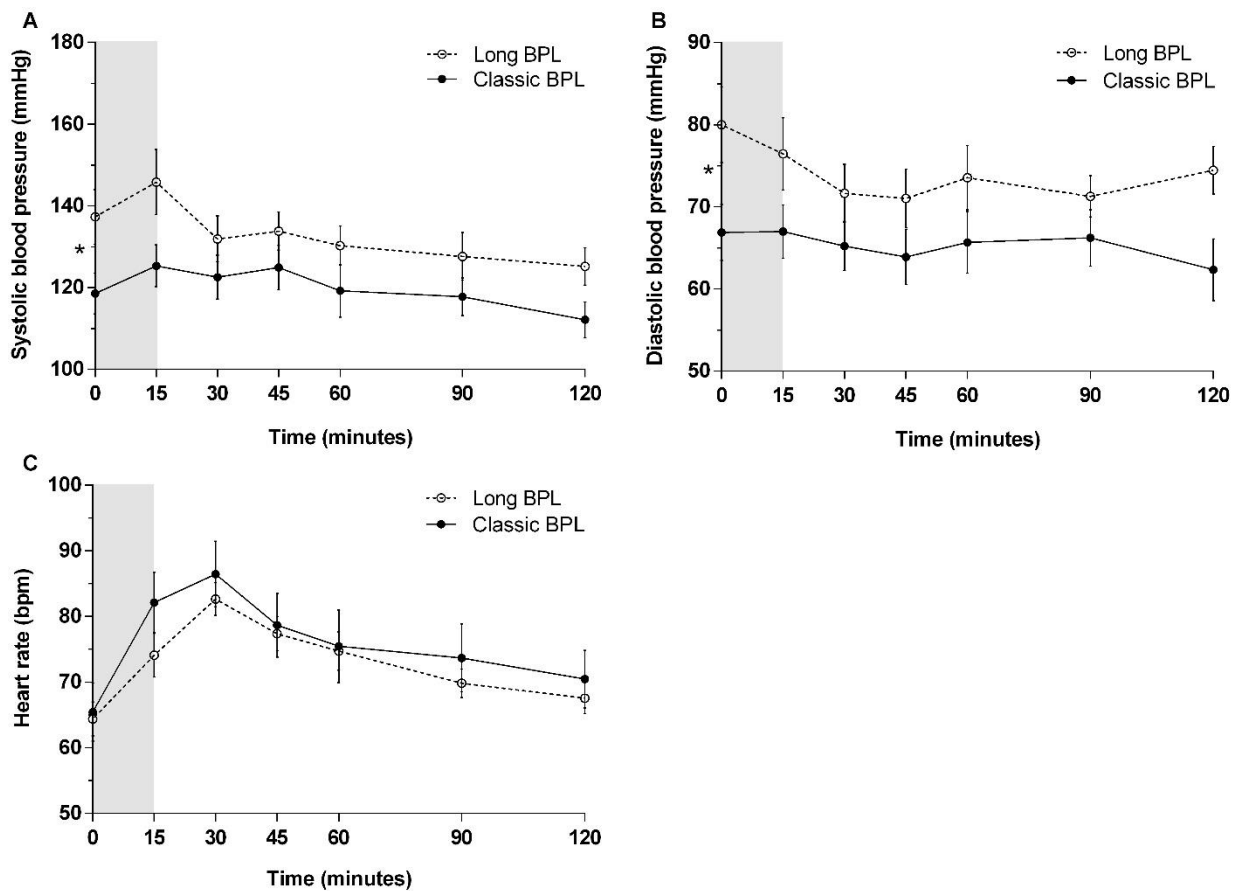
Figure 7. Systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C) in subjects previously submitted to classic BPL and the long BPL RYGB during the mixed-meal study. Data presented as mean ± SEM. *p<0.05.

3. Hormone levels

Fasting glucose, pancreatic and gut hormones levels are shown in Table 8. There were no significant differences between the groups for fasting glucose, pancreatic and gut hormones levels, except for GLP-1 and NT levels which were significantly higher in the long BPL RYGB patients (p=0.01 and p=0.02, respectively). The long BPL RYGB group

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also depicted higher fasting concentrations of TBA, although not statistically significant (p=0.06).

Table 8. Fasting glucose and hormone concentrations in subjects submitted to classic and the long BPL RYGB.

	Classic BPL	Long BPL	p-value
Glucose (mmol/L)	5.1 ± 0.3	4.8 ± 0.5	0.21
GLP-1 (pmol/L)	12.9 ± 7.2	24.8 ± 10.8	**0.01**
GIP (pmol/L)	7.3 ± 2.6	9.5 ± 4.4	0.20
Glucagon (pmol/L)	6.4 ± 2.9	8.4 ± 3.9	0.22
Insulin (pmol/L)	46.0 ± 18.4	41.0 ± 15.7	0.52
C-peptide (pmol/L)	557.8 ± 114.8	484.4 ± 96.9	0.14
PP (pmol/L)	5.6 ± 3.1	7.6 ± 5.8	0.35
PYY (pmol/L)	12.0 ± 7.8	7.4 ± 6.3	0.16
NT (pmol/L)	8.7 ± 5.2	16.5 ± 8.0	**0.02**
TBA (µmol/L)	2.0 ± 0.5	2.8 ± 1.3	0.06

Data presented as means ± SD. p-values for the difference between subjects previously submitted to classic BPL and the long BPL RYGB. Statically significant p-values are presented in bold.

Peak concentrations of the analysed hormones occurred between t=15 and t=45 minutes. Significant differences for the area under the curve (AUC) were observed between the two groups for GLP-1 and NT, being higher in the long BPL RYGB group (Table 9).

Table 9. Peak concentrations and T-AUC of all parameters measured in subjects previously submitted to classic BPL and the long BPL RYGB.

		Classic BPL	Long BPL	p-value
Glucose	**(30') Peak** (mmol/L)	9.5 ± 0.6	9.3 ± 1.4	-
	T-AUC (pmol/L x min)	711 ± 57	735 ± 91	0.50
GLP-1	**(30') Peak** (pmol/L)	152 ± 54	188 ± 65	-
	T-AUC (pmol/L x min)	7801 ± 1817	9543 ± 3399	**0.01**
GIP	**(15'/30') Peak** (pmol/L)	72 ± 24	61 ± 22	-
	T-AUC (pmol/L x min)	4550 ± 1237	3983 ± 1279	0.12
Glucagon	**(30'/45') Peak** (pmol/L)	12 ± 3	15 ± 5	-
	T-AUC (pmol/L x min)	1269 ± 271	1554 ± 372	0.08
Insulin	**(30'/45') Peak** (pmol/L)	1725 ± 883	1196 ± 400	-
	T-AUC (pmol/L x min)	77061 ± 33655	60742 ± 17064	0.10

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C-peptide	**(45') Peak** (pmol/L)	4203 ± 1060	3700 ± 944	-
	T-AUC (pmol/L x min)	280239 ± 64250	255150 ± 50928	0.11
PP	**(15'/30') Peak** (pmol/L)	17 ± 8	14 ± 11	-
	T-AUC (pmol/L x min)	4118 ± 2220	4269 ± 2401	0.91
PYY	**(30'/45') Peak** (pmol/L)	42 ± 16	48 ± 45	-
	T-AUC (pmol/L x min)	3112 ± 703	3780 ± 2968	0.37
NT	**(30') Peak** (pmol/L)	156 ± 54	225 ± 96	-
	T-AUC (pmol/L x min)	12396 ± 3658	17111 ± 7806	**0.02**
TBA	**(30') Peak** (µmol/L)	4 ± 2	4 ± 2	-
	T-AUC (µmol/L x min)	415 ± 109	400 ± 116	0.91

Data presented as means ± SD. The time point at which the peak is reached is between parenthesis and written from the
left to the right for classic BPL and long BPL RYGB subjects, respectively. p-values for the difference in T-AUC in subjects
previously submitted to classic BPL and the long BPL RYGB during the mixed-meal test. Statically significant p-values
are presented in bold.

3.1. Plasma glucose

No differences were found for glucose levels between the two groups (Figure 8A), which
presented similar plasma glucose excursion curves with a peak 15 minutes. There was
no significant difference in the T-AUC between subjects previously submitted to classic
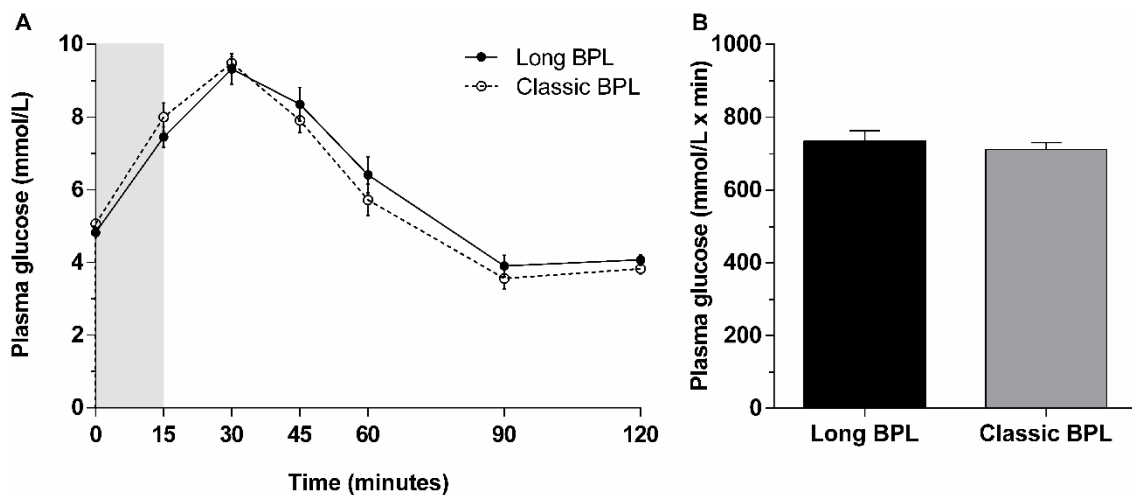BPL and the long BPL RYGB (Figure 8B).

Figure 8. Plasma glucose levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic
BPL and the long BPL RYGB. Data presented as mean ± SEM.

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3.2. Glucagon-like Peptide-1 (GLP-1)

In the long BLP limb group, GLP-1 levels were significantly higher at baseline (p=0.01) and at 45 minutes (t=45, p<0.05) (Figure 9A), as well as for T-AUC (11205 ± 3399 *vs* 7800 ± 1817 pmol/L x min, p=0.01) when compared to the classic BLP group (Figure 9B).

Figure 9. GLP-1 levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM. *p<0.05.

3.3. Glucose-dependent insulinotropic peptide (GIP)

The long BPL RYGB group displayed significantly lower GIP levels at 15 minutes (p<0.01) when compared to the classic BPL RYGB group (Fig. 10A). No differences were found between the groups in the T-AUC for GIP levels (Fig. 10B).

Figure 10. GIP levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM. **p<0.01.

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3.4. Glucagon

Glucagon concentrations were not significantly different between subjects undergoing classic BPL and long BPL RYGB. The secretion profiles were similar, with an increase in glucagon levels up to 30 minutes after meal time with subsequent stabilization until the end of the MMTT (Figure 11A). No significant differences were observed in the T-AUC between the groups (Figure 11B).
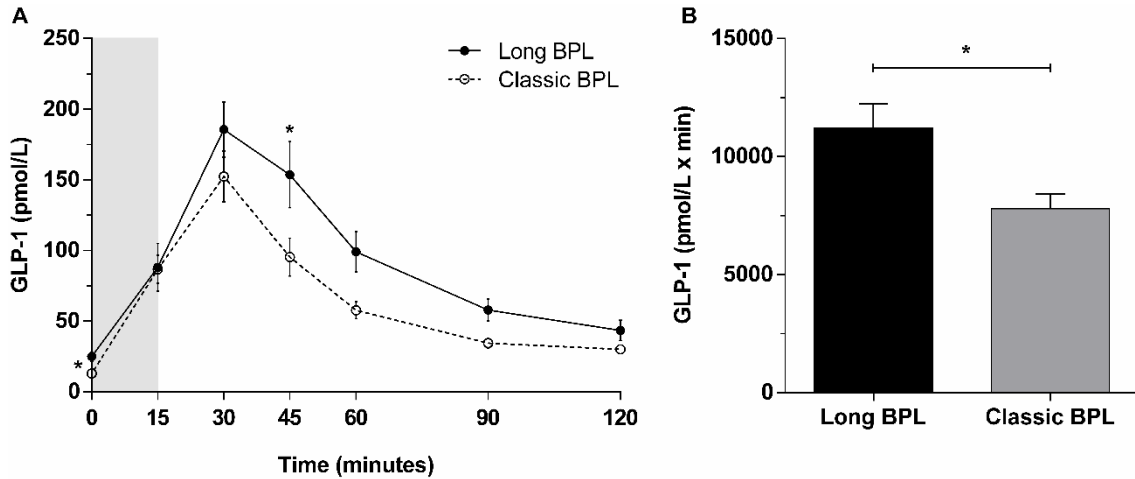

Figure 11. Glucagon levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM.

3.5. Insulin and C-peptide

Both hormone levels displayed a similar increase up to 30 min after the mixed-meal was ingested, with a subsequent decrease after minute t=45 (Figure 12 and 13A). In the long BPL RYGB group, both insulin and C-peptide levels were significantly lower at 30 minutes (p<0.001) after the MMTT as compared to the classic BPL RYGB procedure. No differences were found between the groups in the T-AUC for insulin and C-peptide levels (Figure 12 and 13B).

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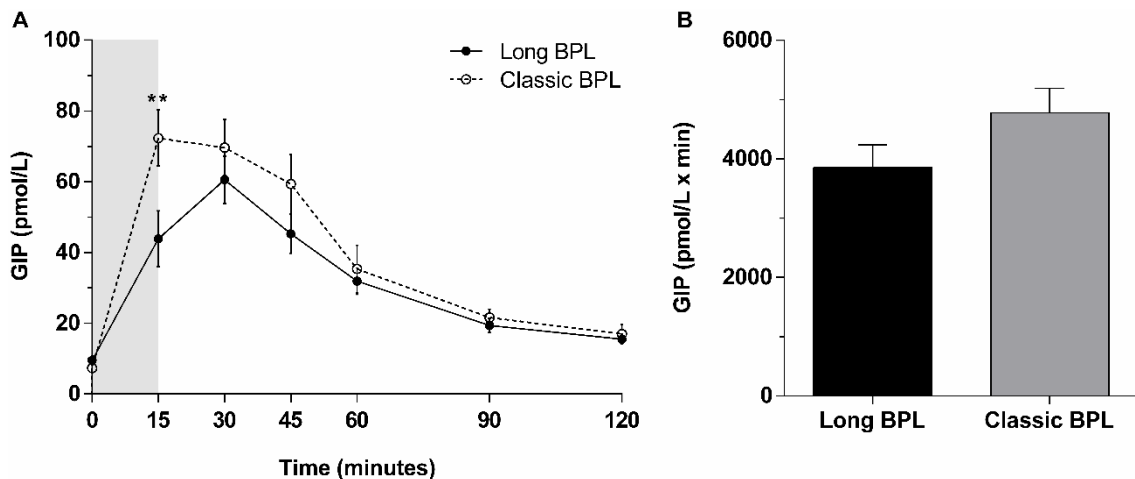biliopancreatic limb variant.

Figure 12. Insulin levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM. ***p<0.001.

Figure 13. C-peptide levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM. ***p<0.001.

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3.6. Pancreatic polypeptide (PP)

There were no significant differences in PP levels between the two experimental groups nor in the T-AUC (Fig. 14A and B).
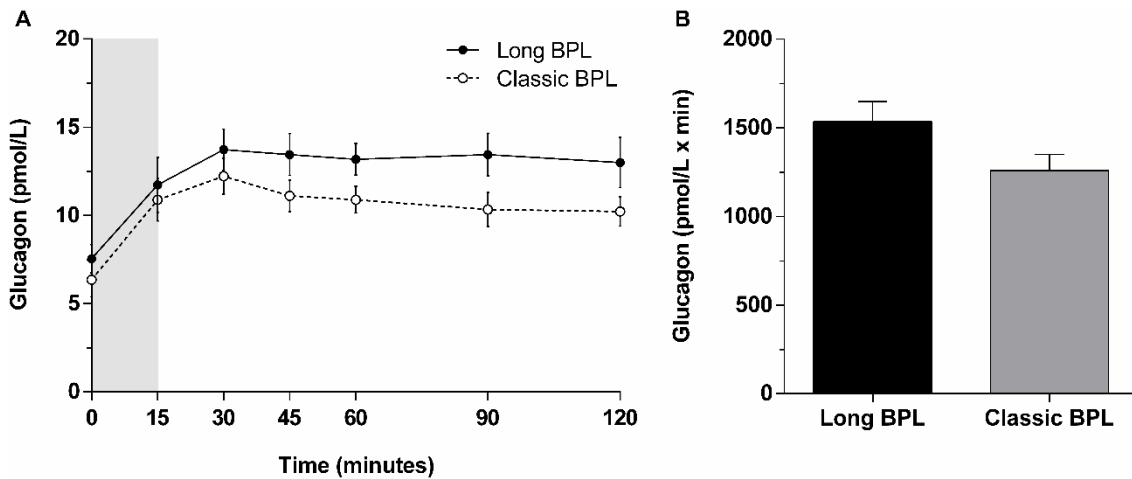

Figure 14. PP levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM.

3.7. Peptide YY (PYY)

There were no significant differences in PYY levels between the two experimental groups (Fig. 15A and B), although PYY levels were higher since t=30 minutes and until the end of the experiment in the long BPL RYGB group when compared to the classic RYGB group.

Figure 15. PYY levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM.

3.8.   Neurotensin

Neurotensin levels were significantly higher at fasting ($p=0.02$) and postprandially at minute $t=45$ ($p<0.05$) and $t=60$ ($p<0.01$) in the long BPL group (Figure 16A). The T-AUC (11205 ± 3399 *vs* 7800 ± 1817 pmol/L x min, $p=0.02$) was also significantly higher for the long BPL group (Figure 16B) when compared to the classic BPL group.
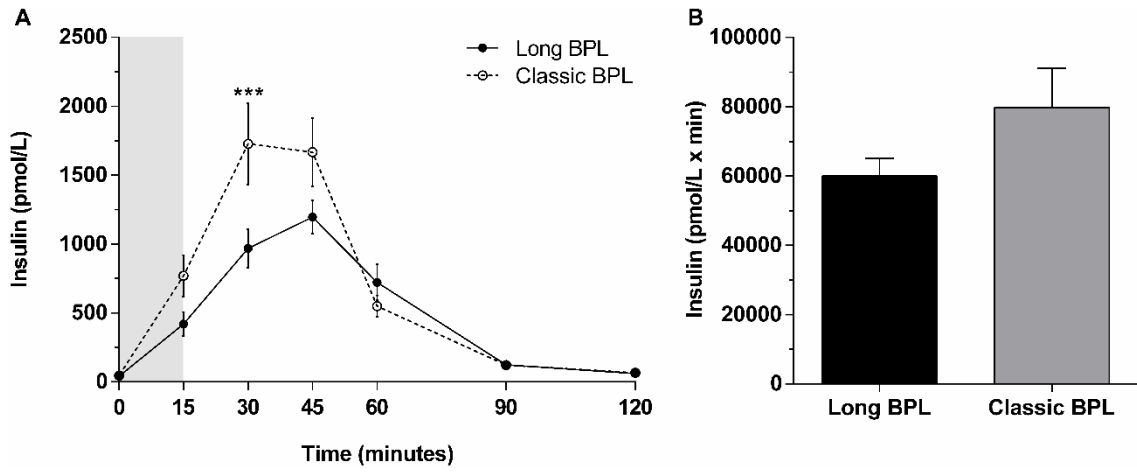

Figure 16. Neurotensin levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM. *p<0.05; **p<0.01.
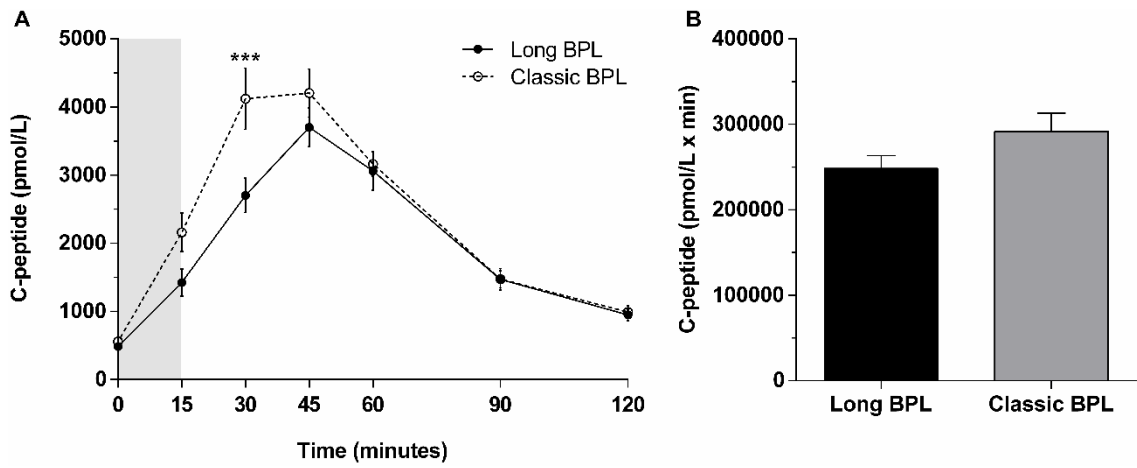
3.9.   Total bile acids (TBA)

There were no significant differences in total bile acids (TBA) levels between the two experimental groups nor in the T-AUC (Fig. 17A and B).
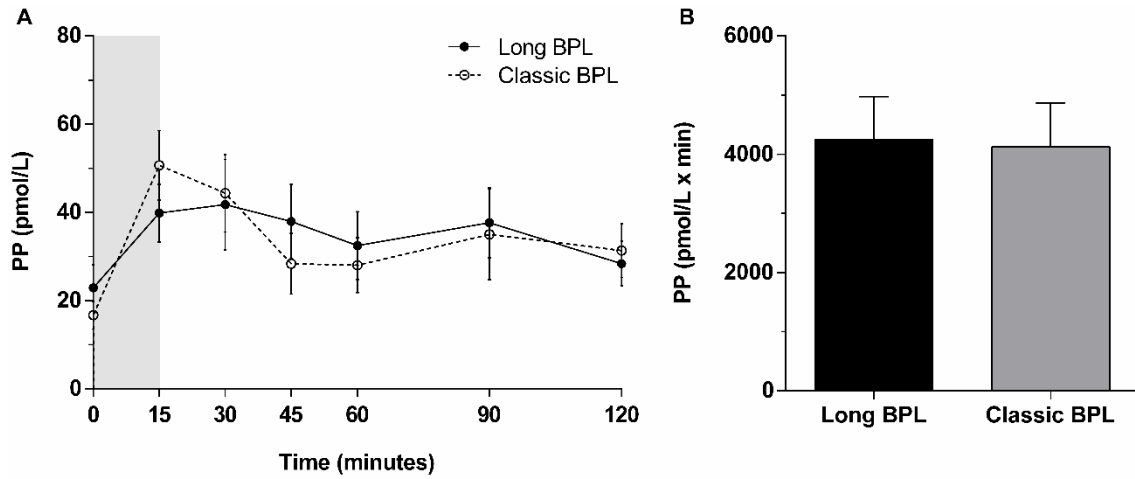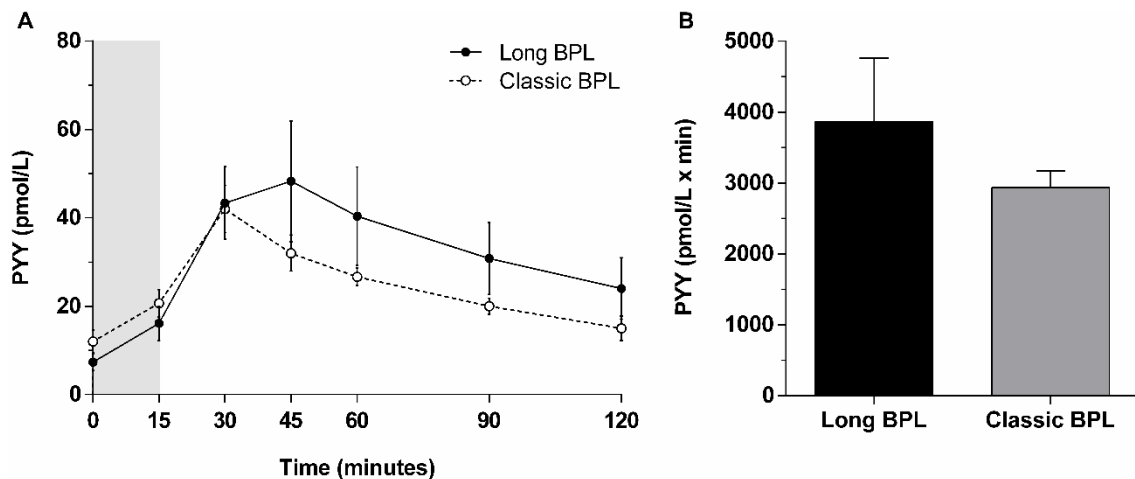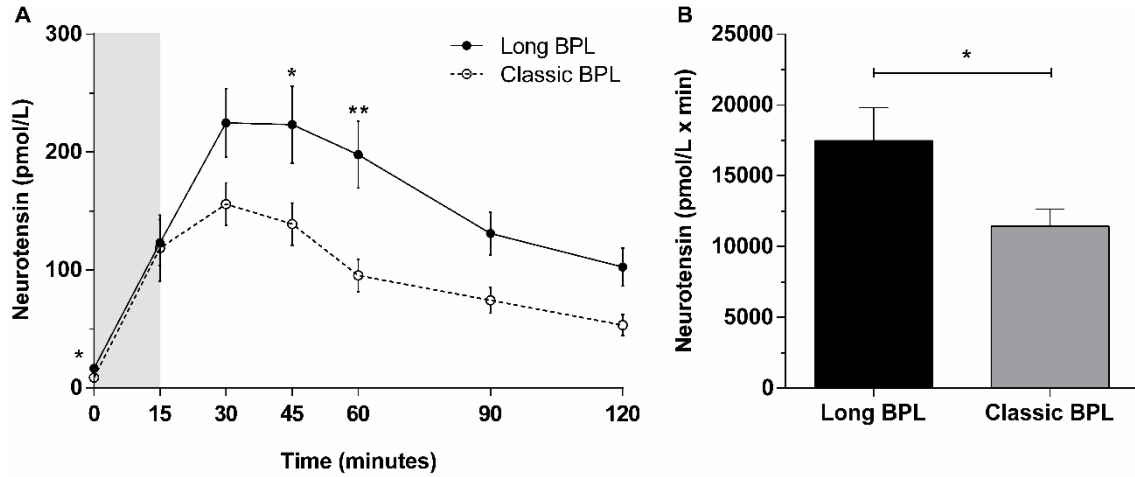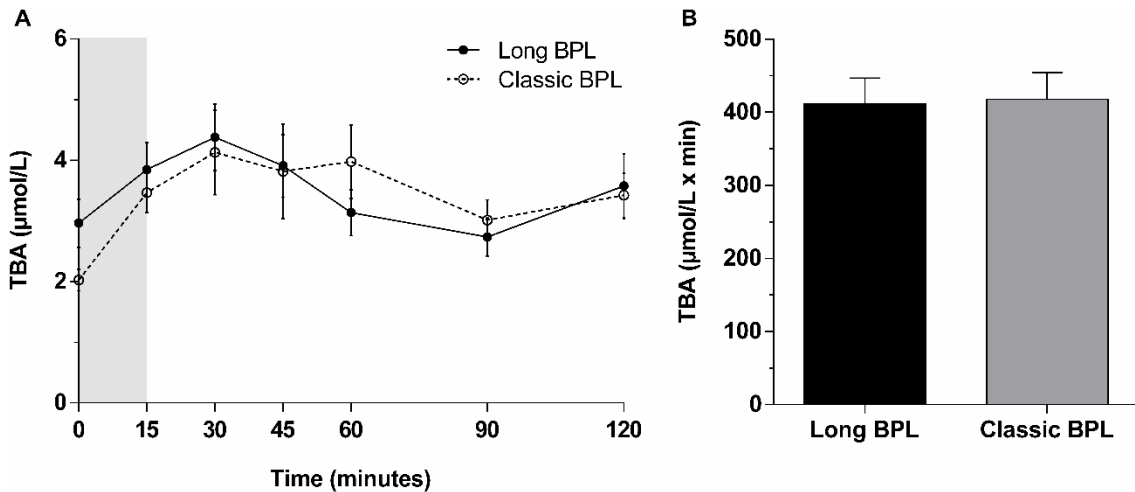

Figure 17. TBA levels (A) and T-AUC (B) during the mixed-meal test in subjects previously submitted to classic BPL and the long BPL RYGB. Data presented as mean ± SEM.

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DISCUSSION

RYGB induces a considerable rearrangement in GI tract anatomy resulting in sustained weight loss and a high rate of T2DM improvement or even clinical remission. The weight reduction and metabolic effects of bariatric surgery have been attributed in a considerable extent to alterations in GI and pancreatic hormone levels secreted by EEC [34].

This study was performed with the ultimate goal of establishing the link between the biliopancreatic limb length of RYGB and the after-meal GI endocrine response, in order to assess its potential contribution for glycaemic homeostasis.

To achieve this aim, formerly-obese and weight stable patients previously submitted to a classic or a longer BPL RYGB were recruited among a larger cohort of post-bariatric subjects. To warrant that the BPL length was the only variable, patients in two study groups were cross-matched to minimize the differences regarding age, BMI before and after the surgery, and post-operative %EBMIL. After RYGB surgery, there was a significant BMI decrease as compared to the pre-operative period in both groups, which demonstrates RYGB efficacy in producing sustained weight loss, as previously widely reported [96]. In addition, we herein demonstrate for the first time that in former-obese and non-diabetic patients, different RYGB BPL lengths induce a distinctive secretion of GI and pancreatic hormones when challenged by a mixed-meal. Moreover, this hormonal secretion profile could potentially contribute for the weight loss maintenance and glycaemic homeostasis.

Previous studies that sought to evaluate the effect of lengthen the BPL of RYGB, were mostly retrospective, focused on weight loss and resolution of obesity comorbidities, suggested a modest weight advantage for RYGB with longer BPL lengths of at least 150 cm [85]. *Kaska et al.* in 93 obese T2DM subjects submitted to RYGB, 51 patients to a short BPL RYGB (50-75cm) and 42 to an extended BPL RYGB (100-150cm) that were followed-up for 2-years, showed that a longer BPL was able to intensify the anti-diabetic effects of RYGB surgery [97]. *Nergaard et al.* comparing 187 patients either submitted to a 200 cm BPL with a 60cm AL (n = 93) or a 60-cm BPL with a 150-cm AL (n = 94) over 7 years, concluded that a longer BPL RYGB allowed better long-term weight loss, with similar co-morbidity resolution and surgical complication rates [98]. These studies

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contrast to other studies focusing on investigating the relationship between different BPL and AL lengths in total weight loss one year after surgery, which found no significant differences [99-101]. Consequently, there is no current consensus on the ideal RYGB limb lengths to optimize the clinical outcomes, while the impact of such modifications in GI and pancreatic hormone secretion profile had not been previously investigated.

Weight loss is long known to have profound effects on glucose metabolism in T2DM subjects [102]. Nevertheless, after RYGB glycaemic control greatly improves even before any major weight loss has occurred [103]. *Jørgensen et al.* reported the acute and long-term effects of RYGB on glucose metabolism in both T2DM and normal glucose tolerance subjects and found that glucose metabolism was radically altered within the first week and up to one-year after surgery in both patient groups [104].

In our study, subjects submitted either to classic or long BPL RYGB depicted similar fasting and postprandial plasma glucose excursion curves, which was relatively predictable considering that all subjects included in the study had no previous medical history of diabetes or glucose intolerance [105]. However, despite the fact that glucose levels were maintained within normal ranges [9], a distinctive profile of fasting and postprandial GI hormones was disclosed suggesting that this could harbour a potential mechanism an additional improvement in glycaemic control after long BPL RYGB if performed in diabetic patients [36, 106].

Insulin and glucagon are two pancreatic hormones known for having classic antagonistic effects on glucose homeostasis. Thus, after meal glucagon suppression along with the increase in glucose, GLP-1 and insulin excursions would be the predicted physiological response. Nevertheless, postprandial glucagon concentrations in subjects previously submitted to RYGB increases and although not statistically significant the glucagon excursion was higher after the long BPL RYGB as compared to the classical surgery. Recent evidence suggests that glucagon secretion may occur not only in the pancreatic α-cells but also in the intestinal L-cells through "pancreatic type processing" of proglucagon peptide as hypothesized by *Jørgensen et al.* and *Falkén et al.* [78, 104]. Considering that glucagon was shown to have additional peripheral effects, by stimulating thermogenesis and energy expenditure, this hormonal response could be one of the contributing mechanisms driving to weight loss maintenance and T2DM control after surgery [107].

Postprandial insulin and C-peptide levels, were lower in the long BPL RYGB group when compared to the classic RYGB group. Post-operative insulin and C-peptide in non-

diabetic obese subjects submitted to RYGB within the first year after surgery, were found to increase when compared to before or earlier after surgery [78, 108]. Given the cross-sectional design, our study does not allow to evaluate changes in insulin secretion response that occurred after surgery as compared to baseline. However, despite similar glucose excursion curves, insulin levels were higher in patients that underwent classic RYGB as compared to the long BPL RYGB group, suggesting that to maintain the same glucose levels more insulin is needed. A possible explanation could be that the long BPL RYGB results in a greater decrease in insulin resistance, however no significant differences in insulin resistance or pancreatic β-cell function as assessed by HOMA-IR and HOMA-β, were found between the two groups both before and after surgery at the time of the mixed-meal. Nevertheless, there was indeed a significant decrease in HOMA-IR after long BPL RYGB group when compared to the pre-operative time suggesting a greater improvement in insulin sensitivity. RYGB in non-diabetic patients has demonstrated to decrease HOMA-IR to values comparable to lean controls [108]. *Bojsen-Møller et al.* found that insulin secretion in response to oral glucose was markedly enhanced postoperatively in T2DM when compared to obese glucose-tolerant subjects. Moreover, as early as 1 week after RYGB there was an increase in hepatic insulin sensitivity but not in peripheral insulin sensitivity in both groups [109].

As incretin-producing cells are unevenly distributed along the human small gut, sectioning the proximal or distal small intestine in order to perform the classic or the long BPL RYGB, will result in a different proportion of EEC at the level of the gastro-enteric anastomosis [110]. Moreover, one could also speculate that this anatomical modification is likely to induce different GI hormone secretion profiles, as a RYGB procedure with a longer BPL allows earlier stimulation of EEC cells that in the normal anatomical disposition predominate in the more distal part of the small intestine, such as the ileum. GLP-1 producing cells density is known to be greater in the distal gut, whereas a RYGB procedure with shorter BPL results in the early arrival of nutrients to the jejunum, where in contrast GIP-producing cells predominate [72].

Fasting and postprandial GLP-1 levels were reported to increase after RYGB as compared to pre-operative values [75, 104]. This GLP-1 increase was attributed to L-cell stimulation of upon the early arrival of nutrients to the distal intestine in result of the anatomical rearrangement. This hypothesis is further corroborated by our results, which in addition to what was previously known, showed that longer BPL RYGB also result in significantly higher fasting and postprandial GLP-1 levels as compared to the classical

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procedure, suggesting that this modification of the surgical procedure is able to boost GLP-1 stimulation.

The increase in postprandial GLP-1 observed after the long BPL RYGB was not associated with a parallel rise in insulin or C-peptide levels, as GLP-1 pancreatic action is characterized by only potentiating insulin secretion in a glucose-dependent manner and no differences were observed in glucose levels. As GLP-1 has other physiological roles in addition to the incretin effect and contribution for the glycaemic control, central nervous system action, such as appetite control could be particularly beneficial for these patients [111].

Reports on post-prandial changes in GIP levels after RYGB surgery were largely inconsistent. Some studies showing an increase [112], no change, or a reduction in GIP levels [113], which could be attributed to differences in the RYGB limb lengths since shorter BP limbs were associated with a higher GIP response [114]. In our study, subjects submitted to the long BPL BGYR had lower postprandial GIP levels when compared to those that underwent the classic procedure. Again, these results support of the hypothesis that the GI hormone response is partially triggered by the arrival of nutrients to the small intestine and depends on the relative distribution of the EEC at the site where the first contact occurs, which for the classical RYGB is the proximal jejunum where a greater proportion of K-cells lies [115]. Being an incretin hormone, the insulin and C-peptide peaks observed shortly after GIP increase in the classic RYGB could be related to the effect of this hormone in the potentiation of the insulin secretion.

PYY is synthesized in L-cells of the most distal part of the small intestine, with GLP-1 and NT as a coproduct [116]. PYY post-prandial response is known to increase significantly within the first week after RYGB [117]. Similarly, cross-sectional studies that compared PYY levels before and after RYGB with nonsurgical controls found that patients who were at least 1 year post-RYGB had higher PYY levels [71, 118]. As observed for GLP-1, a longer BPL would also be expected to boost the PYY response, however no significant differences in fasting or postprandial PYY levels were found between the two experimental groups, although PYY levels were higher in long BPL RYGB group. PYY is thought to contribute for the long-term weight loss maintenance after RYGB, as *le Roux et al.* showed that patients losing more weight had significantly higher PYY levels as compared with those with an inferior weight loss response [117]. Additionally, *Korner et al.* found that the exaggerated postprandial PYY response in weight-stable individuals' 35±5 months after RYGB was associated with a greater weight reduction and to the ability of weight loss maintenance after surgery [118]. *Svane et al.*

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found that GLP-1 and PYY blockade increased food intake in patients previously submitted to RYGB, supporting that these hormones play a role in decreased food intake postoperatively [119]. Therefore, PYY may be directly involved in weight loss regulation and indirectly involved in glycaemic control by promoting weight maintenance.

PP secretion occurs after meals and is predominantly stimulated by proteins. PP is known to regulate exocrine and endocrine pancreatic secretion via central mechanisms [120]. In our study, no significant differences in PP levels between the two experimental groups. Unfortunately, very little is known about the effect of bariatric surgery upon PP secretion or whether PP has any preponderant role in mediating the surgical outcomes. *Nannipieri et al.* found that PP response to a MMTT, 15 days and 1 year after RYGB in T2DM patients, was decreased when compared to pre-surgical values [121]. *Schrumpf et al.* assessed the PP secretion in response to a mixed-meal before and after RYGB and observed a significant increase in PP levels in response to meal intake both before and after operation, thus concluding that the distal part of the stomach and the duodenum play no role in the early-phase of meal-stimulated PP release and only a minor role in the late-phase of meal-stimulated PP release [122]. H*oldstock et al.* found that postprandial PP increased significantly in obese, RYGB operated with a mean postoperative time of 43 months, age-matched lean and young lean subjects, although in the RYGB group PP tended to increase to a lesser extent [123]. As both RYGB procedures bypassed similarly the duodenum, this could be a reason for not observing any differences in PP levels between the groups.

Fasting and postprandial NT levels were significantly higher in the long BPL group, which could also be accounted by the early arrival of nutrients to a more distal part of the small intestine. *Ratner et al.* using a rat model explored the NT plasma concentrations, NT expression throughout the GI tract after RYGB surgery and its influence in food intake using a NT receptor antagonist. Following RYGB, NT was found to be increased in the circulation and in the GI tract, while NT antagonism resulted in a transient increase in food intake in operated rats. The authors thus concluded that peripheral NT should be included among the appetite-regulating hormones [124]. In addition, *Grunddal et al.* showed that NT acts synergistically with GLP-1 and PYY to decrease palatable food intake and inhibit gastric emptying. Thus, NT is a major gut hormone deeply integrated with GLP-1 and PYY, which explains the similar secretion pattern of these hormones [125]. A cross-sectional study including RYGB-operated, lean and obese subjects showed that pre-prandial concentrations of pro-NT, a precursor for the mature but

unstable NT, were higher in the RYGB group, while the postprandial concentrations were higher in their lean and obese counterparts [123].

Despite not being considered classical endocrine mediators, bile acids were recently demonstrated to participate in glucose homeostasis and possibly contribute to the anti-diabetic effect of some bariatric surgery procedures. *Patti et al.* measured serum bile acid levels in a cross-sectional cohort of patients previously submitted to RYGB for obesity and in two groups of control subjects matched for preoperative and postoperative BMI, showing that levels were higher in the surgical group as compared to the other groups [77]. *Dirksen et al.* did not find any differences in fasting or postprandial TBA levels between lean control subjects and 1-year after RYGB regardless presenting good and poor weight loss [75]. Bile acids were demonstrated to induce GLP-1 secretion by activating G protein–coupled bile acid receptors TGR5 on the basolateral membrane of L-cells [126]. No significant differences were found in TBA levels between groups in our study, thus our results do not support for a role of TBA as a contributor for the additional GLP-1 response.

In summary, we sought to analyse some of the GI hormones involved in glucose homeostasis regulation that could contribute to the metabolic improvement associated with RYGB. The gut hormone secretion profile was known to be diverse depending on the type of GI tract anatomical rearrangement produced by RYGB. The main strength of this study was to be first report of the GI and pancreatic hormone secretion profile in weight stable, non-diabetic subjects previously submitted to RYGB with two different BPL lengths. In addition, the fasting and postprandial response to a mixed-meal test after classical RYGB and the long BPL RYGB of hormones that had not been extensively studied before, such as PP and NT, were here described for the first time in these patients. One of the limitations of this study was that despite having assessed a wide array of hormones, the focus was mostly on molecules secreted in the distal intestine, while hormones secreted in the proximal intestine, such as secretin and CCK, were not characterized. Secretin and CCK are known to be involved in a large number of biological actions within the GI tract, namely the stimulation of bile and pancreatic secretions, and gallbladder contraction [127, 128], which could help to explain changes in TBA levels. Somatostatin, a peptide hormone known to reduce production and secretion of insulin and glucagon, as well as inhibit exocrine secretions, is another hormone that would be interesting to measure once different profiles of insulin were found in subjects submitted to classic RYGB and to the long BPL RYGB variant [129]. Moreover, *Rigamonti et al.* suggested that somatostatin infusion inhibits release of PYY in morbid obese women

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[130], but the role overall of this peptide following bariatric surgery in the inhibition of pancreatic hormones and metabolic improvements is still unclear. Another limitation of the study was not having included a control group of non-diabetic subjects matched for gender, age and BMI. Yet, as GI and pancreatic hormone secretion profile is well characterized in healthy individuals [131], our research efforts were mainly concentrated in the analysis of the differences in the endocrine profile as a consequence of the modification of the RYGB BPL length.

In conclusion, the GI and pancreatic hormones secretion profile of subjects who underwent a long BPL RYGB as compared to the classical procedure is characterized by increased GLP-1 and NT postprandial, along with decreased insulin. These distinct hormonal profiles suggest that the RYGB surgical technique could be tailored to obtain a personalized endocrine response in order to optimize the bariatric surgery clinical outcomes.

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FUTURE PERSPECTIVES

Gut hormones and their analogues are promising candidates for obesity and T2DM treatment, due to their relatively safe profile with few non-specific side effects as compared with centrally acting drugs. This study findings further reinforce the therapeutic potential of gut hormones for obesity and T2DM management as a means to replicate using a pharmacological approach the effects that so far are seldom achieved without bariatric surgery.

Further understanding of the mechanisms involved in food intake regulation and improved glycaemic control after RYGB will allow not only to optimize the surgical technique in order to induce the most favourable hormone secretion profile to achieve improved clinical outcomes, but also create the opportunity for novel, more effective and less invasive gut hormone based pharmacological approaches to be developed.

Our future plans include to widen this study by evaluating the changes in GI hormone profile before surgery and at 3 months' intervals until weight stabilization, in order to gain further insight into the endocrine mechanisms underlying the weight loss and anti-diabetic effects, as well as to determine the impact of RYGB BPL length on these clinical outcomes. Moreover, as was shown to RYGB improve glucose tolerance within days after surgery before significant weigh loss has occurred, we would like to evaluate the relative contribution of postprandial insulin clearance in the metabolic outcomes of the two surgical procedures.

By performing this comprehensive assessment, the putative contribution of these hormonal factors in active weight loss, weight maintenance and glycemic control is likely to be disclosed.

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