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"Efficacy of Lisosan G (fermented wheat) on post-prandial hypoglycemia after bariatric surgery"

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Abstract

Introduction: post-bariatric hypoglycemia (PBH) is considered a chronic complication after bariatric surgery that increase cardiovascular risk. Changes in gut microbiota and a dysregulated inflammatory response to a glucose load seems to be the conditions favoring the development of PBH.

Lisosan G (LG) is a fermented powder obtained from whole grains (Triticum aestivum), rich in polyphenols and flavonoids with antioxidant, anti-inflammatory and prebiotic properties.

Aims: The aims of the study were 1) to evaluate the effectiveness of using LG added to the diet in reducing PBH events in patients undergoing RYGB, 2) investigate the mechanism by which LG acts on the gut-pancreas axis.

Methods: Twenty patients self-reporting symptoms/signs of PBH, who had undergone gastric bypass between 2015 and 2018, were enrolled. At baseline, patients underwent clinical examination, blood test and 4-hour oral glucose load test (OGTT). After that, the patients were kept on a free diet with 15 days of continuous glucose monitoring (CGM). The CGM was then repeated for another 15 days, adding LG to the same dietary regimen (5 g LG-powder, bid). At the end of the treatment period, patients repeated the 4-hours OGTT and blood test. Plasma insulin and C-peptide were measured by electrochemiluminescence on a Cobas e411 instrument. Plasma total glucose-like peptide-1 (GLP-1) concentrations were measured by ELISA (Millipore). Insulin sensitivity was assessed by the oral glucose insulin sensitivity index (OGIS).

PBH was defined as a plasma glucose level of $\leq$ 60 mg/dl in presence of typical symptoms.

Results: a marked reduction in PBH episodes recorded by CGM was observed after LG administration (6.5 [5-11] vs 2.5 [2-3], p= 0.009), as well as a reduction in the overall length of hypoglycemia (410 [129-633] vs 39 [20-89], minutes, p=0.003). During OGTT, a marked increase in the blood glucose nadir (44 ± 11 vs 56 ± 10, mg/dl, p= 0.038) was found after LG treatment. Conversely, no difference were observed in fasting glycemia, in the time-to-nadir, as well as in the blood glucose zenith nor the time-to-zenith. After treatment, the peak of GLP-1 was attenuated and total GLP-1 AUC significantly decrease (7.6 ± 4.1 vs 6.5 ± 3.8, nmol/L*min, p= 0.043), as well as potentiation factor ratio (1.5 ± 0.5 vs 0.8 ± 0.4, p= 0.038) and total insulin AUC (57±12 vs 49±9, nmol/m^2, p= 0.041).

Conclusion: LG is effective on reducing frequency, overall duration and severity of PBH episodes. The main mechanism by which LG acts appears to be the attenuation of GLP-1 peak and, consequently, the second phase of insulin secretion in response to the glucose load. Further specific studies are necessary to evaluate the effects of LG on the microbiota and inflammation to better understand the mechanisms of action of this precious supplement.

1. HYPOGLYCEMIA

1.1 Definition of hypoglycemia

Hypoglycemia is a clinical condition defined by the coexistence of three elements which together constitute the so-called Whipple Triad:

- Symptoms/signs of hypoglycemia
- Reduced plasma glucose concentration
- Resolution of symptoms/signs following an increase in blood sugar

The clinical manifestations of hypoglycemia are nonspecific and it is not possible to establish a single glycemic threshold that uniquely defines this pathologic condition, therefore the simultaneous presence of all components of the Whipple Triad must be demonstrated.

Normal fasting blood glucose values are between 70 and 99 mg/dL. The clinical manifestation of hypoglycemia generally appear for values below 60-55 mg/dL, while in subjects with recurrent episodes the threshold tend to drop [1].

1.2 Physiological mechanism of defense against hypoglycemia

In healthy subjects, when blood glucose levels fall, a series of counter-regulatory responses are enacted in order to maintain brain metabolism and to prevent hypoglycemic episodes, and the threshold for their activation is higher than that at which cognitive deficit occur. All of these counter-regulatory mechanisms include an interplay of hormones and neural signals to regulate the release of endogenous insulin, to increase hepatic glucose output, and to modify peripheral glucose utilization. Among the counter-regulatory mechanisms, the regulation of insulin production plays a major role. The decrease in insulin production occurs while the glucose level is in the low-normal range, for blood glucose levels of approximately 81 mg/dL [1-2]. Additional counter-regulatory measures occur once the glycaemia decrease beyond the physiologic range. Among the additional counter-regulatory mechanisms, pancreatic alpha cell secretion of glucagon is the next line of defense against hypoglycemia, which occurs for values around 68 mg/mL; in the absence of adequate levels of this hormone, the role of adrenergic response becomes relevant. On occasions the counter-regulatory mechanisms fail to resolve the hypoglycemia, further counter-regulatory measures are employed in the form of growth hormone and cortisol, especially in prolonged hypoglycemic state [2].

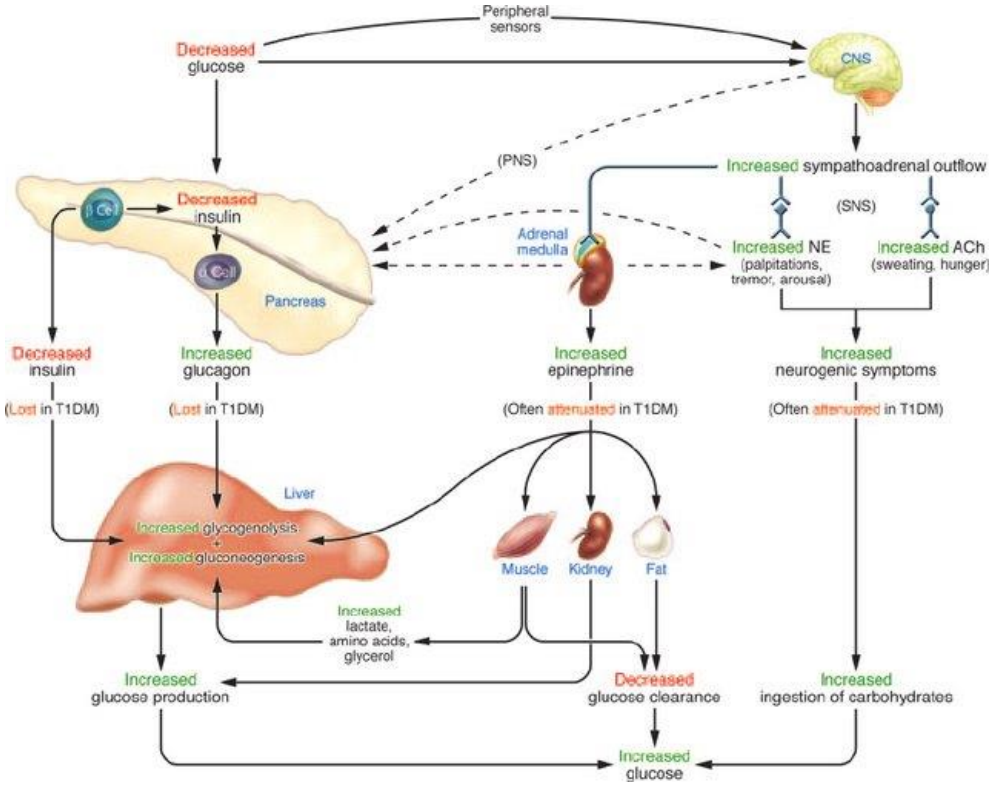


Figure 1. Physiological and behavioral defenses against hypoglycemia in humans. ACh, acetylcholine; NE, norepinephrine; PNS, parasympathetic nervous system; SNS, sympathetic nervous system. From Cryer PE. J Clin Invest 116:14701– 473, 2006.

1.3 Cardiovascular effects of hypoglycemia

During hypoglycemia, the central autonomic nervous system is stimulated as part of the counterregulatory response to hypoglycemia. The autonomic neural stimulation, accompanied by the raise in plasma catecholamines and the diminished energy supply due to the reduction of blood glucose, has important cardiovascular effects. In a clamp study in healthy participants in whom hypoglycemia was induced, a 12-fold increase in plasma epinephrine concentration was observed, with the consequent increased heart rate and cardiac stroke volume and decreased peripheral resistance [3], and acute hypoglycemia provokes a substantial rise in myocardial contractility and cardiac output [4].

Evidence that hypoglycemia can cause myocardial ischemia is accumulating. In a study of 19 subjects with type 2 diabetes and coronary heart disease, in which simultaneous continuous glucose monitoring and Holter ECG recording were applied, hypoglycemic episodes were associated with acute ischemic ECG abnormalities [5]; furthermore, a large retrospective study of 414 subjects presenting with severe hypoglycemia revealed newly diagnosed vascular disease in five patients [6].

Even more important than the association between hypoglycemia and cardiac ischemia is the possible induction of serious cardiac arrhythmias, which can lead to sudden death. Epinephrine induces hypokalemia, which may predispose to the development of a fatal arrhythmia [7]. A very frequent finding during hypoglycemia is QT prolongation, another strong risk factor for severe ventricular arrhythmia and sudden death [6].

Hypoglycemia decrease vascular endothelial function (measured by reactive hyperemia peripheral arterial tonometry) in patients free of abnormal glucose tolerance [8]. The decrease in vascular endothelial function correlated with increases in catecholamine levels at the time of hypoglycemia. The association between hypoglycemia, increased serum levels of biomarkers related to oxidative stress, and endothelial dysfunction is further supported by the finding that, in a recent study, flow mediated dilation was inversely correlated with the number of hypoglycemic events and the time spent in hypoglycemia in patients post bariatric surgery [9]. Both endothelial dysfunction and oxidative stress are important drivers of the atherosclerotic process and predict cardiovascular events [10].

1.4 Symptoms/signs of hypoglycemia

Symptoms of hypoglycemia can be divided into two categories:

- Autonomic
- Neuroglycopenic

The perception of the hypoglycemic state is mainly due to the appearance of neurogenic/autonomic symptoms, usually for blood glucose concentration of about 60 mg/dl. These can be adrenergic, such as palpitations, tachycardia, tremors, anxiety, or cholinergic, such as sweating, paranesthesia, hunger. Neuroglycopenic symptoms are a direct results of the brain's glucose deprivation and typically appear at concentrations of 50 mg/dl. They include weakness, dizziness, amnesia, lethargy, seizures, loss of consciousness up to coma [11].

1.5 Etiology of non-diabetic hypoglycemia

Historically, the causes of hypoglycemia in non-diabetic patients have been classified according to the onset of symptoms during the fasting or post-prandial phase, but for some etiology it is difficult to enter in a specific category. Therefore, the current recommendations envisage an approach based on the clinical characteristics of the patients, healthy or ill-appearing [12].

The most frequent causes in healthy individuals are:

- Insulinoma
- **Post-bariatric hypoglycemia**
- Autoimmune insulin syndrome
- Accidental or factitious hypoglycemia (from abuse of antidiabetic drugs)
- Family disorders (i.e insulin receptor mutations)

The main causes in ill-appearing are:

- Medicines or drugs (insulin, alcohol…)
- Critical health conditions (liver or kidney failure, sepsis…).
- Hormonal deficiencies (GH, adrenal insufficiency)
- IGF-2 paraneoplastic syndrome
- Severe malnutrition

2. BARIATRIC SURGERY

2.1 Introduction

Bariatric surgery is a branch of surgery primarily aimed at the treatment of obesity. Since its inception, over 50 years ago, it has undergone considerable changes and technical innovations, proving capable of reducing and often resolving comorbidities of obesity, guaranteeing sustained weight loss over time, improving quality of life and increasing survival of obese population [13].

One of the greatest innovations in the field of bariatric surgery was certainly the introduction, in 1994, of the laparoscopic technique. The first results on laparoscopic gastric bypass were excellent: at 60 months after surgery, about 80% of patients had maintained the loss of at least 50% of excess weight, the total comorbidities had been reduced by 86% and overall complications were less than 10% [14].

Conventional open bariatric surgery involves one large midline abdominal incision, while the laparoscopic counterpart is typically performed through 5 small incisions. In general, the introduction of the laparoscopic technique in bariatric surgery has reduced complications related to the surgical wound and accelerated patient healing. In fact, compared to the open modality, the minimally invasive approach is associated with reduced postoperative pain, a lower risk of wound infections and the development of incisional hernias and reduced mortality [15].

Currently, approximately 97% of surgeries are performed laparoscopically [16].

2.2 Indications and contraindications.

Classically, since the consensus conference of the American National Institute of Health in 1991, the central element on which the surgical indication is based in the obese patients is the body mass index (BMI). In particular there is an indication in the case of:

- BMI $\geq$ 40 kg/m^2
- BMI $\geq$ 35 Kg/m^2 and at least one comorbidity (type 2 diabetes mellitus, arterial hypertension, obstructive sleep apnea…).

The guidelines of the American Diabetes Association of 2015, as well as other international organizations involved in the treatment of diabetes, stated that bariatric surgery should be considered for adults with BMI $\geq$ 35 Kg/m^2 and T2DM, especially if the latter or other comorbidities are difficult to control with lifestyle interventions or medical therapy [17].
According to the guidelines of the S.I.C.O.B (Italian Society of Obesity surgery) of 2016, intervention is also permitted if:

- BMI between 30-35 Kg/m^2 in the presence of T2DM not controlled with medical therapy.
- BMI < 30 Kg/m^2 not controlled by medical therapy exclusively in the context of prospective controlled clinical studies.

The current indications refer to the severity of obesity and the potential reversibility of the clinical picture, associating the assessment of the BMI, characterized by the aforementioned limits, with the analysis of metabolic, functional and psychological parameters, in an overall balance between the risks and benefits for each single patient.

There are a number of absolute contraindications to bariatric surgery, some of which are common to any type of elective surgery [18].

- High intraoperative risk
- Reduced life expectancy due to cardiopulmonary or other organ failure, or inoperable or metastatic malignancy
- State of pregnancy
- Diagnosis of endocrine disease responsible for secondary obesity

- Not compensated psychiatric illnesses, including bipolar disorder, schizophrenia and psychosis, eating disorders (bulimia).
- Alcoholism and drug addiction

Relative temporary contraindications are:

- Impaired intellectual capacity of inability to understand the surgical procedure or the long - term behavioral changes necessary to ensure the success and safety of the procedure.
- Lack of ability, willingness, or motivation to fulfill post-operative behavioral guidance, which variably includes lifestyle modification, dietary supplementation and follow up.
- Anxiety disorder and/or depression: it is considered a negative predictive index for the outcome of bariatric surgery, but not a contraindication if associated with a supportive psychiatric program.
- Inadequate pharmacological treatment of pre-existing morbid endocrine conditions

2.3 Type of bariatric surgeries: the RYGB.

In the international literature and in the S.I.C.O.B register, the most represented operations are: gastric bypass, sleeve gastrectomy, gastric banding and biliopancreatic diversion. None of them can be considered a "universal" reference since the surgical choice must be linked to the characteristics of the patient, the surgeon's experience and the organizational level of the structure, the assessment of risk/benefit balance in the short-term and in the long term.
Traditionally, interventions are grouped into macro-categories in relation to their mechanism of action:

- Restrictive interventions: based on the reduction of the size of the stomach, which early induce the sense of satiety by decreasing the subsequent caloric intake.
- Malabsorptive interventions: provide for a variable modification of the anatomy of the gastrointestinal tract, such as to cause a certain degree of malabsorption of macronutrients.
- Mixed restrictive-malabsorptive interventions.

However, in recent years it has been observed that the mechanisms of action underlying bariatric surgery are complex and involve multiple neuroendocrine signals, which exert their effects at the

level of the central nervous system and peripheral organs. Therefore, this classification appears in some ways excessively simplistic.

Currently, the most commonly performed intervention globally is the RYGB, followed by sleeve gastrectomy, while in the USA the latter type has become the most frequent. In fact, in 2011 the interventions of RYGB were 35.7% and those of sleeve gastrectomy only 17.8% of the total, while in 2016 the numbers underwent a clear inversion, with 58.1% of sleeve gastrectomy against 18.7% of RYGB [19].

Also in Italy the number of sleeve gastrectomy interventions exceeded that of RYGB, with 9046 cases of the former against 2361 of the latter in 2017 [20].

RYGB involves the creation of a small gastric pouch, with a volume of about 30 ml, obtained by separating the cardia area from the rest of the stomach. Subsequently, the small intestine is dissected approximately 30-50 cm downstream of the ligament of Treitz. The distal stump (Roux loop) is transposed upwards and anastomosed to the gastric pouch, generally passing in front of the colic loops; the proximal stump (biliopancreatic loop) is reconnected with the small intestine about 100-150 cm downstream from the point of section, through a jejuno-jejunal anastomosis. The section of intestine between the anastomosis and the ileo-caecal valve is defined as the common canal: in this sector the chime and the biliopancreatic secretion converge (**Figure 2**).

The mechanism through which RYGB leads to weight loss are various, this being a mixed type of intervention (both restrictive and malabsorptive). In fact, the small neo-stomach can only contain a small amount of food (restrictive component), and at the same time the bypass of the stomach, duodenum and proximal jejunum occurs, causing a variable degree of fat malabsorption (malabsorptive component).

Furthermore, the fact that gastric and biliopancreatic secretions are diverted towards the distal tract of the jejunum causes complex neurohormonal and gut microbiota modifications, which influence the sense of hunger/satiety, insulin sensitivity and glucose homeostasis [21].

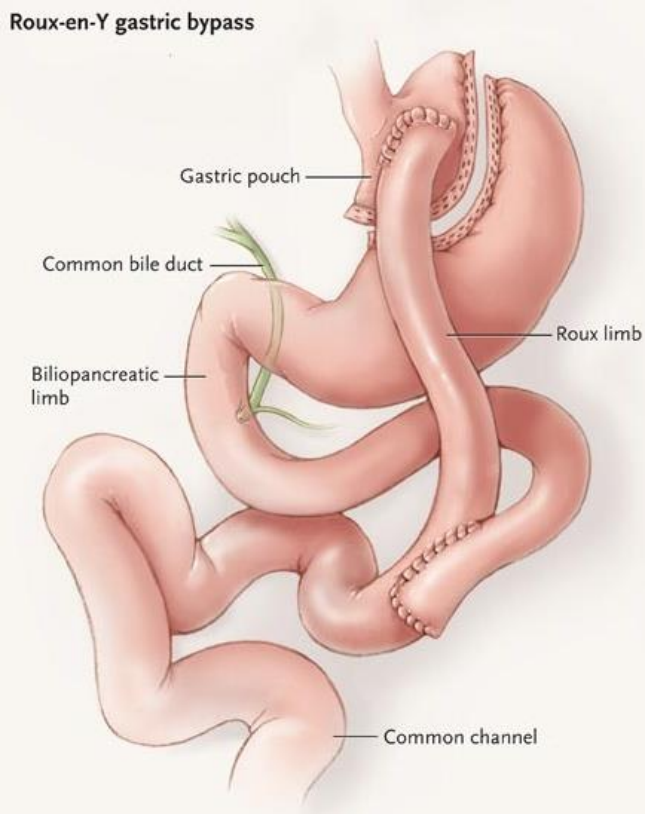


Figure 2. Roux en-Y gastric bypass. The gastric bypass procedure consists of a creation of a small (about 30 ml) thumb-sized pouch from the upper stomach, accompanied by bypass of the remaining stomach (about 400 ml). The stomach is totally divided into two separate parts (also with staples). The small intestine is divided approximately 45 cm (18 in) below the lower stomach outlet and is re-arranged into a Y-configuration, enabling outflow of food from the small upper stomach pouch via a "Roux limb".

3. POST-BARIATRIC HYPOGLYCEMIA (PBH)

Hypoglycemia represent a possible late complication of bariatric surgery: it occurs after a variable period of time from the surgery and typically the symptoms appear in the post-prandial phase [22]. The difficulty in characterizing this complication depends, both on why the reported symptomatology is highly nonspecific, and because following bariatric surgery, another complication may occur- the dumping syndrome- which could be part of a continuum with post-bariatric hypoglycemia.

The dumping syndrome represent a symptomatic phenomenon classically associated with the accelerated discharge of food from the stomach into the gut, due to the alteration of the gastrointestinal anatomy that is the basis of the bariatric surgery. Dumping syndrome can be early, within 1-hour after meal, as a result of an accelerated gastric emptying of hyperosmolar material, which draws fluid from the blood into the gut with consequent diarrhea, abdominal pain and

vasomotor activation such as palpitations, sweating, hypotension. Late dumping syndrome occurs 1–3 hours after a meal and is an incretin-driven hyperinsulinaemic response. This leads to reactive hypoglycemia, which presents with adrenergic and neuroglycopenic symptoms such as tremor, sweating, anxiety, fatigue, hunger and confusion and blurred vision. However, it is not unequivocally established that symptoms in these patients are strictly related to low blood levels of glucose as such, and one study showed that people with "late dumping syndrome" are more accurately recognized by their subjective (for example, perspiration or hunger) than objective (for example, nadir glucose values) symptoms during an oral glucose tolerance test [23].

In summary, PBH can be diagnosed when other causes of hypoglycemia have been ruled out if the following criteria are meet [24]:

1. History of post-prandial onset neuroglycopenic symptoms - between 1-3 hours after meal- in a patient who underwent bariatric surgery at least 6-12 months previously.
2. Plasma hypoglycemia documented at the onset of symptoms, with resolution of the same following an increase in blood glucose.
3. Absence of hypoglycemia after fasting for at least 12 hours.

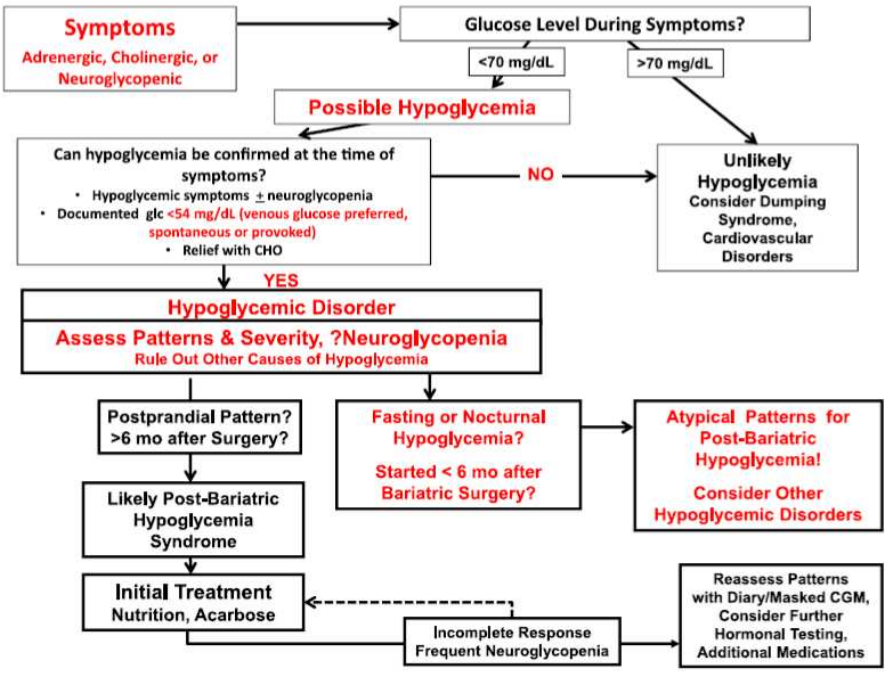


Figure 3. Diagnostic and therapeutic Flow chart for hypoglycemia. Modified from Ahmad Z et al. Prevalence of Dumping Syndrome After Laparoscopic Sleeve Gastrectomy and Comparison with Laparoscopic Roux-en-Y Gastric Bypass. Obes Surg. 2019

3.1 Epidemiology

One-three years after Roux-en-Y-gastric bypass, approximately 30% of patients develop mild to moderate PPHG symptoms, often resolving with dietary modifications [25].
A recent study analyzed the prevalence of hypoglycemia subjecting a cohort of patients to an oral glucose tolerance test 2 years after RYGB: reduced glucose levels (< 50 mg/dl) were found in 32.6% of individuals 2 hours after the load of 75 g of glucose. The two factors correlated with an increased risk of PPHG were the younger age at the time of the surgery and the lower blood glucose values at OGTT pre-surgery [26].
A few years earlier, Pigeyre et al reported a 10.4% of PBH following RYGB one year after surgery. The difference between the results of these two studies could be explained by the gradual increase over time in the frequency of PBH [27].
The difficulty in defining the real prevalence of PBH could be attributed to the differences in the definition of the phenomenon, in the selection criteria of the patients, and in the duration of the follow up. In addition, thanks to the use of continuous blood glucose monitoring devices (CGM), it was possible to observe very low blood glucose levels even in completely asymptomatic patients [28].

3.2 Risk factors for PBH

Risk factors for PBH identified during retrospective epidemiologic studies include female sex, younger age, no diagnosis of diabetes pre-surgery, history positive for hypoglycemic episodes pre-surgery, lower pre-surgery glycated hemoglobin (HbA1C), and greater excess weight loss postoperatively [29].
Interestingly, our previous study demonstrated that individuals who have PBH had lower glucose levels on OGTT pre-surgery [30].
However, differences between those who develop hypoglycemia and those who do not are of relatively small magnitude, and there are no defined diagnostic thresholds at present.
Some authors advise to consider EKG during the preoperative evaluation in order to investigate on the presence of a long QT interval, given the association between long QT caused by mutations in genes KCNQ1 and KCNH1 and postprandial insulin hypersecretion [31].

3.3 Bariatric surgery and glycemic homeostasis

An aspect common to bariatric surgery is the acceleration of gastric emptying following the modification of gastrointestinal anatomy and of gastric innervation.

Specifically, meals are more rapidly transferred from the gastric pouch to the distal intestine, with the consequent higher loads of undigested carbohydrates and the accelerated absorption of glucose into the bloodstream.

It seems that, as a results of RYGB, there are anatomic adaptations with the increase of specific transporters of glucose, which cause a greater absorption [32]. Protein digestion and amino acid absorption also appear to be greater after RYGB: this could help explain the high serum levels of GLP-1 after meal ingestion, since the aminoacid glutamine is a factor that stimulates its secretion [33].

Changes in glucose clearance have been postulated. It has been shown that dietary calorie restriction, even for very short period, is able to increase insulin secretion [34].

Furthermore, following surgery-induced weight loss, it is clearly demonstrated that both diabetic and non-diabetic subjects have a significant improvement in hepatic and peripheral sensitivity to insulin [35]. The latter is detectable in both muscle and adipose tissue, and it is associated with modification of molecular mediators, restoring the activation of kinase β-activity and enhancing insulin signaling [36].

Summarizing these concepts, in 2005 Gumbs et al in a review proposed that bariatric surgery leads to improved glucose metabolism and insulin resistance through caloric restriction in the short-term period and weight loss in the long-term, proving that substantially the short-term improvement (1-3 months following surgery) in insulin resistance precede significant weight loss in both restrictive and bypass operations, while the improvement in insulin resistance 1-year following weight loss surgery correlate with weight loss but not with BMI [37].

Bariatric surgery was observed to have marked effects on post-prandial glycemic profile.

After surgery, due to the more rapid transport of ingested carbohydrates to the intestinal absorbent surface, a positive deflection of blood glucose which exceeds the pre-surgery peak levels is observed [38]. However, glucose clearance is also increased following surgery: despite the high blood glucose peaks elicited by OGTT or MMTT (Mixed Meal Tolerance Test), an improvement in glucose tolerance is noted, with a reduction in area under curve (AUC). Indeed, it is common for patients undergoing RYGB to show significantly lower postprandial blood glucose nadir than fasting levels [39-40].

3.4 Bariatric surgery and the incretinic effect: role of GLP-1.

Glucagon-like peptide-1 (GLP-1) is a 30- or 31-amino-acid-long peptide hormone deriving from the tissue-specific posttranslational processing of the proglucagon peptide. It is produced and secreted by intestinal enteroendocrine L-cells. Among the numerous metabolic effects of GLP-1 are the glucose-dependent stimulation of insulin secretion, decrease of gastric emptying, inhibition of food intake, increase of natriuresis and diuresis, and modulation of rodent β-cell proliferation [41].

In subjects with a normal gastrointestinal tract postprandial GLP-1 levels increase approximately twice the baseline concentration, while after bariatric surgery post-prandial levels of GLP-1 increase by 10 times [42]. This phenomenon is probably caused by the rapid arrival of the chyme in the distal gut, since it has been seen that the release of GLP-1 is more sensitive to the rhythm of entry into the intestine rather than the amount of nutrients per se [43]. In fact, non-diabetic patients underwent bariatric surgery have the same insulin secretion in response to an intravenous glucose stimulus compared to controls of comparable weight, while the insulin-response to an oral glucose load is significantly increase in surgery group [44].

In addition, after bariatric surgery, there is an increasing density along the distal part of the gut of L-type enteroendocrine cells, responsible for the release of GLP-1 [41].

3.5 Pathogenetic hypothesis of PBH.

The first hypothesis involved an abnormal growth of pancreatic β-cells, with consequent insulin hypersecretion. In an attempt to mitigate this effect, patients with sever PBH were recommended to undergo partial pancreatectomy: microscopy examination of surgical specimens described hyperplasia and increased cells volume, with similar aspect similar to a nesidioblastosis [45]. The possibility of islet cells growth was supported by the typical 1-2 years latency observed between bariatric surgery and the development of the hypoglycemic symptoms. However, subsequent analysis of the same samples by other researchers did not confirm the histological picture of nesidioblastosis [46]. Furthermore, the majority of patients undergoing partial pancreatectomy still showed recurrent symptoms, demonstrating that the intrinsic β-cellular hyperfunction is not the predominant mechanism underlying PBH [47].

A recent study corroborated this theory by analyzing a cohort of patients with severe PBH and demonstrating substantial improvement following intervention reconversion [48]. The patients underwent three mixed meal tests (MMTs): the first was done before reversal surgery, the second via

gastrostomy tube in the excluded stomach in the setting of RYGB, and the third several months after RYGB reversal.

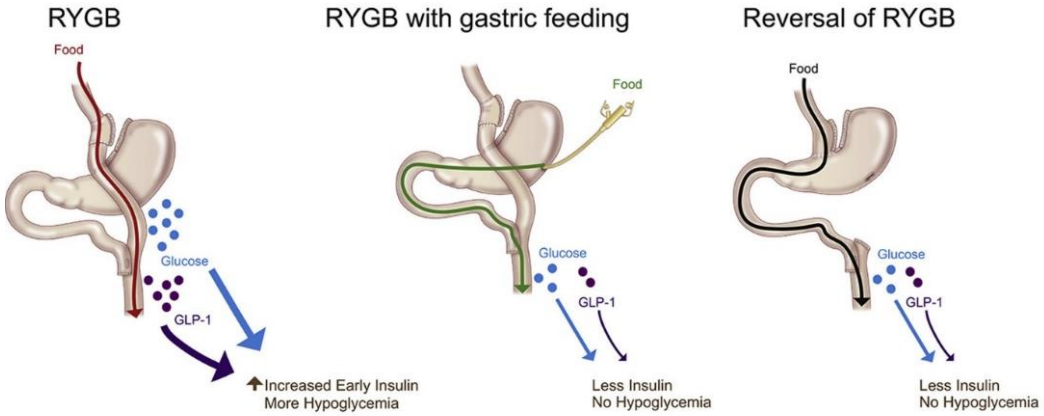


Figure 4a. Main differences in glucose absorption and GLP-1, insulin secretion before reversal surgery, via gastrostomy tube and after reversal surgery. Modified from Davis DB et al. Roux en Y gastric bypass hypoglycemia resolves with gastric feeding or reversal: Confirming a non-pancreatic etiology. Mol Metab. 2018;9:15-2.

A noticeable reduction in post-prandial insulin and GLP-1 levels was observed in MTTs performed with G-tube and after reversal surgery, with the consequent improvement in glucose nadir: it was therefore hypothesized that the mechanism responsible for hypoglycemia was the anatomy alteration resulting in altered glucose, gut, and pancreatic hormone levels and decreased insulin clearance, rather than inherent β-cell hyperplasia or hyperfunction (**Figure 4a-4b**).

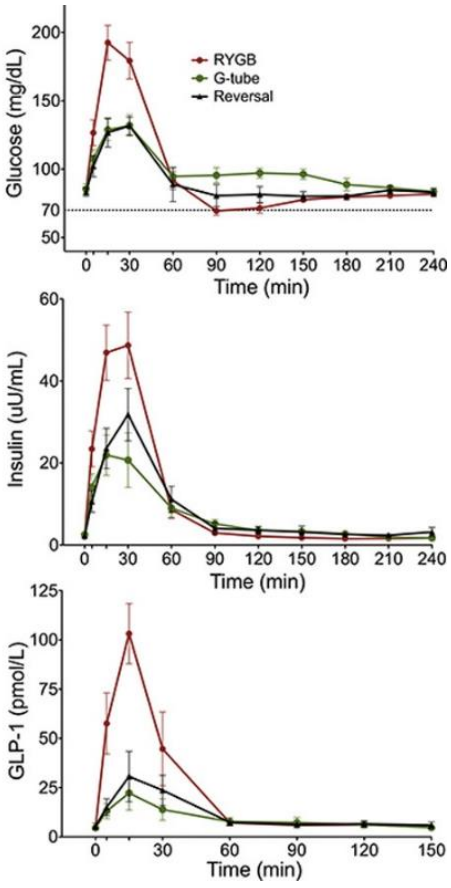


Figure 4b. Main differences in plasma glucose, GLP-1 and insulinemia before reversal surgery, via gastrostomy tube and after reversal surgery. Modified from Davis DB et al. Roux en Y gastric bypass hypoglycemia resolves with gastric feeding or reversal: Confirming a non-pancreatic etiology. Mol Metab.2018;9:15-2.

It is plausible to think that PBH reflects the extreme effects of RYGB on blood glucose in a subset of susceptible individuals. Indeed, patients with PBH have higher zenith of glucose, insulin and GLP-1 during MMTs compared to individuals without PBH underwent bariatric surgery (**Figure 5**).

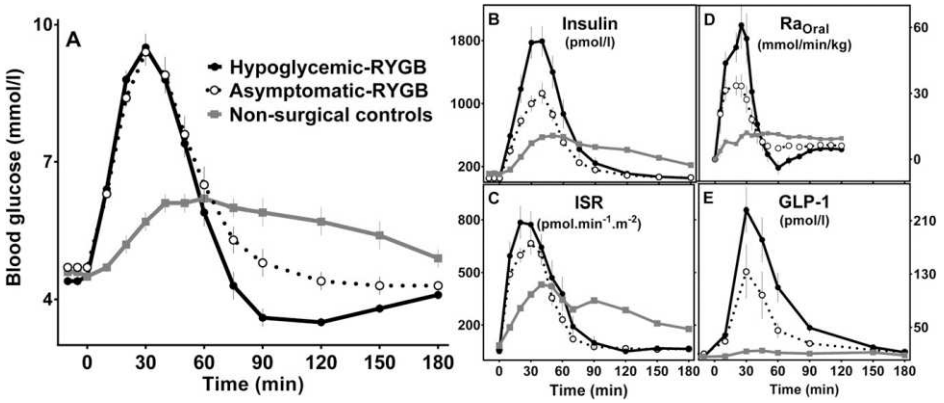


Figure 5. Typical glycemic and hormonal patterns after mixed meal. (A) Blood glucose, (B) plasma insulin, (C) insulin secretory response (ISR), (D) systemic appearance of ingested glucose (Ra_{Oral}) and (E) circulating GLP-1 levels during meal tolerance test in RYGB subjects with (black ● and solid line) and without (black ○ and dashed line) hypoglycemia and nonsurgical controls (gray ▪ and solid line). Reproduced from Salehi J et al. Clin Endocrinol Metab. 2018;103(8):2815-2826.

A postulated mechanism appears to be the considerable post-prandial elevation of GLP-1 levels, resulting in a stimulation of insulin secretion.

Furthermore, chronic exposure to this hormone could lead to the inhibition of apoptosis of the β-cells, resulting in an increase in their number [49]. Regardless of the mechanism, not yet fully understood, the increase in GLP-1 levels is believed to be a causal factor for PBH: in support of this theory, a study shows that treatment with exendin-9, a GLP-1 receptor antagonist, correct the hypoglycemia reducing insulin secretion in subjects affected by PBH [50].

By contrast, it has been suggested that GLP-1 analogs might provide a new treatment option in patients with PPHG [51]; however, it is possible that the constant activation of the GLP-1 receptor mitigates the impact of acute post-prandial excursions of the endogenous hormone.

Furthermore, it has been postulated that, after RYGB, hypoglycemic counter-regulation may be dysfunctional due to the lack of inhibition of insulin secretion or changes in neuronal/sympathetic activity [52].

In a prospective clinical trial twelve obese nondiabetic patients were studied before and 23 weeks after gastric bypass surgery with hyperinsulinaemic-hypoglycemic clamp (stepwise to plasma glucose 2.7 mmol/L). Authors showed a marked post-surgery reduction in levels of glucagon, cortisol, and

catecholamine and the sympathetic nerve responses to hypoglycemia. In addition, growth hormone displayed a delayed response but to a higher peak level supporting the hypothesis of an altered insulin counter-regulation [53].

Recently, a role of inflammasome NLRP3 has been demonstrated by researcher at the University Hospital Basel [54]. The authors suggest that an early peak in blood sugar leading to an inflammatory response mediated by IL-1β with a subsequent exaggerated rise in insulin secretion. Accordingly, treatment with anakinra, which blocks the inflammation mediated by IL-1β, effectively improves complication of bariatric surgery. Moreover, analysis of serum monocytes ex vivo revealed a distinct gene expression pattern characterized by upregulated IL-1β, and a hyper-reactive inflammatory state that has features of an exaggerated response to a meal in patients affected by PBH.

Another potential factor implicated in the pathogenesis of PBH is the alteration of the intestinal microbiota. Numerous studies have shown an increase in *gammaproteobacteria* and a reduction in *firmicutes* after bariatric surgery compared to normal weight controls and non-operated obese patients [55]. Furthermore, the biodiversity and richness of the microbiota is increased. Generally, these changes are observed within 3 months of bariatric surgery and most of them remain stable for at least a year [56].

To investigate the functional role of RYGB-associated microbiota alterations, a group of researchers transferred microbiota from sham- and RYGB-treated fa/fa rats to germ-free mice, observing lower postprandial peak and nadir of glucose in mice that received cecal microbiota from RYGB- versus sham-operated rats. Thus, microbiota alterations induced by RYGB, may partially explain both the improved glucose tolerance and the reduction of glucose nadir after RYGB [57].

The mechanisms by which post-bariatric microbiota alterations leads to metabolic changes and potentially the development of hypoglycemia are not yet known. Two possible mediators have been called into question: bile acids and short-chain fatty acids (SCFAs).

Biliary acids are cholesterol-derived amphipathic molecules produced by hepatocytes constitute about 50% of the organic component of bile. Cholic acid and chenodeoxycholic acids are the main primary BAs synthesized in humans. The primary biliary acids are stored in the gallbladder until secretion in the duodenum. In the intestine, they are transformed into secondary biliary acids by the gut microbiota. Biliary acids regulate their own synthesis and transport via the nuclear farnesoid X receptor (FXR) in the gut [56].

Evidence has shown that biliary acids are involved in the regulation of glucose metabolism by controlling insulin signaling, glucose utilization, hepatic gluconeogenesis [58]. Furthermore, chenodeoxycholic acid intake stimulated GLP-1 secretion in patients after RYGB through activation

of G-protein-coupled bile acid receptor (TGR5) [59]. Alterations in gut microbiota, which play a crucial role in the transformation of primary biliary acid, may influence glucose metabolism. Clinical studies have showed that circulating biliary acids significantly increase following RYGB, with a simultaneous decrease in blood glucose levels [60-61]. Via FXR, biliary acids induce synthesis of fibroblast growth factor 19 (FGF-19) in humans. FGF-19 activates glycogen synthesis and inhibits gluconeogenesis, which subsequently leads to a decrease in circulating glucose concentrations. Activation of FXR in the muscles and adipose tissue leads to improvements in insulin sensitivity [62]. Recently, a clinical study performed proteomic analysis of blood samples in patients with PBH after RYGB to explore the mechanisms contributing to glucose reduction. Authors showed that FGF-19 levels were 2.4-fold higher in patients with PBH compared with controls [63]. Therefore, gut microbiota regulation of biliary acid metabolism might be an important factor in the pathogenesis of PBH. SCFAs are gut microbiota-derived metabolites produced in the colon by fermentation of non-digestible polysaccharides. They act on G-protein-coupled receptors, called free fatty acid 2 (FFA2) and FFA3, which are widely expressed in many tissues, to regulate appetite and host energy expenditure.

It has been reported that SCFAs play an important role in glucose metabolism. Both FFA2 and FFA3 are normally expressed in pancreatic cells, and their activation may result in improvement of insulin secretion. Butyrate (one of the most important SCFAs) increases the production of GLP-1, and it is also involved in reducing appetite [64]; furthermore, SCFAs are able to activate brown adipose tissue, increasing the host energy expenditure [65].

It is a reasonable hypothesis that gut microbiota modification results in changes in SCFAs, which might mediate hypoglycemia after bariatric surgery. In a recent study conducted by Tremaroli et al, germ-free mice colonized with the fecal microbiota collected from humans nine years post-RYGB, reported an increased resting energy expenditure along with increased concentration of the SCFA propionate [66].

On the other hand, the effect of RYGB on increasing energy expenditure is well-established to contribute to hypoglycemia after RYGB [67]; therefore, SCFA changes resulting from altered gut microbiota after surgery may increase host energy expenditure and then contribute to hypoglycemia.

3.6 Treatment of PBH

3.6.1 Nutritional therapy

The goal of PBH therapy is to reduce the frequency and severity of hypoglycemic episodes, thus improving the quality of life and safety of these patients, protecting from long-term consequences, including memory loss and cardiovascular mortality.

Unfortunately, however, with the currently available treatments it is highly unlikely the complete elimination of the disorder in patients with severe PBH.

Nutritional intervention represent a fundamental element in the management of PBH, aiming to eliminate the stimulus for glycemic spikes that my lead to insulin hypersecretion.

Diet itself does not cause PBH, but it can exacerbate the episodes; in fact, high glycemic index carbohydrates are capable of rapidly increasing blood glucose levels and patients with PBH are advised to avoid excessive consumption. This concept was confirmed in a study demonstrated that a high carbohydrate (80 grams), low protein (10 grams) meal led to significant hyperinsulinemia and hypoglycemia, while an isocaloric very low carbohydrate (2 grams), high protein meal (25 grams) prevented excessive insulin secretion or postprandial hypoglycemia [68].

In one study, limiting a test meal to 30 grams of solid carbohydrate or 28 grams of liquid low glycemic index supplement was successful in preventing hypoglycemia in patients with PBH [69].

However, even complete elimination of carbohydrates carries some risk over time, as it may contribute to malnutrition and reduced responsiveness to glucagon [70]. For these reasons, a share of low glycemic index carbohydrates should be guarantee. Authors found that low glycemic index carbohydrates slow the postprandial rise in glucose, resulting in fewer postprandial glucose “spikes” and, therefore, less PBH [71].

Some patients find that avoiding high glycemic index carbohydrates, especially pasta and bread, is very challenging. Patients might consider eating pasta made from 100% whole wheat or protein-supplemented pasta in carefully measured, small quantities.

An optimal dietary plan has not been defined, but there are some general principles to follow. In general, a mixed meal approach is recommended, combining low-glycemic index carbohydrates and in moderate doses with protein and fats, so as to slow down the absorption of nutrients and reduce post-prandial glycemic peak. The main meals should be small, with the addition of balanced snacks between one and other.

The consume of heart-healthy fats should be encouraged. Although glucose excursions were not markedly affected by a fat preload, fats are a source of calories which do not typically trigger insulin secretion [72].

Further recommendations include eating slowly and chewing food completely, avoiding the simultaneous intake of liquids in order not to cause dumping syndrome. Excessive consumption of alcohol, which can inhibit the hepatic release of glucose, is to avoid.

In addition, the consumption of caffeine, which can rapidly increase glycemia via increased hepatic glucose production, decreased glucose uptake into skeletal muscle, and leads to an over-stimulus of insulin release, should be limited [73] (**Table 1**).

Table 1. Nutrition plan for preventing PBH. Reproduced from Suhl et al. Surg Obes Relat Dis. 2017;13(5):888-896

10-Point Nutrition Plan for Preventing Hypoglycemia in Post-Bariatric Hypoglycemia.

1. Control portions of carbohydrate – 30 grams/meal, 15 grams/snack.
2. Choose low-glycemic carbohydrates.
3. Avoid high-glycemic carbohydrates.
4. Include (heart-healthy) fats in each meal or snack – 15 grams/meal, 5 grams/snack.
5. Emphasize optimal protein intake.
6. Space meals/snacks 3–4 hours apart.
7. Avoid consuming liquids with meals.
8. Avoid alcohol.
9. Avoid caffeine.
10. Maintain post-bariatric vitamin and mineral intake.

3.6.2 Pharmacological therapy

Dietary intervention can be implemented with the use of medications. **Acarbose** delays and reduces the absorption of glucose by inhibiting the alpha-glucosidases associated with the brush-border membrane of the small intestine which are responsible for the digestion of complex polysaccharides and sucrose. In healthy subjects, 100 to 200 mg of acarbose significantly inhibits postprandial glucose, and the consequent insulin production, reducing the episodes of reactive hypoglycemia [74]. The main factor that limit tolerance is the development of gastrointestinal side effects such as bloating and abdominal cramps.

Diazoxide activates adenosine triphosphate (ATP) sensitive potassium channels in the pancreatic β cells, increasing potassium (K) efflux. The hyperpolarization of the beta cells inactivates voltage-gated calcium channels (VGCC) resulting in inhibition of insulin release. Excess incretin hormones in PBH enhance insulin secretion through the inhibition of K_{ATP} channels, which is potentially counteracted by diazoxide [75]. Diazoxide is also used for other forms of hyperinsulinaemic hypoglycemia, but it is tainted by important side effects such as nausea, headache, hypotension and fluid retention.

Even somatostatin analogs, such as **octreotide,** effectively reduces the episodes of PBH suppressing both the insulin-release and the secretion of the incretin hormone by binding to 5 SS receptors at levels of L-cells [76]; however, their use is limited by high costs and side effects, which include diarrhea, steatorrhea, cholelithiasis and QT lengthening.

Further therapeutic possibilities are represented by **GLP-1 agonist**, as previously mentioned.

Some theories have been formulated that can explain at least partially these observations, in some respects, paradoxical. It has been observed that in type 2 diabetes patients on GLP-1 treatment, glucagon levels in the hypoglycemic state were elevated compared with controls, and that GLP-1 receptor activation may make β-cells more capable of suppressing insulin secretion at low glucose values [51]. Possibly, the positive effects of GLP-1 analogs in PBH involve the same glucose-stabilizing mechanism as in patients with diabetes receiving GLP-1 active drugs, downregulating insulin secretion and upregulating glucagon levels in low glucose conditions.

Finally, **calcium channel blockers** have been shown to block insulin secretion through inhibition of voltage-gated calcium channels on β-cells and have been used successfully for the treatment of PBH [77].

However, most of these therapies are based on case series or reports and extrapolated from their use in other conditions that cause hypoglycemia. The main limitations of pharmacological therapy are represented by side effects and patient nonadherence, that can limit their utility (**Figure 6**).

3.6.3 Surgical therapy

When non-invasive interventions fail, in selected case of severe PBH, surgery is possible. A G-tube (gastrostomy tube) can be introduced into the portion of the stomach excluded from the alimentary pathway allowing meals to reach the small intestine through the duodenum and proximal jejunum, as they would in the absence of bariatric surgery, in order to normalize the levels of glucose, insulin and incretin response. Low-carb foods can continue to be introduced via the oral route. This approach may be limited by the reduction in the quality of life caused by the use of a tube to feed [22].

A more invasive approach involves the reconversion of RYGB, but this can lead to weight regain, symptoms of slowed gastric emptying such as nausea and vomiting, in addition to the possible persistence of hypoglycemia.

Some authors propose a robotic revisional surgery for the conversion of RYGB into sleeve gastrectomy to mitigate the symptoms of PBH [78].

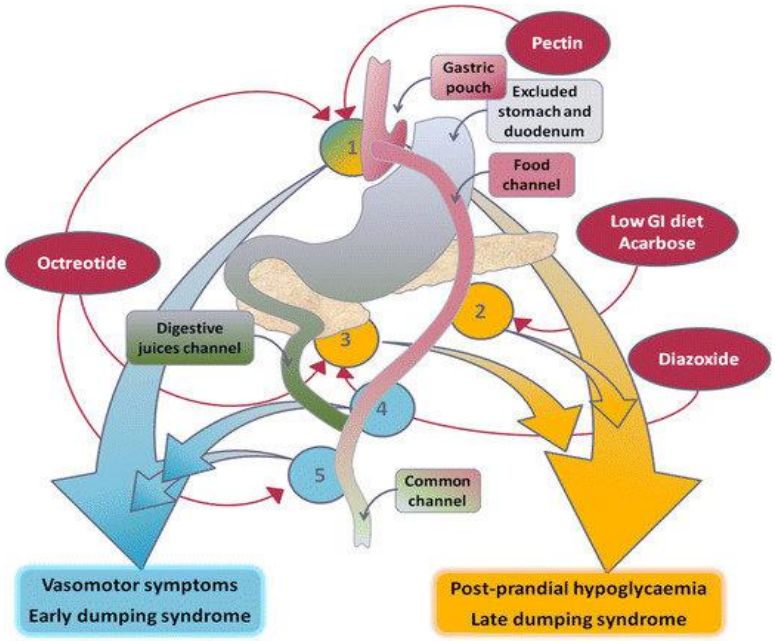


Figure 6. pathophysiology of PBH and mode of action of common therapeutic agents. Reduced gastric volume results in rapid gastric emptying (1) and rapid glucose absorption (2), which induces a hyperinsulinaemic response (3), leading to reactive hypoglycemia. Rapid delivery of hyperosmolar chyme to the upper small bowel (4) induces release of several gut hormones (5). Pectin, increasing meal viscosity may help to slow down gastric emptying. Acarbose, can slow down the absorption of sugars. Diazoxide inhibits insulin release from β cells in the pancreatic islets while octreotide inhibit the secretion and release of insulin and vasoactive gut hormones. Modified from Narayan et al. Obes Surg. 2016;26(10):2523-9).

4. LISOSAN G (WHEAT LYSATE)

4.1 Fermented foods.

Fermented foods and beverages are defined as foods produced through controlled microbial growth that enzymatically transforms certain components [79]. They have been produced and consumed since the origin of human civilization and some of them are basic elements of the human diet. It is increasingly understood that fermented foods can have enhanced functional properties due to transformation of substrates and formation of bioactive end-products [80].

Historically, fermentation has been exploited for its ability to prolong food storage, as the accumulation of antimicrobial metabolites inside it reduces the risk of contamination by pathogenic

microorganism. It is also used to improve the organoleptic properties of some otherwise inedible foods. The interesting aspect in the field of medicine is the fact that fermented foods show beneficial properties on health, and research in this direction is shedding more and more light on the possible mechanisms underlying these characteristics.

Fermented foods contain large quantities of potentially probiotics microorganism, capable of resisting adverse conditions in the intestinal environment, competing with pathogenic bacteria in order to maintain a healthy microbiota, and producing molecules involved in some important physiological functions; furthermore, these microorganisms could convert some components of ingested food into biologically active metabolites or reduce the concentrations of toxins [81].

It has been also observed that some bioactive peptides contained in fermented foods are involved in glucose metabolism, cardiovascular function, nutrient balance, protection from oxidative stress and in the gut-brain axis crosstalk [80-82].

These peptides are often inactive when contained within the protein from which they are released by means of microbial enzymatic action. For example, it has been shown that the phenolic compounds contained in red wine extracted by fermentation of grape must have important antioxidant properties, are involved in the modulation of lipid metabolism, inhibition of platelet aggregation and protection against carcinogenesis [83].

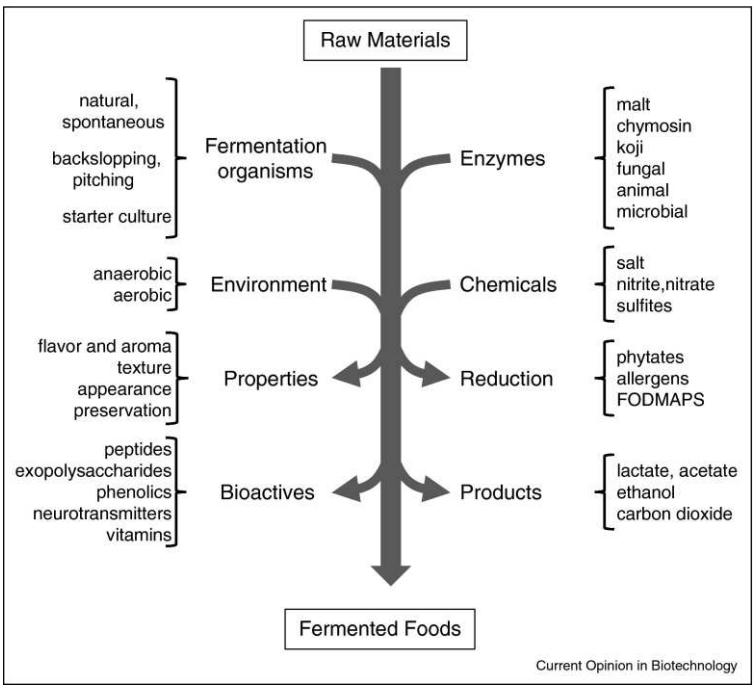


Figure 7. Overview of the transformative nature of fermented foods. Raw materials are fermented in specific conditions. Fermentation then creates novel and potentially health promoting compounds in foods, while removing those with negative health potential. Reproduced from M. Marco et al. Curr Opin Biotechnol. 2017; 44:94-102).

4.2 Fermented wheat

Cereals represent the main source of sustenance for humans globally, especially in developing countries. Whole cereals are a rich source of fiber, vitamins, minerals and phytochemicals such as phenols, carotenoids, beta-glucans and others [84]. A product of wheat fermentation that is slowly emerging in the scientific landscape is Lisosan G® (LG), an organic what lysate (Triticum aestivum), which has been certified by the Italian Ministry of Health as a food supplement [85].

It is produced through a fermentation process at a temperature below 36°C, which avoids the degradation of enzymes. In particular, the whole wheat grains are coarsely ground, after which germ is separated from the bran. Water is added and some specific starter microbial cultures are inoculated to start the fermentation process. Generally these are *lactobacilli* and natural yeasts. Once the first fermentation phase is over, the mixture is dried to obtain dry powder.

The goal of these processes is the lysis of macromolecular compounds, which makes the active component accessible to the enzymes and transporters present on the intestinal wall, in order to allow their absorption [84].

4.3 Components and proprieties of Lisosan G.

Lisosan G (LG) is a wheat lysate which contains not only the typical components of cereals, but after fermentation it is enriched with bioactive substances such as phenolic compounds and alpha-lipoic acid, a natural antioxidant that has shown a protective effect against vascular damage induced by reactive oxygen species (ROS) [86] (**Table 2**).

Both in vitro and in vivo studies have shown that LG is highly effective in protecting and improving the bioactivity of different cell types, from hepatocytes to microvascular endothelial cells, through the control of both oxidative and inflammatory processes [87-88]. In particular, in a study conducted on healthy rabbits, LG was found to improve the lipid profile and reduces the blood reactive oxygen metabolites inducing the antioxidant enzymes in the liver and kidney of the treated rabbits [89].

In vitro studies on human cells have shown that LG supplement exerts a protective effect against the microvascular endothelial cells exposed to oxidized LDL, reducing the inflammatory processes [90]. Furthermore, after exposure of endothelial progenitors' cells (EPCs) to H_2O_2, LG pre-treated cells showed a lower senescence than untreated EPCs. LG significantly decreased H_2O_2-induced ROS generation and increased glutathione peroxidase-1 and superoxide dismutase-2 (SOD-2) expression.

The authors concluded that probably the mechanism underlying the increase in survival and adhesion capacity, and the reduction of senescence, consist in the activation of the nuclear factor signaling pathway (erythroid-derived-2)-like 2 [87].

More recently, a protective effect has been described against the EPCs exposed to LPS-induced oxidative stress [91]. LG protected EPCs from inflammation as well as from LPS-induced oxidative and endoplasmic reticulum (ER) stress reducing ROS levels, downregulating proinflammatory and proapoptotic factors. In particular, the positive effects of LG was likely to be associated with reduced nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which is an oxidant-sensitive transcription factor responsible for regulating gene expression of factors involved in inflammatory responses.

A protective effect was observed on mouse retinal cells exposed to oxidative stress, with a reduction in apoptosis and the extension of vascular lesions. The effects of LG were studied in cultured explants of mouse retinas challenged with oxidative stress or in retinas of streptozotocin treated rats. LG extensively inhibited apoptosis, VEGF expression, and oxidative stress both in retinal explants and in STZ rats [92].

Finally, it has been recently demonstrated that orally administered LG protects DBA/2J mice from glaucoma, improving the retinal ganglion cells (RGCs) function by protecting against oxidative stress, inflammation marker expression and RGCs loss. All four LG metabolites – nicotinamide, gallic acid, 4-hydroxybenzoic acid and quercetin- were found in the retina. These findings are consistent with neuro-enhancing and neuroprotective effects of LG in glaucoma.

Table 2. Composition of Lisosan G, lysate of organic wheat fermented. Lisosan G contains a consistent percentage of antioxidants, polyunsatured fatty acids, polyphenols. Reproduced by www.agrisan.it

Main components of Lisosan G			
Proteins	174 g/kg	Copper	0.01 g/kg
Lipids	147 g/kg	Selenium	57 µg/kg
Glucids	372 g/kg	Linolenic acid	3 g/kg
Polysaccharides	72 g/kg	Linoleic acid	33 g/kg
Cellulose	26 g/kg	Oleic acid	7.4 g/kg
Phosphate	13 g/kg	Tocoferols	0.02 g/kg
Sulphur	1.9 g/kg	Vitamin B1	3.8 µg/kg
Magnesium	4.1 g/kg	Vitamin B2	0.9 µg/kg
Iron	0.1 g/kg	Vitamin B6	2.2 µg/kg
Zinc	0.13 g/kg	Nicotinamide	1.3 µg/kg

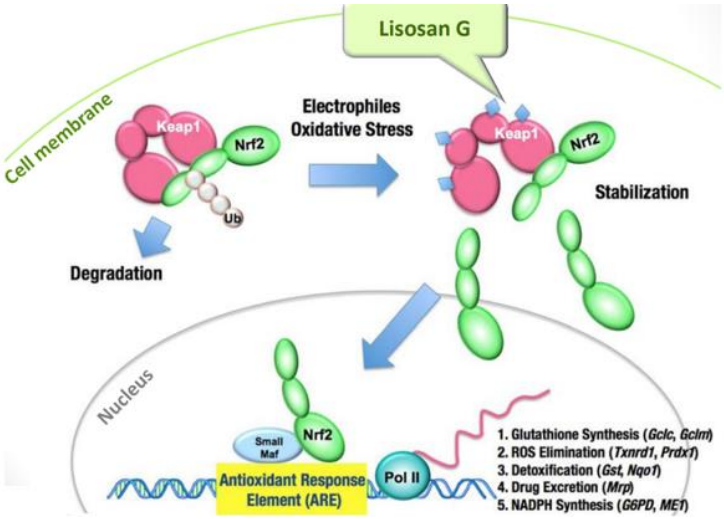


Figure 8. The antioxidant response of Lisosan G. Lisosan G is an effective inducer of ARE/Nrf2-regulated antioxidant and detoxifying genes.

5. LIPOSOMES

5.1 Definition of liposomes

Liposomes are small artificial vesicles of spherical shape that can be created from natural phospholipids that are biologically inert and feebly immunogenic. The size and the hydrophobic/ hydrophilic characteristics make liposomes effective systems for drug delivery. Liposome properties differ considerably with lipid composition, size and the method of preparation (**Table 3**).

Generally, liposomes are definite as spherical vesicles with particle sizes ranging from 30 nm to several micrometers, and this is due to the fact that phospholipids form closed structures when they are hydrated in aqueous solutions. In particular, they consist of one or more lipid bilayers surrounding aqueous units, where the polar head groups are oriented in the pathway of the interior and exterior aqueous phases. Drugs with different lipophilicities can be encapsulated into liposomes: strongly lipophilic drugs are entrapped almost totally in the lipid bilayer, intensely hydrophilic drugs are located entirely in the aqueous compartment, and drugs with intermediary features effortlessly partition between the lipid and aqueous phases, both in the bilayer and in the aqueous core.

On the other hand, self-aggregation of polar lipids is not limited to conventional bilayer structures which rely on molecular shape, temperature and preparation conditions but may self-assemble into various types of colloidal particles [93].

Because of their biocompatibility, biodegradability and aptitude to trap medications [94] and simplify site-specific drug delivery to tumor tissues [95], liposomes have increased rate as a drug-delivery system. Many studies have been conducted on liposomes with the goal of decreasing drug toxicity and/or targeting specific cells [96-97].

The liposomes, in fact, form a barrier around their contents, which is resistant to enzymes in the digestive tract, such as alkaline solutions, digestive juices, bile salts, and intestinal flora. The contents of the liposomes are, therefore, protected from oxidation and degradation. This protective phospholipid shield remains undamaged until the contents are delivered and release at the designated targets [98] (**Figure 9).**

Table 3. Advantages and disadvantages of liposome. Reproduced by Akbarzadeh A et al. Nanoscale Res Lett. 2013; 22;8(1):102.

Advantages of liposome	Disadvantages of liposome
Liposomes increased efficacy and therapeutic index of drug (actinomycin-D)	Low solubility
Liposome increased stability via encapsulation	Short half-life
Liposomes are non-toxic, flexible, biocompatible, completely biodegradable, and non-immunogenic for systemic and non-systemic administrations	Sometimes phospholipid undergoes oxidation and hydrolysis-like reaction
Liposomes reduce the toxicity of the encapsulated agent (amphotericin B, Taxol)	Leakage and fusion of encapsulated drug/molecules
Liposomes help reduce the exposure of sensitive tissues to toxic drugs	Production cost is high
Site avoidance effect	Fewer stables

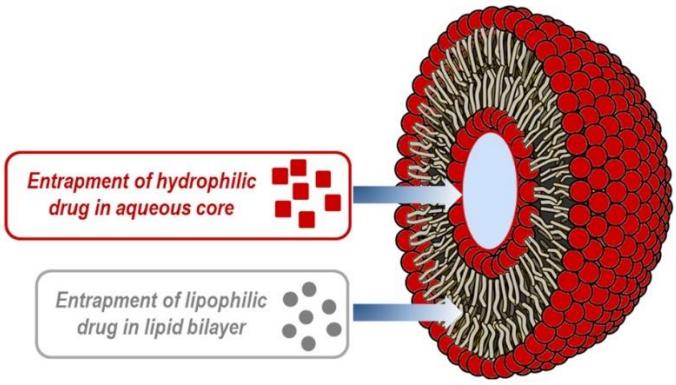


Figure 9: Liposome structure with hydrophilic core (aqueous) and hydrophobic double layer.

5.2 Classification of liposomes

The liposome size can vary from 0.025 μm (very small) to 2.5 μm (large) vesicles, and they can have one or bilayer membranes. The vesicle size is the main parameter that impact on the circulation half-life of liposomes.

Size and number of bilayers affect the amount of substance encapsulation into the liposomes. On the basis of these two parameters, liposomes can also be classified in two categories, multilamellar vesicles and unilamellar vesicles. Unilamellar vesicles are in turn divided into two categories, large unilamellar vesicles and small unilamellar vesicles (SUV) [99]. In unilamellar liposomes, the vesicle has a single phospholipid bilayer sphere enclosing the aqueous solution, while in multilamellar liposomes, vesicles have a structure similar to an onion. Classically, several unilamellar vesicles will form on the inside of the other with smaller size, making a multilamellar structure of concentric phospholipid spheres separated by layers of water [100].

5.3 Preparation of liposomes

5.3.1 Sonication

Sonication is the most extensively used method for the preparation of SUV. MLVs are sonicated either with a bath type sonicator or a probe sonicator under a passive atmosphere. The main disadvantages of this method are low internal volume/encapsulation efficacy, possible degradation of phospholipids and compounds to be encapsulated, metal contamination from probe and elimination of large molecules [101].

There are two sonication techniques.

a) Probe sonication. The tip of a sonicator is directly engrossed into the liposome dispersion. The energy input into lipid dispersion is very high in this method. The coupling of energy at the tip results in local hotness; therefore, the vessel must be engrossed into a water/ice bath. Throughout the sonication up to 1 h, more than 5% of the lipids can be de-esterified.

b) Bath sonication. The liposome dispersion in a cylinder is placed into a bath sonicator. Controlling the temperature of the lipid dispersion is usually easier in this method, in contrast to sonication by dispersal directly using the tip. The material being sonicated can be protected in a sterile vessel, dissimilar the probe units, or under an inert atmosphere [97].

5.3.2 French pressure cell: extrusion

The French pressure cell produces fairly homogeneous SUVs or MLVs (depending on lipid composition) by passing a lipid suspension under extreme pressures through a small orifice [101]. Trapping efficiencies up to 25% can be achieved although this value decreases somewhat with increasing molecular weight of the trapped solute. An important feature of the French press vesicle (FPV) technique is that proteins do not appear to be significantly affected during the process as they are in sonication [102]. An interesting observation is that FPVs appear to retain entrapped solutes significantly longer than do SUVs produced by sonication or detergent removal [103]. The drawbacks of the method are that the high temperature is difficult to attain, and the working volumes are comparatively small (about 50 mL as the maximum) [97].

5.3.3 Solvent dispersion method

5.3.3.1 Solvent vaporization

A solution of lipids dissolved in diethyl ether or ether-methanol mixture is gradually injected to an aqueous solution of the material to be encapsulated at 55°C to 65°C or under reduced pressure. The consequent removal of ether under vacuum leads to the creation of liposomes. The main disadvantages of the technique are that the population is heterogeneous (70 to 200 nm) and the exposure of compounds to be encapsulated to organic solvents at high temperature [104].

5.3.3.2 Ethanol injection

A lipid solution of ethanol is rapidly injected to a huge excess of buffer. The MLVs are at once formed. The disadvantages of the method are that the population is heterogeneous (30 to 110 nm), liposomes are very dilute, the removal all ethanol is difficult because it forms into azeotrope with water, and the various biologically active macromolecules could become inactivated in the presence of low quantities of ethanol [105].

5.3.3.3 Reverse phase evaporation method

Reverse-phase evaporation is based on the creation of inverted micelles. These inverted micelles are shaped upon sonication of a mixture of a buffered aqueous phase, which contains the water-soluble molecules to be encapsulated into the liposomes and an organic phase in which the amphiphilic molecules are solubilized. The slow elimination of the organic solvent leads to the conversion of these inverted micelles into viscous state and gel form. At a critical point in this process, the gel state collapses, and some of the inverted micelles were disturbed. The excess of phospholipids in the environment donates to the formation of a complete bilayer around the residual micelles, which results in the creation of liposomes. Liposomes made by reverse phase evaporation method can be made from numerous lipid formulations and have aqueous volume-to-lipid ratios that are four times higher than multilamellar liposomes. Briefly, first, the water-in-oil emulsion is shaped by brief sonication of a two-phase system, containing phospholipids in organic solvent, for example a mixture of isopropyl ether and chloroform with aqueous buffer. The organic solvents are detached under reduced pressure, resulting in the creation of a viscous gel. The liposomes are shaped when residual solvent is detached during continued rotary evaporation under reduced pressure. With this method, high encapsulation efficiency up to 65% can be obtained in a medium of low ionic strength for example 0.01 M NaCl [97].

5.4 Detergent removal methods

5.4.1 Dialysis

The detergents at their critical micelle concentrations have been used to solubilize lipids. As the detergent is detached, the micelles become increasingly better-off in phospholipid and lastly combine to form large unilamellar vesicles. The detergents were removed by dialysis. Dialysis is a technique

which allow the separation of particles in a liquid on the basis of differences in their ability to pass through a membrane. The procedure of extrusion of crude liposome dispersions through controlled-pore polycarbonate membranes is used to control the upper limit of liposome diameter and to remove the detergents. Subsequent dialysis, using the same type of membrane, can remove the majority of liposomes smaller than a predetermined size and detergents. The pattern of dialysis of a liposome preparation is a function of the size-frequency distribution (as well as the membrane pore size) [106].

5.4.2 Detergent removal of mixed micelles (absorption)

Detergent absorption is attained by shaking mixed micelle solution with beaded organic polystyrene adsorbers. The great benefit of using detergent adsorbers is that they can eliminate detergents with a very low critical micelle concentrations, which are not entirely depleted [107].

5.4.3 Gel-permeation chromatography

In this method, the detergent is depleted by size special chromatography through gel filtration. The liposomes do not penetrate into the pores of the beads packed in a column. They percolate through the inter-bead spaces. At slow flow rates, the separation of liposomes from detergent monomers is very good. The swollen polysaccharide beads adsorb substantial amounts of amphiphilic lipids; therefore, pre-treatment is necessary. The pre-treatment is done by pre-saturation of the gel filtration column by lipids using empty liposome suspensions [108].

5.4.4 Dilution

Upon dilution of aqueous mixed micellar solution of detergent and phospholipids with buffer the micellar size and the polydispersity increases dramatically and as the system is diluted beyond the mixed micellar phase boundary, a spontaneous transition from polydisperse micelles to monodisperse

5.5 Medications loading in liposomes (encapsulation)

Medications can be encapsulated during liposome formation (passively) or after liposome formation (actively). Hydrophobic drugs can be directly combined into liposomes during vesicle formation, and the amount of uptake and retention is governed by drug-lipid interactions. Trapping effectiveness of 100% is often achievable, but this is dependent on the solubility of the drug in the liposome

membrane. Passive encapsulation of water-soluble drugs depends on the ability of liposomes to trap aqueous buffer containing a dissolved drug during vesicle formation. Trapping effectiveness (generally <30%) is limited by the trapped volume delimited in the liposomes and drug solubility. On the other hand, water-soluble drugs that have protonatable amine functions can be actively entrapped by employing pH gradients, which can result in trapping effectiveness approaching 100% [109].

5.6 Lyophilization of liposomes

Freeze-drying (lyophilization) involves the removal of water from products in the frozen state at very low pressures. The process is normally used to dry products that are thermo-labile and would be demolished by heat-drying. It has been showed that that leakage of entrapped materials may take place during the process of freeze-drying and on reconstitution, but when liposomes are freeze-dried in the presence of adequate amounts of trehalose (a carbohydrate commonly found at high concentrations in organism) retained as much as 100% of their original substances. Freeze-driers range in size from small laboratory models to large industrial units available from pharmaceutical equipment suppliers [97-109].

5.7 Mechanism of transportation through liposome

In vivo and in vitro studies have shown that liposome can interact with cells by four different mechanisms: 1) endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; 2) adsorption by specific interactions with cell-surface components, electrostatic forces, or by non-specific weak hydrophobic; 3) fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal content into the cytoplasm and finally 4) the transfer of liposomal lipids to cellular membranes without any association of the liposomes contents. It is often difficult to determine what mechanism is operative and more than one may operate at the same time [110].

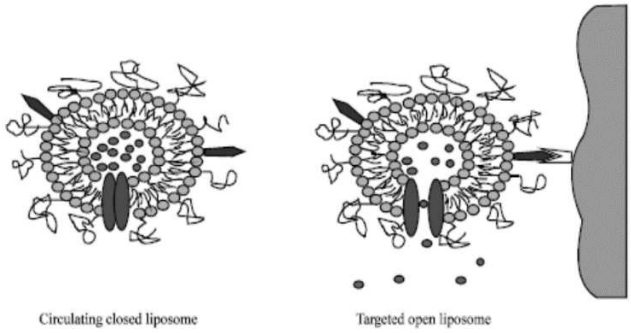


Figure 10. Mechanisms of transportation through liposomes.

5.8 Applications of liposomes in medicine and pharmacology

Applications of liposomes in medicine and pharmacology can be divided into diagnostic and therapeutic applications of liposomes containing various markers or drugs, and their use as a tool, a model, or reagent in the basic studies of cell interactions, recognition processes, and mode of action of certain substances [109]. Unfortunately, many drugs have a very narrow therapeutic window, meaning that the therapeutic concentration is not much lower than the toxic one. In several cases, the toxicity can be reduced or the efficacy can be enhanced by the use of a suitable drug carrier which alters the temporal and spatial delivery of the drug, such as its biodistribution and pharmacokinetics. This is one of the most important applications of liposomal system. It is clear from many pre-clinical and clinical studies that drugs, for instance antitumor drugs, parceled in liposome demonstration reduced toxicities, while retentive enhanced efficacy. Advances in liposome design are leading to new applications for the delivery of new biotechnology products, for example cloned genes, and recombinant proteins. A vast literature define the viability of formulating wide range of conservative drugs in liposomes, frequently resultant in improved therapeutic activity and/or reduced toxicity compared with the free drug. As a whole, changed pharmacokinetics for liposomal drugs can lead to improved drug bioavailability to particular target cells that live in the circulation, or more prominently, to extravascular disease sites, for example, tumors [110].

5.9 Eudragit® L100 Liposomes

Eudragit is the brand name for a diverse range of polymethacrylate-based copolymers. It includes anionic, cationic, and neutral copolymers based on methacrylic acid and methacrylic/acrylic esters or

their derivatives [111]. Eudragits have glass transition temperatures between 9 to 150 C, and they are non-biodegradable, non-absorbable, and nontoxic. Depending on the functional groups used in the polymer, Eudragit® formulations can be tuned to the timing of drug release, immediate, delayed or sustained. With gastrointestinal targeting or time-controlled release, it is possible to define gradual release profile specifics.

Specifically, Eudragit® L 100 is anionic copolymerization product of methacrylic acid and methyl methacrylate. The ratio of free carboxyl groups to the ester is approximately 1:1 in Eudragit® L 100 having a mean relative molecular mass of about 135.000 Da and apparent viscosity of 50–200 mPas [112]. Eudragit® L100 has the characteristics of protecting the drug against the action of the enzymes and gastric fluids, which are in fact very acidic (pH = 1-2), while it is soluble in intestinal fluid from pH 6-7, allowing the drug release in mid to upper small intestine (especially jejunum) [113].

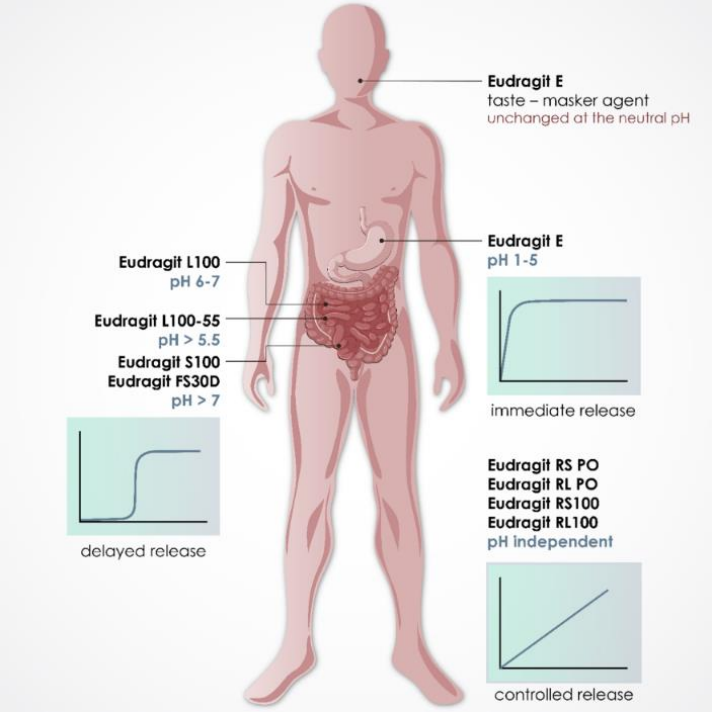


Figure 11. Predictable drug release sites from dosage forms produced with Eudragit polymers, according to their physicochemical properties. Reproduced by Dos Santos J et al. Pharmaceutics. 2021; 8;13(9):1424.

6. STUDY PROTOCOL

6.1 Background and Aims.

PBH is considered a chronic complication after bariatric surgery. Nutritional approach is considered the main treatment for this complication, impacting on glucose absorption, insulin secretion and gut microbiota. In fact, bariatric surgery induces profound changes in gut microbiota, with a post-operatively increase in Bacteroides and a decrease in Firmicutes, although the results are not always in agreement [114].

In a recent RCT study, the investigators showed that a macrobiotic diet, with its high fiber load, was effective in increasing the production of SCFAs by the gut microbiota in 12 patients affected by reactive hypoglycemia [115]. The authors speculated that the increased bioavailability of SCFAs could be relevant in reactive hypoglycemia, thanks to their capacity to counterbalance the metabolic deregulation in persons with impair glucose homeostasis.

Among the molecules studied, preclinical and clinical data suggest that dietary polyphenols and flavonoids present prebiotic properties in order to modify the gut microbiota. Although the precise mechanisms deserve further clarification, polyphenols and flavonoids have been shown ability to modulate microbiota function, promoting the transition to a healthy microbiota and favoring the production of SCFAs and the improvement of the gut barrier integrity [116-117].

A recent study shows that one of the pathophysiologic mechanisms of PBH is the glucose-induced IL-1β response by hyper-reactive monocytes to a meal which leads to an insulin overshoot [54]; starting from this new observation, the anti-inflammatory properties induced by polyphenols and flavonoids may be effective in counteracting the effect of PBH [114].

In order to overcome the low bioavailability of polyphenols, several different approaches have been developed, aiming to improve solubility and transport of dietary polyphenols throughout the gastrointestinal tract and reach the targeted intestinal regions. Although more research is still needed, the biotechnological progresses achieved during the last years open up good perspectives to improve the use of dietary polyphenols modulating gut microbiota.

For all these reasons, the aims of this project are:

- **In vivo, clinical study (1st part)**:

1) to evaluate the effectiveness of using LG added to the diet in reducing PPHG events in patients undergoing RYGB.

2) to investigate the mechanism by which LG acts on the gut-pancreas axis.

- **In vitro (2nd part)**:

to evaluate the phenolic content and the antioxidant properties of LG extract and LG extract encapsulated in Eudragit-liposomes. Eudragit-liposomes are in fact expected to protect LG from acidic degradation in the stomach, allowing pH-driven absorption in the intestine, and facilitate the entry of LG in the cells, potentiating its bioactivity.

6.2 First part of the study protocol (clinical study, in vivo).

6.2.1 Materials and methods

Study participants and protocols.

In this prospective study, twenty consecutive patients, who had a routine assessment (before surgery) as part of metabolic pre-surgery work-up (3-hour OGTT), self-reporting symptoms/signs of PPHG, after RYGB were recruited. We excluded patients with heart failure III-IV NYHA class, liver failure or NASH, chronic diarrhea (including inflammatory bowel disease). All participants had a detailed medical history and a complete physical examination. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Committee. Informed written consent was obtained from all subjects before enrollment.

At **T1**, after an overnight fast, patients were admitted to our outpatient unit (Medicine 1 Unit, Azienda Ospedaliera Universitaria Pisana). Demographic, anthropometric and clinical parameters were recorded. Blood pressure was measured by a digital electronic manometer with a suitable cuff according to arm circumference, after sitting for >10 min. Venous blood and urinary samples were

collected for standard biochemistry and circulating levels of inflammatory markers, and finally a 4-hour oral glucose load test (OGTT) was performed.

After that, the patients were discharge and kept on a free diet with 2 weeks of continuous glucose monitoring (CGM). During these 2 weeks the patients were instructed to keep a nutritional diary precisely noting the quantity, type of foods and times of intake. At the same time, the patients were required to perform common daily activities and report any structured physical activity.

After this period, the CGM was then repeated for another 2 weeks, adding LG to the same dietary regimen (5 g LG-powder, twice daily) (**T2**). During this period the patients repeated the same dietary regimen recorded in the nutritional diary, at the same quantities and at the same times. Similarly, they were required to repeat the same amount of daily physical activity, respecting times and modalities.

Finally, at the end of the treatment period, patients returned to our outpatient Clinic (**T3**), and they repeated the same evaluation of the baseline, with clinical evaluation, blood and urinary sample and 5-hours OGTT. Thirty minutes before OGTT they took 5 grams of LG in 20 ml of still water (**Figure 12**).

Oral Glucose Tolerance Test (OGTT)

After an overnight fast (12 h), all subjects consumed an oral glucose load consisting of 150 mL of 50% dextrose solution. Blood samples were collected through an indwelling cannula at times −15, 0, 15, 30, 45, 60, 90, 120, 180, 240, 300 min during the test to measure plasma glucose and insulin. Blood samples were centrifuged for 15 min (3000× *g* at 4 °C) and frozen at −20°C before analysis.

Measurements

Plasma glucose was measured immediately by the glucose-oxidase technique (Beckman Glucose Analyzer II, Fullerton, CA, USA). Plasma insulin and C-peptide were measured by electrochemiluminescence on a Cobas e411 instrument (Roche Diagnostics, Monza, Italy). Plasma total GLP-1 concentrations were measured by ELISA (Millipore).

Beta-cell function modelling

Insulin sensitivity was estimated as the oral glucose insulin sensitivity index (OGIS), which calculates plasma glucose clearance rate (ml min^{-1} m^{-2}) at a level of hyperinsulinemia in the range that would be achieved during a standard (240 pmol min^{-1} m^{-2}) hyperinsulinaemic–euglycemic clamp, against

which this index has been validated in individuals with normal glucose tolerance, impaired glucose tolerance and overt diabetes [118].

Insulin secretion rate (ISR) was estimated via C-peptide deconvolution using the Van Cauter's model of C-peptide kinetics [119]

Beta-cell function was assessed by mathematical modelling of the plasma glucose, insulin and C-peptide concentrations measured during the frequently sampled OGTT, as previously described [120]. The beta-cell function model consists of three blocks: (1) a model for fitting the plasma glucose concentration profile, the purpose of which is to smooth and interpolate plasma glucose concentrations; (2) a model describing the dependence of insulin (or C-peptide) secretion on glucose concentration; and (3) a two-exponential model of C-peptide kinetics in which the model variables are individually adjusted to the individual's anthropometric data. The mean slope of the insulin secretion/plasma glucose dose–response function is taken to represent beta-cell glucose sensitivity (β-GS, in pmol min^{-1} m^{-2} mmol l^{-1}). This parameter can be modulated by several factors (*i.e.*, non-glucose substrates, gastrointestinal hormones and neurotransmitters), which are collectively modeled as a potentiation factor whose value is set to be a positive function of time, and to average the value 1 during the duration of the 2-h OGTT. The ratio of the values at 100–120 min *vs* 0–20 min (potentiation factor ratio) [121].

Continuous glucose monitoring (CGM)

Flash glucose monitoring device was applied on the back of upper arm (*Freestyle Libre, Abbott Diabetes Care, Oxon, UK*). The flash glucose sensor is an arm worn sensor intended to last for 14 days. The component not directly attached to the patient was the handheld reader and/or mobile phone app which displays current and historical glucose data. Education and training about insertion and initiation of the sensor as well as how to use flash-glucose monitoring data for treatment optimization was also provided. Encouragement was also provided to download data at home to identify pattern recognition. This session, designed to meet the needs of the individual, was conducted by a professional diabetes educator member of the study team.

Based on the international consensus, coefficient of variation (CV%) was calculated by dividing standard deviation of glucose by mean glucose from CGM.

PBH was defined as a plasma glucose level of ≤ 60 mg/dl in presence of typical symptoms or < 50 mg/dl also without symptoms.

Statistical analysis

Continuous variables are presented as means ± standard deviations (SD) and nominal variables are reported as counts and/or percentages. Variables with a skewed distribution are presented as median [interquartile range].
The significance of the differences between two independent samples was tested using the non-parametric Mann–Whitney tests or by means of Student t test, as appropriate. Categorical data were analyzed by the Chi-square test. Repeated measures ANOVA was employed for time series.
Statistical tests were performed using JMP Pro 15.3.0 (SAS Institute Inc., Cary, NC, USA) using a two-sided α level of 0.05.

6.2.2 Results

Impact of bariatric surgery on clinical and metabolic parameters

Two patients enrolled in the study could not tolerate LG due to bloating and diarrhea and dropped out. Eighteen patients were ultimately included in the study.
The cohort consisted predominantly of women (78%), the mean age was 41 ± 7 at time of the time of the surgery, while at the development of PBH was 44 ±8. Only three patients had diabetes before surgery and in remission after surgery, while hypertension was present in 8/18 (39%) before surgery. BMI, fasting glucose, HbA_{1c} and insulin sensitivity significantly improved after surgery. The prevalence of hypertension was reduced from 39% (8/18) to 11% (2/18), with a significant reduction in systemic blood pressure. The kidney function remained stable (**Table 4**).

Impact of LG on episodes and overall length of time of hypoglycemia.

Pre-LG, the episodes of hypoglycemia were mainly diurnal (94% of the total) and occurred 112 ± 20 minutes after the main meals. A marked reduction in PBH episodes was observed after LG administration during the 15 days period recorded by CGM (6.5 [5-11] vs 2.5 [2-3], p= 0.009).
There was also a significant decrease in the overall length of time with glycemia ≤60 mg/dl (410 [129-633] vs 39 [20-89], minutes, p=0.003), and a marked reduction in the time spent in severe hypoglycemia, defined as a blood glucose <50 mg/d, during the treatment period (52 [20-105] vs 2 [0-10], minutes, p=0.001) (**Figure 13**).

In addition to the positive impact on PBH, during treatment with LG we also assisted to a significant improvement in the glycemic variability, with a significative reduction in the coefficient of variation (%CV) (36.5 ± 4.5 vs 24.1 ± 3.9, p= 0.019).

Glucose tolerance and insulin sensitivity.

Fasting plasma glucose, fasting plasma insulin were not affected by LG treatment. Similarly, insulin sensitivity remained stable before and after LG administration under fasting condition as measured by the OGIS index (514 ± 55 vs 534 ± 44 mL/min^{-1} · m^{-2}) (**Table 5**).

Impact of LG on glucose, insulin and incretin secretion during OGTT.

Before surgery, the patients had higher fasting glycemic value and nadir compared to post-surgery (95 ± 9 vs 86 ± 5 mg/dl, p=0.039 and 74 ± 10 vs 44 ± 11, mg/dl, p< 0.0001, respectively). On the contrary, the glucose zenith was significantly lower (**Table 5**).

After surgery, OGTT performed post-LG treatment showed a marked increase in the blood glucose nadir compared to OGTT pre-LG (44 ± 11 vs 56 ± 10, mg/dl, p= 0.038). Conversely, no difference was found in the time-to-nadir (142 ± 21 vs 154 ± 34, minutes) as well as in the blood glucose zenith (234 ± 39 vs 226 ± 45, mg/dl) nor the time-to-zenith (40 ± 16 vs 45 ± 15, minutes) (**Figure 14, A**).

Compared to post-surgery, fasting insulin before surgery was higher (119 ± 49 vs 50 ± 18, pmol/L, p= 0.009), while total insulin AUC during glucose load was lower (38±17 vs 57±12, nmol/m^2, p= 0.039). After surgery, no differences were shown in fasting insulin pre- and post-LG administration, while insulin AUC during OGTT significantly reduced post-LG treatment (57±12 vs 49±9, nmol/m^2, p= 0.041) (**Figure 14, B**).

After surgery, baseline and glucose-stimulated insulin secretion estimated by C-peptide deconvolution improved. However, there were no differences in basal and total insulin secretion pre- and post-LG administration (**Figure 14, D** and **Table 5**), while we assisted to a significant decrease in the potentiation factor after therapy with LG (1.5 ± 0.5 vs 0.8 ± 0.4, p= 0.038) (**Figure 14, D**).

Furthermore, among model-derived parameters of β-cell function, the β-cell glucose sensitivity improved after surgery, but did not show any variation after LG-therapy (**Table 5**).

Basal GLP-1 concentrations were similar before surgery, Pre-LG and Post-LG, while bariatric surgery markedly potentiated GLP-1 secretion during OGTT (5.0 ± 2.9 vs 7.6 ± 4.1, nmol/L × min, p=0.003). After surgery, the time to GLP-1 zenith was similar before and after LG, while the total

GLP-1 AUC was significantly attenuated by LG treatment (7.6 ± 4.1 vs 6.5 ± 3.8, nmol/L*min, p= 0.043) (**Figure 14, C**).

Table 4. Baseline anthropometric and laboratory features of patients with PBH enrolled in the study, before RYGB and at the time of enrollment in the study.

Parameters	Before surgery	Pre-LG (T1)	P value
Age	41 ± 7	44 ± 8	**<0.0001**
Gender (F/M)	14/4	23.8 ± 2.7	-
BMI	42.4 ± 4.6	23.8 ± 2.7	**<0.0001**
SBP (mmHg)	137 ± 10	133 ± 8	**0.034**
DBP (mmHg)	85 ± 5	82 ± 4	**0.042**
Gycemia (mg/dl)	95 ± 9	87 ± 10	**0.039**
HbA1c (%)	5.6 [5.5-6.08]	5.3 [4.5-5.5]	**0.011**
Creatinine (mg/dl)	0.69 ± 0.12	0.74 ± 0.13	ns
AST (UI/L)	21 [17-32]	16 [12-21]	ns
ALT (UI/L)	37 [20.5-55]	13 [11.25-20.75]	**0.032**
Gamma GT (UI/L)	29 [15-38]	11 [8-17]	**0.021**
Cholesterol (mg/dl)	191.67 ± 32.33	172.43 ± 28.06	ns
HDL (mg/dl)	44.6 ± 11.1	65.5 ± 12.9	**0.016**
LDL (mg/dl)	119.6 ± 45.5	91.4 ± 24.9	ns
Tryglicerides (mg/dl)	152 [96.25-262]	70 [53-120]	**0.042**

Table 5. Changes in glucose and insulin metabolism induced by LG in patients with PBH.

	Before Surgery (T0)	Pre-LG (T1)	Post-LG (T3)	p-value T0 vs T1	p-value T1 vs T3
Glucose Control					
Fasting glucose (mg/dl)	95 ± 9	86 ± 5	85 ± 6	0.039	ns
Nadir Glucose (mg/dl)	74 ± 10	44 ±11	56 ± 10	< 0.0001	**0.038**
Mean Glucose (mg/dl)	132 ± 23	126 ± 14	121 ± 18	ns	ns
Zenit Glucose (mg/dl)	174 ± 23	224 ± 19	208 ± 21	< 0.0001	ns
Fasting insulin (pmol/L)	119 ± 49	50 ± 18	57 ± 17	0.009	ns
Mean Insulin (pmol/L)	416 [283-504]	586 [443- 949]	451 [337- 741]	ns	ns
AUC Insulin (nmol/m^2)	38±17	57±12	49±9	0.039	**0.041**
Insulin sensitivity					
OGIS (ml min^{-1} m^{-2})	331 ± 90	514 ± 55	534 ± 44	0.002	ns
Beta-cell function					
Fasting ISR (pmol m^{-2} min^{-1})	119 ± 41	97 ± 29	99 ± 29	0.032	ns
Total ISR (nmol/m^2)	65 ± 39	94 ± 27	81 ± 21	0.041	ns
β-GS (pmol min^{-1} m^{-2} mM^{-1})	35 ± 28	91 ± 37	93 ± 34	0.007	ns
Potentiation factor (ratio)	0.5 ± 0.3	1.5 ± 0.5	0.8 ± 0.4	0.031	**0.038**
Incretin					
Fasting GLP-1 (pM)	26 [18-44]	29 [20-42]	34 [24-52]	ns	ns
GLP-1 AUC (nmol/L × min)	5.0 ± 2.9	7.6 ± 4.1	6.5 ± 3.8	0.001	**0.043**

Figure 12. Study design. OGTT: oral glucose tolerance test; CGM: continuous glucose monitoring.

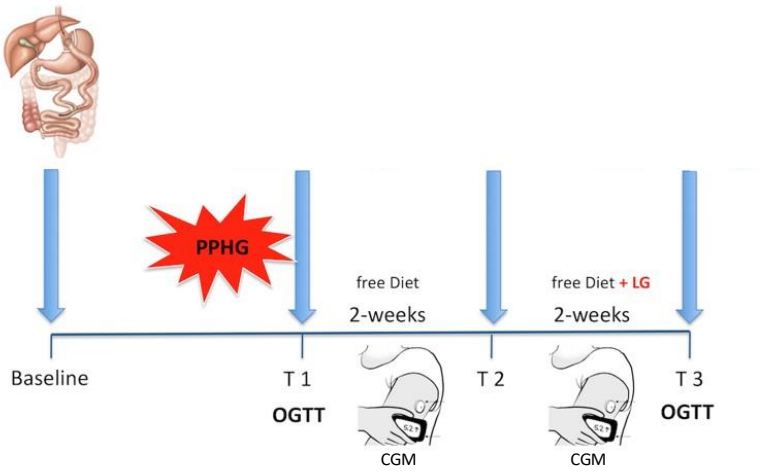


Figure 13. Overall length of time of PBH during CGM monitoring before (PRE) and after (POST) LG. administration. A= number of hypoglycemic episodes. B= total time of PBH. C= total time spent in severe hypoglycemic condition (blood glucose < 50 mg/dl).

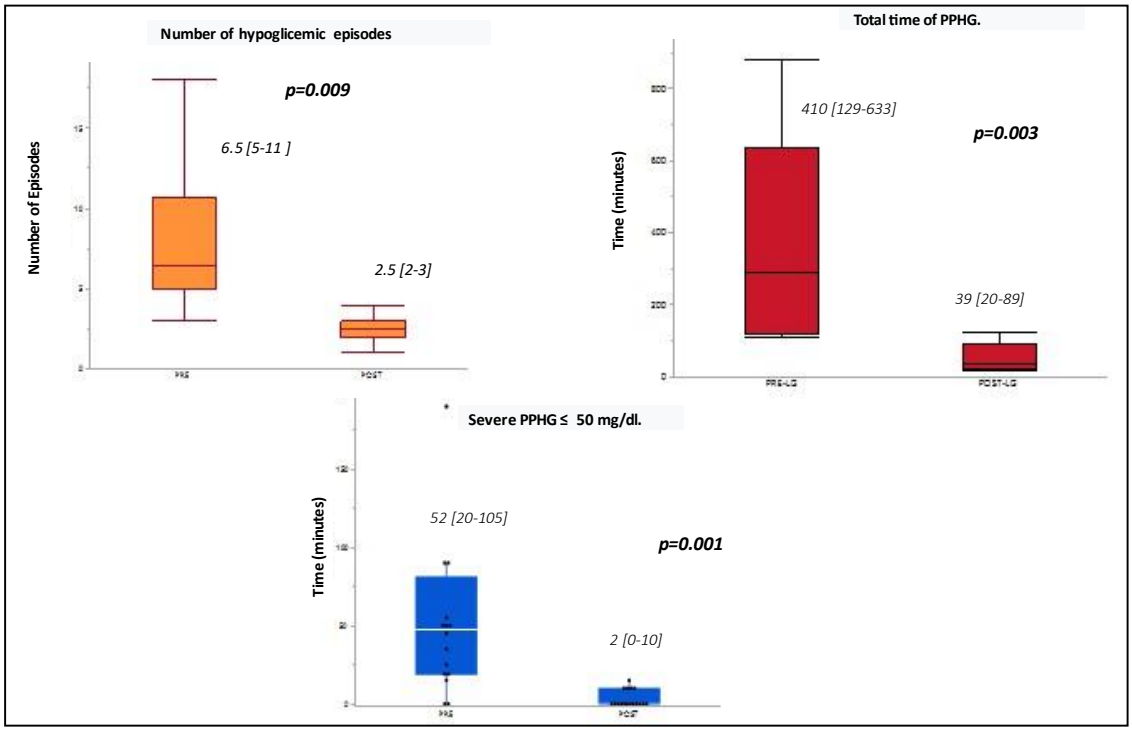
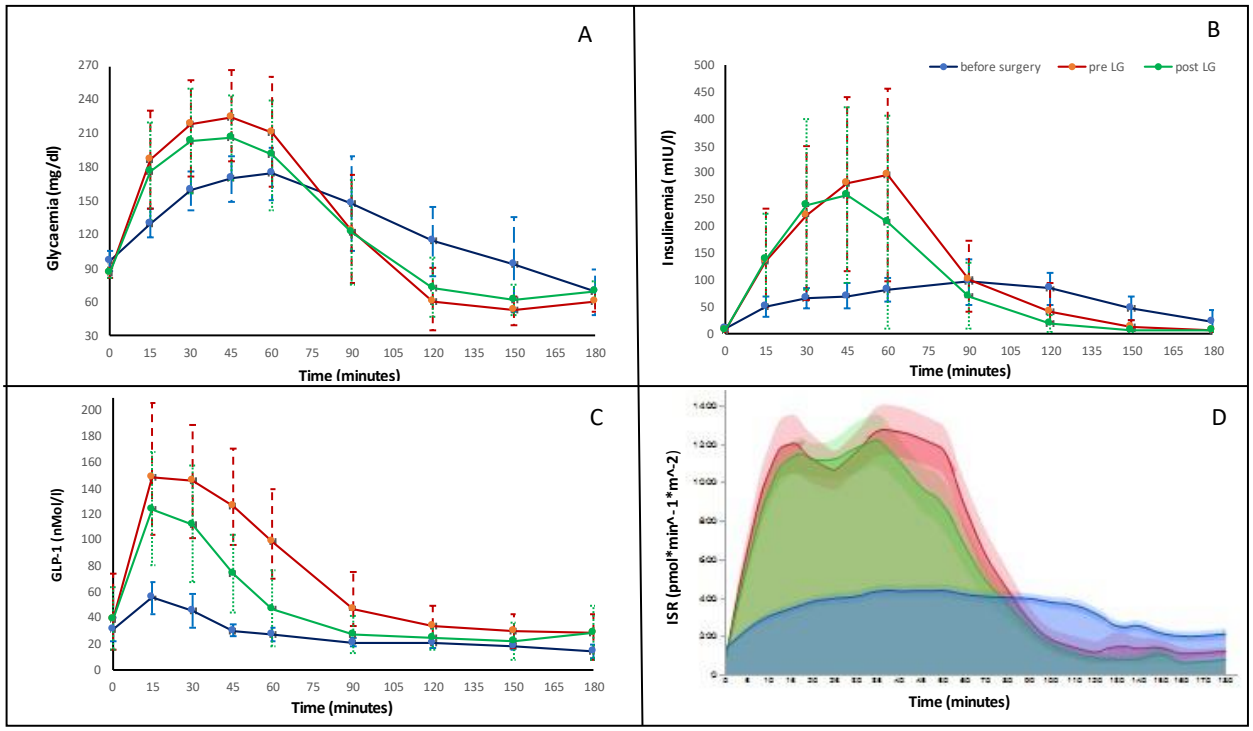


Figure 14. Plasma glucose, insulin, GLP-1 and insulin secretion during OGTT in patients affected by PBH before surgery (blue line), pre-LG (red line) and post-LG (green line). Plots are means ± SEM.



6.3 Second part of study protcol (project of nanosystem LG-based, in vitro).

6.3.1 Materials and methods

LG produced by fermenting and drying *Triticum aestivum* whole wheat grains flour, was kindly supplied by Agrisan srl (Larciano, PT, Italy). LG aqueous extract was obtained by dispersing LG powder in water (8.3 mg mL^{-1}), sonicating and centrifugating at 2300×*g* for 10 min, at 4 °C (Jouan CR3i centrifuge, Newport Pagnell, UK). Then, the supernatant was collected, filtered (0.2 μm), and stored in the dark at 4 ± 2 °C until use.

Liposome preparation and characterization

To produce eudragit-liposomes, Lecinova® and Eudragit® were weighed in a vial, dispersed in a 50:50 LG aqueous extract:water blend, and sonicated (15 cycles, 5 s on/2 s off + 20 cycles, 3 s on/2 s off) with a Soniprep 150 (MSE Crowley, London, UK).

The average diameter, polydispersity index (PI, a measure of the width of size distribution), and zeta potential of the liposomes were determined via dynamic and electrophoretic light scattering using a Zetasizer nano-ZS (Malvern Panalytical, Worcestershire, UK). Prior to analysis at 25 °C, the samples (n > 10) were diluted with water (1:100).

The storage stability of the formulations was assessed by analyzing the mean diameter, PI and zeta potential of the vesicles for 3 months at 4 ± 2 °C. The vesicle dispersions were purified from the non-encapsulated LG active compounds by dialysis. The dispersions (1 mL) were loaded into 12–14 kDa MWCO Spectra/Por® membrane tubing (Spectrum Laboratories Inc., DG Breda, The Netherlands) and dialysed for 2 h against water (2 L) to allow the removal of the non-encapsulated compounds. After disruption of unpurified and purified liposomes with 20:80 methanol:water, the entrapment efficiency (E), expressed as the percentage of gallic acid detected in unpurified samples, was determined using an Alliance 2690 HPLC system (Waters, Milan, Italy). Gallic acid was quantified using a Sunfire C_{18} column (3.5 μm, 4.6 × 150 mm, Waters), an acetonitrile:water:acetic acid (94:5.8:0.2 %v/v) mobile phase, and an 0.3 mL/min flow rate. The absorbance at 259 nm (A_{259}) was measured for gallic acid quantification.

Phenolic contents

Total phenolics were estimated as Folin-Ciocalteu (FC) reducing capacity [122] and expressed as mg of gallic acid equivalents per g of dry weight (mg GAE/g dw). Briefly, 100 µL of LG formulations or gallic acid solution, used as a reference, were added to 500 µL of Folin-Ciocalteu reagent (0.2 eq L^{-1}, diluted in water) and kept for 5 min in the dark at room temperature. Thereafter, 400 µL of 0.7 mol L^{-1} sodium carbonate was added. After 2 h of incubation in the dark at room temperature, A_{760} was measured.

Antioxidant activity evaluation

The antioxidant activity of LG formulations was assessed as a function of their ability to scavenge $DPPH^{\bullet}$, a stable nitrogen-centered free radical. 2 ml of $DPPH^{\bullet}$ methanolic solution (25 µmol L^{-1}) was mixed with 40 µL of LG Eudragit-liposomes or LG aqueous extract and stored in the dark at room temperature. After 30 min, A_{517} was measured against blank. The degree of discoloration of the violet-colored $DPPH^{\bullet}$ solution, which depends on the radical scavenging/antioxidant capacity and the concentration of a sample, is quantified as a decrease in absorbance (A).
The percent antioxidant activity (AA) of the samples was calculated according to equation:

$$AA = \left(\frac{A_{DPPH} - A_{sample}}{A_{DPPH}}\right) x\ 100$$

The antioxidant activity was expressed also as Trolox Equivalents (TE). TE values were calculated using a calibration curve built with different concentrations (0.5–3 mg mL^{-1}) of Trolox (antioxidant reference). Results were expressed as µg TE mL^{-1} solution.
The antioxidant capacity of LG formulations to reduce Fe^{3+} ferric iron to Fe^{2+} ferrous iron was determined by the FRAP (Ferric Reducing Antioxidant Power) assay (PMID: 31622804). Briefly, a mixture of 300 mmol L^{-1} acetate buffer (pH 3.6), 20 mmol L^{-1} $FeCl_3 \cdot 6H_2O$, 10 mmol L^{-1} TPTZ in 40 mmol L^{-1} HCl was added to each sample. After 30 min at room temperature, A_{593} was measured against blank. The results were expressed as µmol Fe^{2+} L^{-1} using a ferrous iron standard curve.

Statistical analysis

Results, expressed as mean values ± standard deviation (SD), were analyzed by one-way analysis of variance (ANOVA) with Dunnett's test and unpaired Student's t-test for single comparisons. p values ≤ 0.05 were considered statistically significant

6.3.2 Results

Vesicle preparation and characterization

In the present study, a vesicular formulation was developed for the oral delivery of LG extract. Eudragit-liposomes were produced to protect LG extract from degradation by stomach acid and allow pH-driven absorption in the intestine, since Eudragit® L100 dissolves above pH 6, and to enhance its antioxidant activity at cellular level. Eudragit-liposomes were made of Lecinova®, a cheap dietary supplement of gluten-free, non-GMO soy lecithin containing phospholipids, ω-3 and ω-6 fatty acids, vitamin E and B6, marketed as an aid to lowering cholesterol. Therefore, it is worth noting that, in addition to functional properties, including acidic protection and improvement of the bioavailability of the incorporated compounds from LG extract, the produced liposomes have a food-grade composition, ideal for oral administration. LG Eudragit-liposomes were produced via an easy, organic solvent-free, and scalable method, and the main physico-chemical and technological features were evaluated. As displayed in **Table 6**, light scattering results show that LG Eudragit-liposomes were relatively small in size (∼130 nm). These liposomes were also characterized by good homogeneity (PI 0.24) and negative zeta potential (∼−30 mV), due to the charge of soy lecithin and Eudragit.
The entrapment efficiency (E%) was calculated based on the content of gallic acid, one of the most abundant components of LG extract. E% was 75% and did not diminish significantly (70% ± 2.6; $p > 0.05$) during a three-month-storage period. The stability of the formulations was assessed for three months by analyzing mean diameter, PI, and zeta potential of the vesicles, as well. No statistically significant changes were found among the three examined parameters.

Phenolic contents

LG Eudragit-liposomes contained comparable phenolics content to the LG extract, which indicates that the encapsulation process preserved LG bioactive compounds (9.2 ± 0.2 vs 9.3 ± 0.2, LG extract vs Eudragit-Liposomes respectively, $p = ns$).

In vitro antioxidant assays

The antioxidant activity of LG formulations was assessed by the DPPH assay, which is based on the reduction of the free radical. **Table 7** reports the results of the assay. It is worth noting that the

antioxidant activity of LG extract in Eudragit-liposomes was essentially the same and corresponded to ~300 μg mL^{-1} of Trolox equivalents. Conversely, the ferric reducing capacity of LG extract in Eudragit-liposomes, corresponding to~215 μmol L^{-1} of Fe^{2+} equivalents, was significantly higher than that of LG extract (~162 μmol L^{-1} of Fe^{2+} equivalents, $p < 0.001$; **Table 7**).

Table 6. Characteristics of LG Eudragit-liposomes. Mean diameter (MD), polydispersity index (PI), zeta potential (ZP), and entrapment efficiency (E) are reported. Each value represents the mean ± SD (n > 6).

LG eudragit-Liposomes	**Value**
MD (nm)	132 ± 4.4
PI	0.24 ± 0.03
ZP (mV)	-29 ± 1.9
E (%)	75 ± 3.7

Table 7. In vitro antioxidant activity of LG extract in the vesicle formulations in comparison with an aqueous solution (50:50 %v/v). DPPH results are expressed as AA (%) and as μg TE mL^{-1} concentrations, whereas FRAP results are expressed as μmol $Fe^{2+}L^{-1}$.

	DPPH		**FRAP**
Formulation	**AA (%)**	**μg TE mL^{-1}**	**μmol $Fe^{2+}L^{-1}$**
LG extract	0.24 ± 0.03	0.24 ± 0.03	0.24 ± 0.03
LG eudragit-Liposomes	-29 ± 1.9	-29 ± 1.9	-29 ± 1.9

6.4 Discussion

This study represent a pilot study on 20 patients enrolled with the aim of evaluating the action of the Lisosan G fermented wheat-based supplement with respect to episodes of reactive hypoglycemia after bariatric surgery. The results show that treatment with LG is associated with:

1. a significant improvement in PBH, as evidenced by the reduction in the frequency, overall duration and severity of hypoglycemic episodes, in real life;

2. an increase in the glycemic nadir during OGTT
3. a reduction in the AUC of insulin and of GLP-1 during OGTT

To our knowledge, this is the first pilot study to date that evaluates the relationship between intake of fermented wheat-based supplement and reduction of PPHG episodes after bariatric surgery. In 2018, a previous case report [123] on only 2 patients demonstrated through CGM monitoring how the administration of cornstarch was capable of reducing postprandial glycemic peaks, the overall duration of hypoglycemic episodes and both daytime and daily glycemic fluctuations.

In the study of Lembo et al [123], both patients had RYGB surgery and started to experience hypoglycemic symptoms with tachycardia, sweating, and neuroglycopenic symptoms from two to five years after the intervention and, according to our observation, their 5-days CGM record evidenced the classical pattern of rapid increases in glucose concentration after meals followed by sharp falls. Notably, the two selected patients had been selected on the basis of the significant severity of symptoms, and they spent about 11% of their entire CGM recording with blood sugar levels below 55 mg/dl, while our cohort included patients with varying degrees of PBH severity, with a mean percentage of the time <60 mg/dl of about 4% of the entire recording.

However, in the present study, for the first time a relatively large cohort of patients was enrolled, with a longer period of CGM reaching two full weeks. Furthermore, we evaluated the PBH events both during the execution of common daily activities through CGM and during a stress test through OGTT, in order to obtain more information deriving from the beta-cell modeling and entero-hormones release.

The administration of LG demonstrate the benefit in reducing the overall duration of hypoglycemic episodes, with a median ranging from 410 minutes to 39 minutes during treatment, and it stabilize the glucose variability, reducing significantly the glycemic circadian excursions.

In a recent meta-analysis including all major bariatric surgery operations, authors surprisingly highlighted a quite significant rate of nocturnal hypoglycemia, suggesting that PBH should not be viewed as an exclusively postprandial phenomenon. Specifically, in this metanalysis the total weighted mean prevalence (WMP) of PBH was 54.3% (95%CI: 44.5%–63.8%), while the WMP of nocturnal PBH was 16.4% (95%CI: 7.0%–34%) [124]. To date, no information are available on the mechanism related to nocturnal PBH.

Actually, we find a pretty negligible rate of nocturnal hypoglycemia (about 6% of total time spent in hypoglycemia), and this finding is in line with a recent observation demonstrating that PBH is frequently post-prandial and symptomatic after RYGB, while it is mostly nocturnal and asymptomatic

after sleeve gastrectomy [125].

Our data broaden the knowledge in the literature concerning the phenomenon of PBH, demonstrating that nutritional therapy with nutraceuticals acquires an important clinical impact in terms of improvement of quality of life and personal safety, given the demonstrated association between reactive hypoglycemia and the increased risk of cardiovascular diseases and cognitive decline [9-10]. However, the mechanisms by which LG acts will need to be elucidated.

From our OGTT data, LG does not appear to modify glucose absorption, as evidenced by the fact that the blood glucose curves did not differ in time-to-zenith and in the value of glycemic zenith.

On the contrary, the higher levels of glucose nadir seems to be the result of an attenuated second phase of insulin secretion. In fact, during OGTT we assisted to a significant reduction of the potentiation factor, representative of the second phase of insulin secretion, which leads to a reduction of the total insulin AUC.

The mechanism by which LG attenuates the second phase of insulin secretion and consequently improve the PBH, is probably through the reduction of the GLP-1 peak in response to glucose load.

Overall, a significant increase in GLP-1 peak during the OGTT is observed after bariatric surgery; in addition, the GLP-1 peak appears to be excessively elevated in patients with PBH, and the present results are in agreement with was previously described [30].

After LG treatment, the GLP-1 peak is higher compared to the pre-surgery condition, but at the same time it is attenuated compared to GLP-1 peak before LG treatment.

About that, the profile and the AUC of GLP-1 after administration of LG return very similar to those of our previously published study involving post-bariatric patients without hypoglycemia [126].

So, we could speculate that LG restores an excessive and aberrant response of GLP-1 to the glucose load that occurs in patients affected by PBH.

Although the biological mechanisms underlying the favorable impact of LG on PBH remain to be completely elucidated, we could hypothesize that the reduction of GLP-1 secretion could be mediated by an action of LG on the intestinal microbiota.

In fact, LPS are fragments from the cell wall of Gram-negative, mostly anaerobic, gut bacteria, inhabiting the human gastrointestinal tract, and a recent study demonstrate that plasma GLP-1 levels are rapidly increased by lipopolysaccharide (LPS) administration in mice via a Toll-like receptor 4 (TLR4)-dependent mechanism [127].

It is known that a balanced diet, rich in fibers, keeps balanced microbiota and an intact intestinal barrier function, and that prebiotics and probiotics are examples of dietary manipulation of the gut microbiota [128]. So, we could speculate that LG leads to beneficial changes in the gut microbiota

and, in turn, to decreased plasma LPS levels, improving metabolic endotoxemia and reducing GLP-1 stimulation.

For these reason future studies of our research group will focus on the evaluation of microbiota changes through the characterization of volatile and non-volatile metabolite of saliva samples by head space gas chromatography mass spectrometry, before and after the administration of LG.

The importance of this study derives from the limited presence of therapeutic options of comparable efficacy and not burdened by heavy side effects and from the results obtained in a free diet regimen.

A potential limit, on the other hand, could be seen in the use of OGTT which represents a maximal and non-physiological stimulus for the patient, which we have overcome by combining the OGTT values with continuous glycemia monitoring (CGM) in order to be able to record blood excursions in response to daily physiological stimuli.

From a clinical point of view, the results of this study support the nutraceutical approach as a useful complement to the nutritional and pharmacological approach to improve the progress of hypoglycemic episodes.

In order to implement the effectiveness of the LG extract, which includes many known antioxidant molecules such as gallic acid, vanillic acid, quercetin and, at the same time, to improve patients' compliance in daily intake, part of future research will be aimed at the construction of nano-systems capable of improving the LG pharmacokinetics qualities.

The *in vitro* results of our study showed that the encapsulation in liposomes made it possible to preserve the antioxidant properties of LG aqueous extract.

Further *in vivo* studies are needed to demonstrate a significant role in enhancing the transport of bioactive ingredients of LG across biological membranes.

In conclusion, the present pilot study shows that LG administration exerts a favorable effect on the episodes of reactive hypoglycemia of patients undergoing bariatric surgery, significantly reducing the frequency, overall duration and severity of episodes. The effect is observed with a daily integration of LG 5 gr powder 10 minutes before main meals. Further specific studies are necessary to evaluate the effects of LG on the microbiota, inflammation and entero-hormones to better understand the mechanisms of action of this precious supplement.

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