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Abstract

Introduction: Refeeding syndrome (RS) is a life-threatening metabolic complication of nutritional support. Prolonged fasting, frequently observed in malnourished patients referred for percutaneous endoscopic gastrostomy (PEG), induces histological and ultrastructural changes in the intestinal mucosa, potentially influencing RS development. Mixed-Meal Tolerance Tests (MMTT) combined with metabolomics allow dynamic assessment of serum glucose, gastrointestinal hormones and cellular metabolites, which may help to elucidate RS pathogenesis and assess patient risk. **Objective:** The present study aims to perform MMTT in PEG-fed patients to evaluate enteroendocrine hormone responses and metabolomic profiles following a prolonged fasting period and subsequent enteral refeeding. **Methods:** This prospective, single-center study includes adults referred for PEG after at least one month of oral intake below 50% of energy needs. The MMTT will be performed at PEG placement and after 3–6 months of enteral nutrition. Serial blood samples will be collected from baseline up to 120 minutes post-meal to measure serum glucose, insulin, C-peptide, electrolytes and gastrointestinal hormones (GLP-1/GIP/Ghrelin/Peptide YY), and for metabolomic profiling by spectroscopy. Clinical and nutritional data will be prospectively recorded. Statistical and multivariate analyses will assess metabolic and hormonal changes over time and their potential association with patient refeeding syndrome risk and clinical outcome. The study was approved by the institutional ethics committee, and patient informed consent will be obtained. **Conclusion:** This study integrates MMTT and metabolomic profiling to characterize hormonal and metabolic responses during fasting and refeeding in high-risk PEG patients. Findings are expected to reveal underlying mechanisms of RS, identify potential predictive biomarkers, and improve individualized risk stratification and nutritional management.

Keywords: Refeeding Syndrome; Gastrointestinal Hormones; Metabolomics; Mixed-Meal Tolerance Tests; PEG.

1. Introduction

Refeeding syndrome (RS) is a potentially life-threatening metabolic complication that occurs when nutrition is reintroduced after prolonged fasting or malnutrition, leading to electrolyte imbalances – especially low serum phosphate, magnesium and potassium – and fluid shifts that may cause organ dysfunction. Although RS has been traditionally reported in hospitalized high-risk and critical ill patients, its prevalence is probably underestimated, especially in ambulatory individuals needing artificial feeding [1,2]. Actually, only a few years ago standardized criteria to diagnose and stage RS were published for the first time in the literature by the American Society for Parenteral and Enteral Nutrition updating the classic high-risk RS risk criteria described by the National Institute for Health and Clinical Excellence (NICE) [3,4].

Some studies suggest that refeeding hypophosphatemia is more common in enteral than parenteral feeding in adult hospitalized patients [5]. However, in patients undergoing percutaneous endoscopic gastrostomy (PEG) for long-term enteral nutrition due to chronic disease with prolonged dysphagia, a particular high-risk subgroup, clinical and laboratory signs of RS are seldom identified [6]. In fact, chronic starvation induces histological, ultrastructural and hormonal changes in the duodenal mucosa of patients with high-risk of RS as it was already demonstrated in two recent prospective controlled studies [7,8]. These changes include shortening of the villi with or without mucosal atrophy, ultrastructural evidence of autophagy and increased tissue expression of glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) [7,8]. GLP-1 and GIP are the two primary incretin hormones secreted from the intestine after food ingestion to stimulate insulin secretion from pancreatic beta cells [9,10]. This incretin overexpression may constitute an adaptative response to maintain insulinogenic activity for essential anabolic processes during long-term low food supply. Actually, although the impaired absorption expected from the histologic and ultrastructural changes in the duodenum may probably avoid RS, the enteroendocrine adaptative response of the duodenal mucosa may paradoxically increase its risk after enteral PEG refeeding [8].

Gastrointestinal hormones are bioactive peptides released by the gut in response to nutrient ingestion, playing a key role in regulating digestion, absorption, appetite regulation, and systemic metabolic homeostasis [11]. Beyond incretins, ghrelin and peptide YY (PYY) are other gastrointestinal hormones with complementary roles: ghrelin, produced mainly in the stomach, stimulates appetite, whereas PYY, secreted primary in the distal intestine, promotes satiety and slows gastric emptying, together regulating energy balance and postprandial metabolism [11,12].

Mixed Meal Tolerance Tests (MMTT) are standardized clinical procedures in which a subject ingests a mix-nutrient meal containing carbohydrates, proteins and fats, followed by serial measurements of serum glucose, insulin and other metabolic hormones over time. These tests are used to assess postprandial metabolic responses, including beta-cell function, insulin sensitivity and gastrointestinal hormone secretion [13-15].

Metabolomic analysis is the systematic study of small-molecule metabolites within biological samples, that reflects a functional snapshot of cellular metabolism and its response to physiological and pathological changes.

Combining MMTT with metabolomic analysis provides a dynamic framework to investigate the metabolic shifts occurring during nutritional reintroduction. By capturing real-time changes in glucose, insulin, electrolytes, gastrointestinal hormones, and a wide range of metabolites, this approach may reveal the biochemical pathways involved in RS including disturbances in carbohydrate oxidation, phosphate utilization, and energy metabolism [16-17]. Such integrated functional analysis complement the morphological and immunohistochemical study of duodenal mucosa since it helps to elucidate and clarify the mechanisms underlying electrolyte imbalances and metabolic stress, which ultimately may improve early detection and prevention of RS-related complications in individual high-risk patients.

1.1. Research questions:

A) How is the kinetics of serum concentration of glucose, insulin, electrolytes and gastrointestinal hormones (GLP-1, GIP, ghrelin and PYY) during fasting and refeeding?

B) Which serum metabolites may predict RS development in a high-risk patient population submitted to enteral nutrition?

1.2. Objectives:

A) To define the kinetics of serum glucose, electrolytes and gastrointestinal hormones during fasting and refeeding.

B) To perform MMTT and metabolomic analysis to patients referred for endoscopic gastrostomy after a significant period of low ingestion.

C) To repeat the same evaluation after 3-6 months of long-term enteral nutrition with adequate energy supply.

D) To analyze the evolution of serum metabolites during fasting and refeeding aiming to identify potential predictive biomarkers of RS development in high-risk patients.

2. Methods

2.1. Design:

Single center, observational and prospective study conducted in a tertiary hospital.

2.2. Population and Recruitment:

A convenience sampling will be applied to recruit ambulatory patients referred to PEG for long-term enteral nutrition. Adult patients (age above 18 years) with oral ingestion below 50% of energy daily needs for a minimum period of 1 month and absence of any form of artificial nutrition (oral supplements, tube feeding or parenteral nutrition) before PEG were selected to participate in the study. Patient aged below 18 years, quantified food ingestion above 50% of energy daily needs during the last month before PEG and/or presenting oncologic disease, diabetes mellitus or an expected survival after PEG below 3 months were excluded. The evident inability to provide credible anamnestic data or the impossibility to have an appropriate clinical and nutritional follow-up were additional exclusion criteria. Table 1 summarizes patient inclusion and exclusion criteria for the study.

2.3. Data collection:

For each patient included in the study, several clinical features will be recorded:

1. Gender and age.
2. Height, weight and Body Mass Index (BMI). BMI will be calculated as weight/height² (kg/m²) or, if patients could not stand up for height evaluation, BMI will be estimated using the mid-upper arm circumference (MUAC) and regression equations described by Powel-Tuck and Hennessy [18], as this method has been previously used and proved to provide reliable BMI estimation in PEG patients [19].
3. Anthropometry, including MUAC, calf circumference (CC), and triceps skinfold thickness (TSF). Mid-arm muscle area (MAMA) will be calculated from MUAC and TSF.
4. Underlying disease and clinical indication for PEG.
5. A dietary history referring to the month prior to PEG. Energy-protein requirements will be calculated according to the patient´s clinical data.

Subsequently, the following standardized methodology will be applied:

1. Placement of a peripheral venous catheter with extension line.
2. PEG will be performed by two gastroenterologists using the “pull” method described by Gauderer–Ponsky [20] with patients under deep sedation.
3. Blood sampling prior to the procedure for one EDTA K2-Gel tube (7.5ml) and one serum tube (biochemistry). The serum tube will be used for the required serum assays performed at the hospital clinical pathology department, while the EDTA K2-Gel tube will be used to obtain plasma, which will be stored for subsequent metabolomic analysis at the university research laboratory.

4. The extension must be flushed with heparinized saline between samplings. An initial sample will be collected at time 0 (immediately before the meal), followed by administration of the test meal as a bolus through the PEG tube over less than 15 minutes. The administered meal should be of low volume and standardized for all patients (Fortimel®, 125ml, 300Kcal, and 18g protein). Blood samples will then be repeated at 15, 30, 45, 60, 90 and 120 minutes after the meal, with strict adherence to the scheduled times. Patients should remain supine with the head of the bed elevated at 45 degrees. All samples must be delivered to the laboratory within 30-60 minutes after collection for further processing.
5. Capillary blood glucose and vital parameters (heart rate and blood pressure) will also be assessed at the same time points.
6. EDTA K2-Geal tubes must be placed on dry ice until centrifugation. Samples should be centrifugated at 2500g for 12 minutes at 4ºC. The plasma from each tube should then be pipetted into microtubes of approximately 1ml (totaling 3-4ml per time period) and stored at -80ºC whenever possible (alternatively at -20ºC) until analysis. Each tube must be properly labeled with the coded patient identifier and the corresponding sampling time point for further data registration.
7. Serum assays performed in the hospital laboratory will include measurements of glucose, serum electrolytes (phosphorus, magnesium and potassium), insulin, c-peptide, GLP-1, GIP, ghrelin and PYY at each sampling point. The tubes designated for metabolomic analysis after initial centrifugation should be frozen as explained above until be transported to ICBAS-UP for subsequent analysis.

Those mixed meal tests will be performed on all patients at the time of PEG placement (baseline) and repeated after 3-6 months of ambulatory enteral nutrition. During these periods every patient will undergo an individual adapted diet calculated by the dietitian, administered through the tube, aiming to reach the expected energy and macronutrient needs according to the underlying clinical condition. The use of oral nutrition supplements will be allowed if the individual diet is not sufficient to fulfill the nutritional plan.

Before MMTT all patients must undergo eight hours of mandatory fasting as it is already required for PEG procedure and tube replacement.

The study will be finished after 15 patients were recruited and completed follow-up at both time periods.
The patient flowchart is described in figure 1.

2.4. Statistical Analysis

The statistical analysis will be performed using the Statistical Package for Social Sciences (IBM SPSS® Statistics, version 29.0). Continuous variables will be expressed as the mean and standard deviation or medians and interquartile ranges. Categorical variables will be reported as total and relative frequencies. Normality will be assessed using the Kolmogorov Smirnov test. Inferential analysis will be performed to study the evolution of

serum concentration of electrolytes, glucose and hormones during the Mixed Meal Tests comparing with the reference values defined for the normal population. The comparison between the evaluation performed at baseline and after 3-6 months of enteral nutrition will also be accomplished. Multivariate analysis will also be used to investigate the association between hormone expression and other clinical data such as patient survival and refeeding syndrome development.

2.5. Metabolomic analysis:

Plasma samples will be mixed and incubated with cold methanol (1:2 v/v) for protein precipitation. After that, samples will be centrifuged and the supernatant collected and dried in a vacuum concentrator. Samples will then be resuspended in a fumarate solution in D20 (2 mM, pH 7). Sodium fumarate (2 mM; 6.50 ppm) will be used as an internal reference. Spectra will be acquired using NOR5X3INSB optimizer inserts (Norell, Landisville, NJ, USA) on a Bruker Ascend 600 MHz Avance III HD spectrometer equipped with a 5mm CPP BBO 600S3 BB-H F-D-05 Z probe. The Bruker TopSpin X software (Bruker Corporation, Billerica, MA, USA) will be used for processing the spectra and metabolite quantification (Acetate, Acetoacetate, Alanine, Citrate, Creatine, Formate, Glutamine, Glycine, Isoleucine, Lactate, Leucine, Proline, Serine, Valine). Peaks will be assigned through comparison with reference spectra using Chenomx (Chenomx Inc., Edmonton, Canada) and the Human Metabolome Database (HMDB) V 5.0 (128). Initial data structure will be evaluated by principal component analysis (PCA), followed by partial least squares discriminant analysis (PLS-DA) to identify metabolite contributions to class separation. Loadings of pair-wise comparison PLS-DA models will be calculated by multiplying the variable weights (w) with the respective standard deviations and will be color-coded according to the size of variable importance on projection (VIP) values. VIP>1 will be considered relevant to group separation.

2.6. Ethics and Confidentiality:

This research will be conducted in accordance with the Helsinki Declaration and was already approved by the institutional Ethics Committee. All the participants or their caregivers must agree with all study procedures and sign the informed consent form. Participation will be entirely voluntary, and patients may decide to leave the study at any time. All data collected from participants will be strictly anonymous and confidential.

3. Discussion

The current study aims to perform MMTT and metabolomic analysis to patients referred for PEG with chronic reduced ingestion or established malnutrition, a population defined as high-risk of RS, in order to define the evolution of serum glucose, insulin, electrolytes, gastrointestinal hormones (GLP-1, GIP, ghrelin and PYY) and several cellular metabolites during fasting and immediate refeeding. This analysis will contribute to unveil some of the underlying pathogenic mechanisms involving RS, especially with regard to enteroendocrine adaptative changes, to identify potential predictive biomarkers for RS development, and to optimize patient risk stratification and overall clinical management. The repetition of both analyses in each patient after 3–6 months of adjusted enteral nutrition will enable a comparison between the period of long-term fasting and the period of adequate intake following the nutritional intervention, thereby contributing to the validation of the abnormal findings anticipated in the first phase of the study.

Indeed, the current strategies used in clinical practice to stratify the risk of refeeding syndrome (RS) are becoming clearly insufficient, as several studies have reported their limited accuracy in predicting this condition [5]. Most of these criteria rely on body mass index, unintentional weight loss, duration of nutritional deprivation, low serum electrolyte concentrations (potassium, phosphorus, and magnesium), and a history of alcohol use or specific drug consumption. More recent evidence has incorporated body composition–based criteria derived from bioimpedance analysis and considers underlying diseases or comorbidities in patient risk stratification [3,4]. The identification of general biomarkers based on hormonal or metabolite profiles may enable a more precise stratification of patients at risk for RS, while the integration of the mixed-meal tolerance test (MMTT) could further refine individualized risk assessment in specific clinical contexts.

Previous studies conducted by our group demonstrated that prolonged fasting induces alterations in the duodenal mucosa, including changes in the tissue expression of gastrointestinal incretins, with potential implications for immediate nutrient absorption following nutritional reintroduction and for the development of refeeding syndrome (RS) [7,8]. The current protocol will include a functional analysis aimed at determining whether the previously observed immunohistochemical patterns correlate with serum concentrations after a controlled “refeeding simulation.” It is expected that serum GLP-1 and GIP concentrations will increase immediately after refeeding, supporting the previously reported accumulation of both incretins within intestinal cells during prolonged fasting [8]. A similar pattern is anticipated for PYY, whereas an inverse response is expected for ghrelin, whose serum levels typically decrease following meal ingestion [11].

In patients at high risk for RS, metabolomic assessment is expected to demonstrate a signature consistent with prolonged catabolic adaptation and impaired substrate flexibility, including increased levels of ketone bodies such as acetoacetate, alterations in amino acid pools involved in gluconeogenesis and protein turnover like alanine, glutamine, glycine, serine and proline, disruptions in branched-chain amino acids such as leucine, isoleucine and valine, and shifts in intermediates of energy metabolism such as citrate, lactate, formate and creatine. Together, these disturbances reflect impaired oxidative capacity and sustained reliance on fat and

protein breakdown prior to refeeding, thereby providing a biochemical signature that may help to identify patients most vulnerable to metabolic collapse during early nutritional rehabilitation [21,22].

A less pronounced and less marked response to early refeeding is anticipated after 3–6 months of enteral nutrition via PEG, as most hormonal and metabolic imbalances are expected to be corrected with the progressive normalization of nutrient supply. This hypothesis would be consistent with the normalization of the histological and ultrastructural alterations observed in the duodenal mucosa of similar patients after a comparable period of nutritional support, as previously reported in our recent study [7].

This study protocol has some limitations that should be acknowledged. The main weakness is its uncontrolled design. Indeed, obtaining an appropriate control group to perform the MMTT in metabolically healthy individuals is challenging, both for ethical reasons and because of the inherent variability in normal postprandial hormonal and metabolic responses. To mitigate this issue, each patient will serve as his or her own control, with baseline results compared to those obtained after a period of presumed adequate nutrition during the second evaluation. Another limitation is the interindividual variability in patients' diets between the two study visits. Standardizing dietary intake over a 3–6-month period would be virtually impossible unless nutrition were exclusively formula-based, which would be social and economically unsustainable and would not reflect the real-life clinical management of PEG-fed outpatients.

In conclusion, the present study integrates the assessment of gastrointestinal hormones and metabolomic profiling following Mixed-Meal Tolerance Tests in patients at high risk for refeeding syndrome, as stratified by the classic risk criteria. To the best of our knowledge, this is the first study to apply this methodology in this specific patient population, aiming to elucidate the enteroendocrine and metabolic responses to fasting and refeeding. These findings are expected to complement morphological and immunohistochemical analyses and may contribute to advancing and redefining current approaches to RS risk evaluation.

FIGURE 1

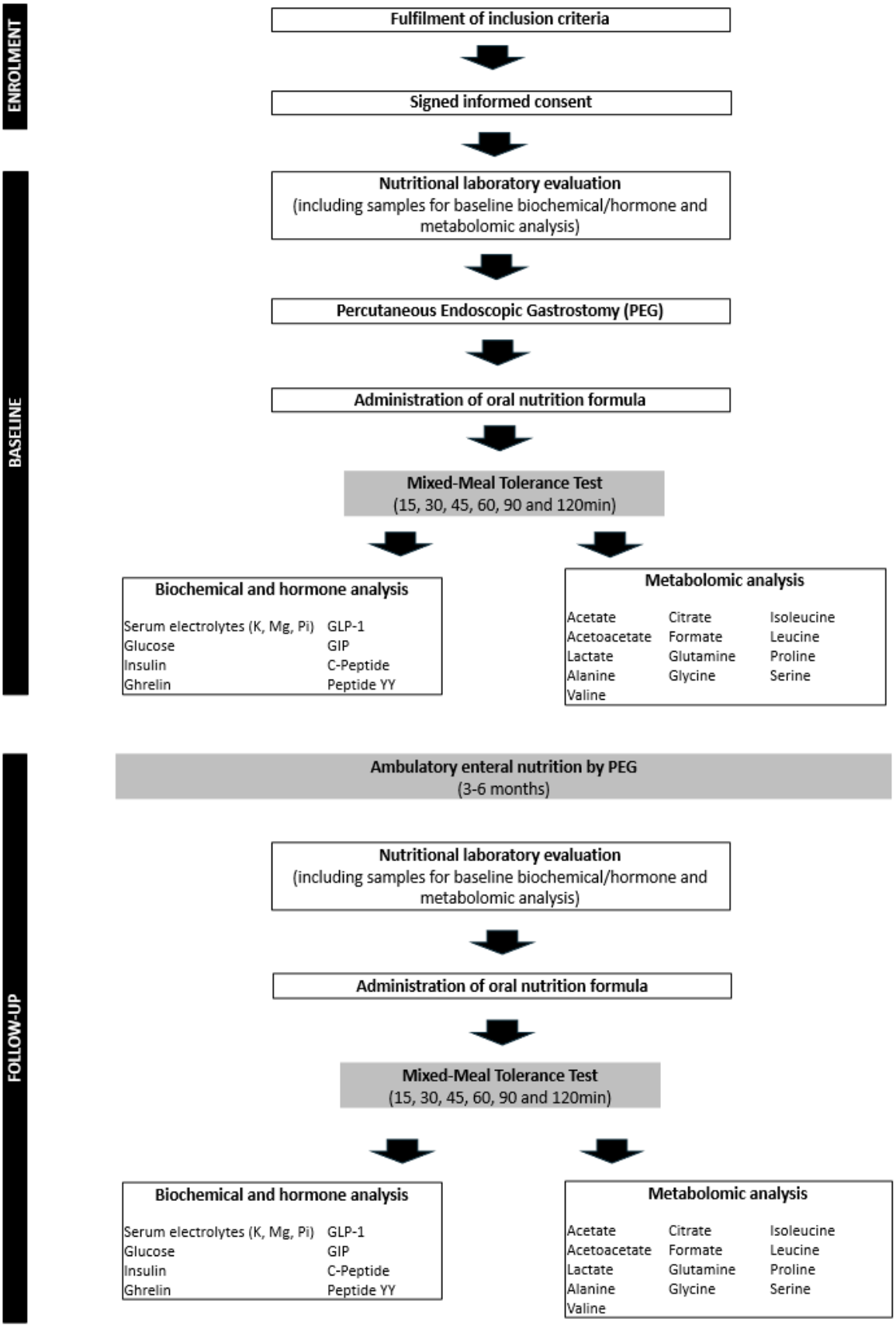


Figure 1 – Study flow diagram

TABLE 1

Inclusion Criteria
Age above 18 years.
Oral ingestion below 50% of energy daily needs for ≥ 1 month.
Absence of artificial nutrition before PEG placement.
Exclusion Criteria
Age below 18 years.
Quantified food ingestion above 50% of energy daily needs during the last month.
Oncologic disease.
Diabetes mellitus.
Expected survival below 3 months.
Inability to provide credible anamnestic data.
Impossibility to have an appropriate clinical and nutritional follow-up.

Table 1 – Summary of patient inclusion and exclusion criteria

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