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The SURMetaGIT study: Design and rationale for a prospective pan-omics examination of the gastrointestinal response to Roux-en-Y gastric bypass surgery

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Abstract

Objective: To describe the protocol of the **SUR**gically induced **Meta**bolic effects on the Human **G**astro**I**ntestinal **T**ract (SURMetaGIT) study, a clinical pan-omics study exploring the

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gastrointestinal tract as a central organ driving remission of type 2 diabetes mellitus (T2DM) after Roux-en-Y gastric bypass (RYGB). The main points considered in the study's design and challenges faced in its application are detailed.

Methods: This observational, longitudinal, prospective study involved collection of gastrointestinal biopsy specimens, faeces, urine, and blood from 25 obese women with T2DM who were candidates for RYGB (20 patients for omics assessment and 5 for omics validation). These collections were performed preoperatively and 3 and 24 months postoperatively. Gastrointestinal transcriptomics; faecal metagenomics and metabolomics; plasma proteomics, lipidomics, and metabolomics; and biochemical, nutritional, and metabolic data were assessed to identify their short- and long-term correlations with T2DM remission.

Results: Data were collected from 20 patients before and 3 months after RYGB. These patients have nearly completed the 2-year follow-up assessments. The five additional patients are currently being selected for omics data validation.

Conclusion: The multi-integrated pan-omics approach of the SURMetaGIT study enables integrated analysis of data that will contribute to the understanding of molecular mechanisms involved in T2DM remission after RYGB.

Keywords

Roux-en-Y gastric bypass, type 2 diabetes mellitus, gene expression, proteomic, metabolomic, lipidomic, metagenomic, microbiota, faecal water

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Introduction

Obesity, a condition characterized by excessive body fat accumulation, has reached epidemic levels worldwide.[1] Excessive adipose tissue is associated with several obesity-related comorbidities that may lead to early death, including dyslipidaemia, type 2 diabetes mellitus (T2DM), and hypertension.[1,2] Because primary care involving dietary and lifestyle modifications often cannot be implemented in the long term, bariatric surgery (BS) is the most effective treatment currently available for severe obesity.[2,3]

Compared with primary clinical care, BS substantially reduces morbidity and mortality associated with excessive body weight.[4] The American Society for Metabolic and Bariatric Surgery reported that approximately 179,000 BS procedures were performed in 2013, 34.2% of which were performed using Roux-en-Y gastric bypass (RYGB).[5] RYGB combines stomach restriction with intestinal malabsorption, enabling effective and sustainable weight loss.[6]

Obese patients with T2DM may experience short-term glucose homeostasis after RYGB, often before significant weight loss; normal postoperative plasma glucose levels were observed in 30% of patients after an average of 2.8 days of hospitalization.[7] A meta-analysis showed full remission of T2DM after BS in 76.8% of 22,094 patients.[8] Higher rates (83%) of T2DM control and remission have been obtained with procedures that include gastrointestinal (GI) bypass, such as RYGB.[7,9] However, other studies have revealed long-term T2DM recurrence rates ranging from 17% to 68%, the main predictors of which include a longer preoperative T2DM duration, a small amount of excess weight loss, and postoperative weight regain.[10–12]

The restrictive and malabsorptive aspects of RYGB may explain the postoperative body weight loss, but they do not fully

explain the metabolic effect of RYGB on short-term T2DM remission or long-term recurrence. We hypothesized that RYGB-induced anatomic GI changes modify GI metabolic functions, thereby affecting systemic metabolism. The small intestine is a neuroendocrine organ that expresses more than 30 gene-encoding hormones under several cell-signalling pathways and produces hundreds of bioactive peptides that participate in food control and glycaemic homeostasis.[13,14] Experimentally, RYGB-induced anatomic changes have been shown to modify intestinal gene-encoding hormone expression, in turn affecting glucose metabolism.[15] Another consequence of intestinal anatomic changes is alteration of the resident microbiota, which appears to influence a wide range of diseases including obesity and T2DM.[16] After RYGB, increased richness of the intestinal microbiota was observed and found to be associated with changes in white adipose tissue gene expression.[17] This observation suggests a possible role of intestinal bacteria as drivers of postoperative metabolic effects.

Multifactorial mechanisms can thus influence the metabolic effects potentially induced by RYGB, implying the need for a multi-integrated study approach. We are conducting a prospective clinical study to examine whether T2DM remission after RYGB is driven by postoperative changes in GI "omic" functions. The **SUR**gically induced **Meta**bolic effects on the Human **G**astro**I**ntestinal **T**ract (SURMetaGIT) study considers the human GI tract to be a central organ driving RYGB-induced metabolic effects, including not only TD2M remission and recurrence but also improvement or worsening (e.g., micronutrient deficiency) of other obesity-associated comorbidities. The main challenges faced in the development of our protocol were minimization of patient risk associated with biopsy collections and identification of key associations between postoperative GI transcriptomic changes and local and systemic responses related to surgically induced metabolic effects. With the intent of facilitating researchers' performance of studies involving multi-integrated analysis, this article describes the design of the SURMetaGIT protocol and discusses how we have overcome these challenges. We also present the initial results of the patient selection and data collection, which reflect the success achieved by the practical application of this protocol.

Patients and methods

Study design

The SURMetaGIT study is an observational, longitudinal, prospective study testing the general hypothesis that RYGB-induced anatomic alterations modify GI function as an adaptive mechanism that affects systemic metabolism. The study protocol comprises several steps to conjunctly investigate associations between potential RYGB-induced changes in GI gene expression and postoperative biomarkers related to improvement or worsening of obesity-associated comorbidities, mainly T2DM remission. The SURMetaGIT study was approved by the local ethics committee (CAPPesq1011/09) and registered at Plataforma Brasil (19339913.0.0000.0068) and www.clinicalTrials.gov (NCT01251016). The protocol is currently being implemented; the steps completed and those still in the process of completion are shown in Figure 1.

Patient selection

Patients were recruited from the Surgical Gastroenterology Department of the Hospital das Clínicas at the Medical School of the University of São Paulo (HC-FMUSP) according to the following inclusion criteria: female sex (approximately 80% of patients undergoing BS at HC-FMUSP are female), age of 18 to 60 years,

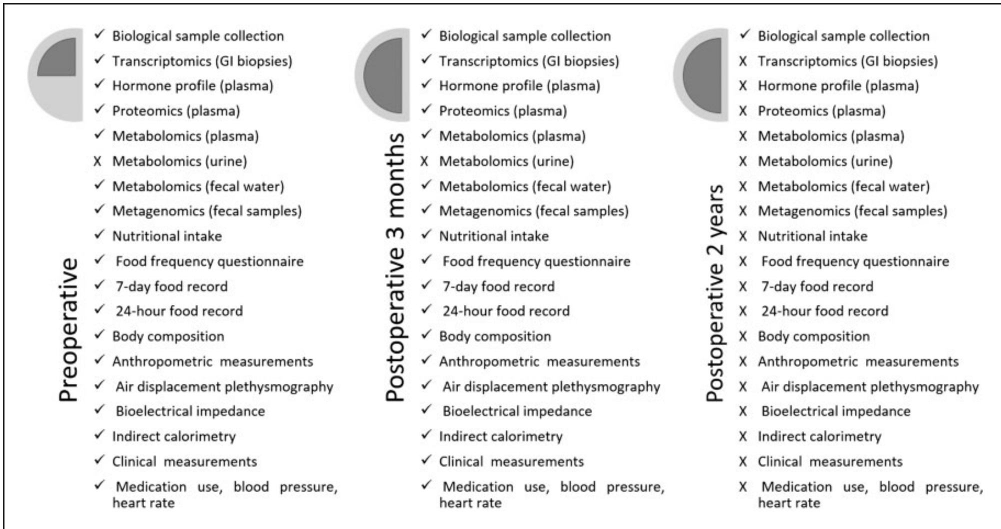

Figure 1. Methodological steps of the SURMetaGIT study. √ Completed steps; x steps in process of completion.
GI, gastrointestinal.

established diagnosis of T2DM (fasting glucose concentration of ≥ 126 mg/dL and glycated haemoglobin level of ≥6.5%) and/or use of an oral antidiabetic drug, body mass index of ≥35 kg/m^2, candidacy for RYGB, absence of a diagnosis of GI *Helicobacter pylori* infection, and interest in and availability for study participation. The exclusion criteria were refusal to participate in the study, use of insulin, diagnosis of thyroid or hepatic disease, candidacy for another BS procedure, antibiotic use in the month preceding faecal material collection, probiotic and/or prebiotic use, dementia and other cognitive and behavioural problems, and current or recent participation in another interventional study protocol. Written informed consent was obtained from each patient prior to participation in the trial.

Primary and secondary endpoints

The primary endpoints of the SURMetaGIT study are potential changes in the expression of GI genes that may affect glucose homeostasis and contribute to the short-term remission of T2DM observed following RYGB. To determine whether such changes occur, the protocol involves assessment of the transcriptomic profiles of GI biopsy specimens before and at 3 and 24 months after RYGB to test their correlations with the following systemic and local data obtained at the same time points: a) systemic (plasma or serum) biochemical and hormonal markers of glucose homeostasis, b) plasma proteomic profiles, c) plasma lipidomic profiles, d) plasma and 24-h urine metabolomic profiles, e) faecal microbiota profiles, and f) faecal water profiles.

The study was designed to examine several hypothesis-generating secondary endpoints related to the improvement or worsening of obesity-associated metabolic comorbidities other than T2DM in the short term and T2DM recurrence in the long term. For instance, obesity is associated with nutritional deficiencies and changes in body composition that may affect health and can be aggravated or improved after RYGB.[18,19] We thus planned to examine potential

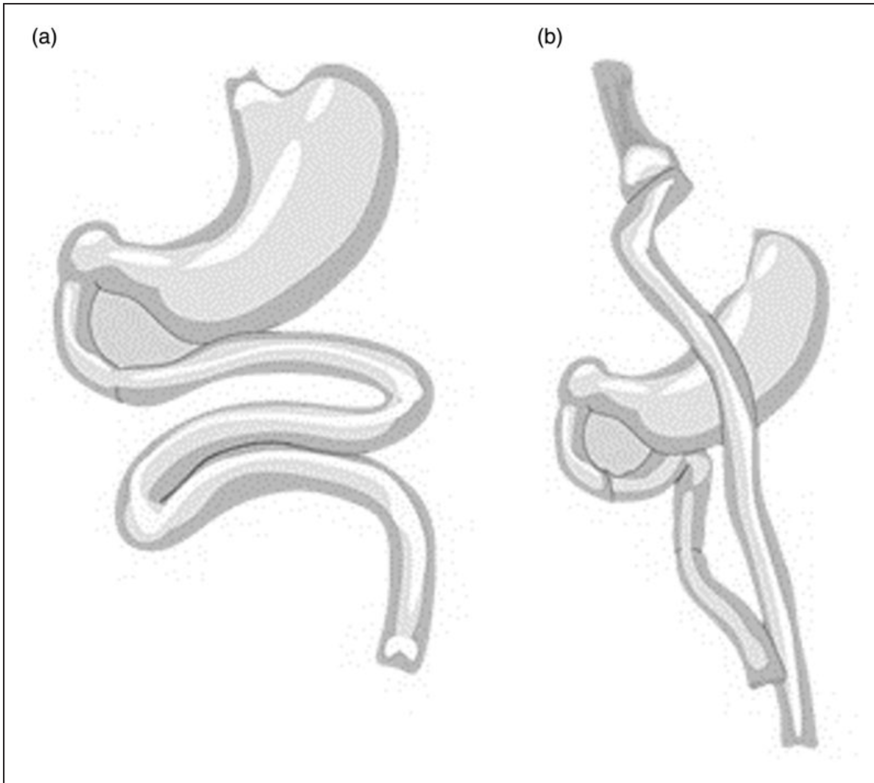

Figure 2. Anatomic changes induced by Roux-en-Y gastric bypass (RYGB) surgery. (a) Intact gastrointestinal tract and (b) anatomic rearrangement of gastrointestinal tract after RYGB.

associations of postoperative changes in GI gene expression with the following nutritional, metabolic, body composition, and energy expenditure markers assessed at the same time points: a) nutritional intake, b) compartmental body composition, c) indirect calorimetry, and d) other clinical measurements.

BS. All patients underwent standardized RYGB without silicon rings and with biliopancreatic (50 – 60 cm) and alimentary (100 – 120 cm) limbs. RYGB reduces the stomach volume by creating a proximal gastric pouch with a capacity of approximately 30 mL, excluding the rest of the stomach, duodenum, and proximal jejunum from the flow of nutrients. After RYGB, about 95% of ingested food bypasses the stomach, the entire duodenum, and a short portion of the jejunum (Figure 2).

Double-balloon enteroscopy for GI biopsies. Double-balloon enteroscopy (DBE) was performed at the Gastrointestinal Endoscopy Unit of HC-FMUSP about 1 week before and 3 and 24 months after RYGB. Before DBE, the patients fasted for 12 h and abstained from diabetes medication for 3 to 5 days. Briefly, each patient was placed in the left lateral position under deep sedation, achieved with 1 µg/kg of fentanyl (Fentanest; Cristália, São Paulo, Brazil), 5 min before anaesthetic induction. For anaesthesia, we applied our institution's standardized protocol for severely obese individuals as follows: dexmedetomidine (Precedex; Hospira, Lake

Forest, IL, USA) was administered at 2 μg/kg/h for 15 min under supplementary oxygen *via* nasal catheter (2 L O_2/min), after which the examination was initiated. Thereafter, dexmedetomidine administration was reduced to 0.4 μg/kg/h, followed by administration of propofol (Diprivan; AstraZeneca, London, England) at approximately 70 to 100 μg/kg/min when necessary. The dosages required for deep sedation and anaesthesia were calculated based on the ideal corrected weight (ideal weight plus 30% of the difference between actual and ideal weight) preoperatively and actual weight postoperatively. The safety of the enteroscopic procedure was monitored continuously by electrocardiography, pulse oximetry, and blood pressure assessment. After establishment of anaesthesia, the enteroscope (EN-450T5; Fujifilm, Tokyo, Japan) was inserted orally and advanced through the GI tract (including the excluded stomach in the postoperative period), with systematic pleating of the small bowel on the enteroscope.[20] A flexible hose and two balloons (TS-13140™; Fujifilm) were inflated and deflated sequentially, allowing the instrument to progress through the small intestine with simultaneous visualization. These actions limited intestinal stretch and improved transmission of the manoeuvres to position and stabilize the end of the enteroscope. GI mucosal biopsy specimens (approximately 15 – 20 mg each of the stomach cardia, stomach fundus, duodenum, jejunum, and ileum) were then collected and stored immediately in liquid nitrogen and then at −80°C. The GI biopsy sites were marked preoperatively with India ink (Spot; GI Supply, Camp Hill, PA, USA), allowing sample collection from the same sites in the postoperative period.

Primary endpoint analyses. **GI transcriptomics.** For transcriptomic analysis, total RNA was extracted from approximately 15 to 20 mg of tissue using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA concentrations were measured with a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA), and quality was assessed with the RNA integrity number using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). For each sample, 300 μg of total RNA was used for microarray analyses conducted with the GeneChip 1.0 ST Array for Humans (Affymetrix, Santa Clara, CA, USA). Sample preparation, processing, and hybridization were conducted according to the instructions provided in the GeneChip expression analysis technical manual. Array quality was checked using boxplots, correlation analysis, and principal component analysis (PCA). Expression values were obtained using the robust multi-array average data pre-processing method.[21] The combat method (http://jlab.byu.edu/ComBat/Abstract.html) was used to remove batch effects.[22] The significance of microarrays[23] and rank products[24] methods were analysed to select differentially expressed genes using the criterion of $P < 0.05$ (corrected for the false discovery rate).[25] All analytical tools are available in the R Bioconductor program (http://www.bioconductor.org/). Real-time reverse-transcription quantitative polymerase chain reaction was performed to validate the significance of changes in some genes of interest identified by microarray analysis using TaqMan gene expression assays (Life Technologies, Carlsbad, CA, USA).

Biochemical and hormonal markers of glucose homeostasis. The concentrations of glucose, insulin, glucagon, and C-peptide; lipid profiles (low-density lipoprotein cholesterol, very-low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides); and concentrations of GI hormones (glucagon-like peptide 1, glucose-dependent insulinotrophic peptide, peptide YY, and ghrelin) were measured in plasma or

serum samples collected several weeks before surgery; at 3, 15, 30, 45, 60, 75, and 90 days after RYGB; and at 1 and 2 years after RYGB. The glycated haemoglobin concentration was also measured before, 3 months and at 1 and 2 years after RYGB. All blood samples were collected after 12-h fasts, and collection was performed 30, 60, 90, and 120 min after oral intake of 200 mL of a liquid formula diet (Ensure; Abbott, Abbott Park, IL, USA). The glucose, glycated haemoglobin, insulin, and C-peptide concentrations and lipid profiles were analysed at the Clinical Laboratory of HC-FMUSP using enzymatic methods (glucose and lipid profiles), liquid chromatography (LC) (glycated haemoglobin), and electrochemiluminescence (insulin and C-peptide). GI hormones were analysed using the multiplex technique (Merck Millipore, Billerica, MA, USA). At the time of initial blood collection, additional samples were collected to obtain plasma (EDTA tubes, centrifugation at 878 g, 4°C, 10 min) and serum (dry tubes, centrifugation at 2800 rpm, 20°C, 10 min) for proteomic, metabolomic, and lipidomic analyses. For GI hormone and proteomic assessments, plasma samples were treated with a protease inhibitor (Complete Mini; Roche, Indianapolis, IN, USA).

Plasma proteomics and lipidomics. We planned to correlate the activity of differentially expressed GI genes with plasma proteomic and lipidomic data obtained by mass spectrometry (MS) at the systemic level. Global plasma lipid profiles were assessed (shotgun lipidomics) before and after RYGB by matrix-assisted laser desorption/ionization MS. Shotgun proteomic analyses were performed for protein profiling using nano-scale LC tandem MS (LC-MS/MS), a well-established technique for peptide identification from complex mixtures (i.e., protein digests). We planned to further validate the identification of proteins of interest by shotgun analysis using MS, with a targeted approach when necessary.[26]

Plasma and urine metabolomics. Plasma obtained from EDTA-treated blood samples was aliquoted in polypropylene microtubes and stored at −80°C until metabolomic analysis. Next, 24-h urine samples were collected in appropriate containers, the supernatants were removed (15 min of centrifugation at 1500 g, 4°C), and the remaining samples were aliquoted in polypropylene microtubes and stored at −80°C until metabolomic analysis. We planned to assess the plasma and urine metabolomic profiles using global (to describe a large number of metabolites) and targeted approaches, enabling the collection of qualitative and quantitative data. The following analyses were also planned: global metabolomic analysis, performed as described previously[27,28]; targeted metabolomic analysis, described in detail in patent US 2007/0004044 (http://www.freepatentsonline.com/20070004044.html); metabolite quantification, performed using a reference and appropriate internal standards; and metabolite panels comprising 183 metabolites (40 acylcarnitines; 19 proteinogenic amino acids, ornithine, and citrulline; 19 biogenic amines; sum of hexoses; 76 phosphatidylcholines; 14 lyso-phosphatidylcholines; and 15 sphingomyelins). The web-based analytical pipeline Metabo Analyst 2.0 (www.metaboanalyst.ca) and the ROC Curve Explorer & Tester (http://www.roccet.ca/ROCCET/) were used for data upload and importation, respectively. The MetaboAnalyst protocol was used to normalize data for univariate and multivariate analyses.[29]

Metagenomics. The patients performed self-collection of faecal samples at home using a specimen collection system (Fisherbrand Commode Specimen Collection System; Fisher Scientific, Hampton, NH, USA), as described previously.[17] The samples were frozen immediately at −20°C and transported to our laboratory by a motorcycle courier service specializing in the transport of biological samples under temperature

control. At the laboratory, the faecal samples were aliquoted immediately (without thawing) and stored at −80°C until DNA extraction and storage at −20°C, as described previously[30] and as detailed in International Microbiome Standard IHMS-SOP06 (http://www.microbiome-standards.org). Library preparation for the MiSeq Sequencing System (Illumina, San Diego, CA, USA) involved amplification of the V3 – V4 region of 16 S ribosomal DNA genes, as described previously.[17,31] Amplification relied on MolTaq 16 S and the corresponding master mix. The polymerase chain reaction mix contained 10 ng of DNA, 1 µL of dNTPs (10 mM), 1.25 µL each of forward and reverse primer (20 µM), and 0.5 µL of Taq in a total volume of 50 µL. The program was set to 94°C for 60 s, followed by 30 cycles at 94°C for 60 s, 65°C for 60 s, 72°C for 60 s, and final extension at 72°C for 10 min. Sequencing was performed using MiSeq technology and the Genopole Toulouse Midi-Pyrenees genomics platform (http://get.genotoul.fr/). The resulting sequences were assigned to taxonomic levels ranging from phylum to genus using the Ribosomal Database Project (http://rdp.cme.msu.edu/). To identify metagenomic changes after RYGB, we planned to further cluster these sequences into operational taxonomic units or phylotypes at 97% identity using QIIME software[32] and the CD-HIT[33] program.

Metabolomics of faecal water. In microtubes (Eppendorf, Hamburg, Germany), 100-mg stool samples with 500 µL ultrapure water were homogenized at 25°C for 5 min and then centrifuged at 25,200 g for 10 min. The supernatants of faecal water were aliquoted (1 mL) and stored at −80°C. Eventual residues were further extracted with ice-cold methanol following the same first-step extraction procedure. Analysis of faecal water metabolites was performed using LC-MS (HPLC Agilent 12906550 and iFunnel Q-TOF; Agilent Technologies) with subsequent metabolite confirmation by LC-MS/MS. Chromatographic separation was performed using a C18 column (ZORBAX Extend-C18, 2.1 × 50 mm, 1.8 u; Agilent Technologies). Spectra were accumulated and processed using mass hunter qualitative analysis. To assess faecal metabolomic changes, we chose to perform PCA (3:11 Pirouette software; Infometrix, Bothell, WA, USA) with data from a database matrix based on the relative intensity of ions (m/z values) in the profiles obtained by LC-MS using Lab Solutions software (Shimadzu, Kyoto, Japan) and Excel (Microsoft, Redmond, WA, USA).[34] Other discriminant techniques available for the analysis of metabolomic datasets, including partial least-squares discriminant analysis [35] and orthogonal partial least-squares discriminant analysis[36], may be used.

Secondary endpoint analyses. **Nutritional intake.** Nutritional intake was assessed several weeks before, 3, 12 and 24 months after RYGB. Two trained dietitian oriented the patients as described previously.[37] The types and amounts of food and beverages consumed were assessed using three nutritional tools: 24-h food records, 7-day food records, and food frequency questionnaires. The amount of food consumed was recorded in terms of cooking units (e.g., tablespoons, cups) as guided by the *Consumo Alimentar: Visualizando Porções* (*Food Consumption Book: Viewing Portions*).[38] Data were used to calculate total calories and macronutrient and micronutrient consumption with Virtual Nutri Plus software (www.virtualnutriplus.com.br).[39] The following data sources were chosen to determine the food nutritional composition: the food chemical composition table developed by Philippi[40] and the Brazilian food chemical composition table.[41] We planned to analyse pre- and postoperative nutritional consumption using recommendations for daily nutrient intake from the dietary reference intakes of the Food and Nutrition

Board, Institute of Medicine,[42] as a reference.

Anthropometric measurements. Anthro pometric measurements were performed preoperatively, every 15 days for 3 months, and 1 and 2 years after RYGB. Body weight was measured using an electronic platform scale with the patient standing in the centre of the scale platform while barefoot and wearing only light clothes. Height (cm) was measured using a stadiometer (Sanny; American Medical do Brasil, São Paulo, Brazil) with the patient standing barefoot, heels together, spine erect, and arms extended next to the body. The body mass index (weight/height^2) was also calculated.[43] The circumferences of the waist (narrowest diameter between the xiphoid process and iliac crest or near the umbilical region) and hip (widest diameter over the greater trochanters) were measured by adjusting a tape in the horizontal plane, and the waist-to-hip ratio was calculated.[44,45]

Body composition. Body composition was assessed by bioelectric impedance analysis (QuadScan4000; Bodystat, Douglas, Isle of Man, British Isles) and air-displacement plethysmography (ADP) (BOD POD BC system device; Life Measurement Instruments, Concord, CA, USA) in the preoperative period and 3, 12 and 24 months after RYGB. The tetrapolar body bioelectric impedance analysis equipment generated electric currents of 5, 50, 100, and 200 kHz with a calibrated signal applied to the skin *via* adhesive electrodes placed on the right-side limbs. Resistance and reactance were used to calculate the total body water, fat mass, and fat-free mass.[46,47] For ADP, Boyle's law ($P1.V1 = P2.V2$, where P is pressure and V is volume) was used to determine body volume and to calculate body fat and fat-free mass by applying densitometry principles ($d = m/v$) and using Siri's equation: $BF\% = (4.95/D - 4.5) \times 100$, where BF is body fat and D is density. All ADP measurements and calculations were performed automatically by the system's software, based on air volume and pressure variations inside the equipment chamber when empty and when occupied by the patient.[46,47] During ADP evaluations, the patients wore only underwear and a cap to keep the hair contained and remained in a seated position inside the chamber. The patients removed metallic objects such as earrings, rings, chains, and piercings for both assessments performed to estimate body composition.

Indirect calorimetry. Indirect calorimetry was used to estimate the resting energy expenditure (REE) preoperatively and at 3 months and 1 and 2 years after RYGB. The patients were assessed after a 12-h overnight fast and were instructed to refrain from any unusual physical activity in the 24-h period before REE measurement. After the patients had rested for 30 min in a recumbent position, their oxygen consumption and carbon dioxide production were measured continually for 35 min using a ventilated hood and open circuit under a canopy (Deltatrac Monitor II MBM-200; Datex-Engstrom Division, Instruments Corp., Helsinki, Finland), as described previously.[48] The REE was calculated without using urinary urea nitrogen, according to the Weir equation.[49] Before each REE measurement, the monitor was calibrated using mixed reference gases of known composition.

Postoperative follow-up: other clinical measurements. Outpatient follow-up was performed immediately postoperatively, every 2 weeks for 3 months, and 12 and 24 months after RYGB. Data on medication use, blood pressure, and heart rate were obtained.

SURMetaGIT study data were obtained during 24 scheduled medical visits. The procedures are detailed in Table 1 and Figure 3.

Statistical analysis

Sample size calculation. A sample of 20 participants was considered to be adequate for

Table 1. Schedule of study procedures.

Action/ examination	V1 Pre-op	V2 Pre-op	V3 Pre-op	V4 Pre-op	V5 Pre-op	V6 Pre-op	V7 Pre-op	V8 Post-op 3 d	V9 Post-op 15 d	V10 Post-op 30 d	V11 Post-p 45 d	V12 Post-op 60 d	V13 Post-op 75 d	V14 Post-op 3 m	V15 Post-op 3 m	V16 Post-op 3 m	V17 Post-op 3 m	V18 Post-op 1 y	V19 Post-op 1 y	V20 Post-op 1 y	V21 Post-op 2 y	V22 Post-op 2 y	V23 Post-op 2 y	V24 Post-op 2 y
Informed consent	X																							
Biopsy collection						X										X							X	
Blood collection		X			X		X	X	X	X	X	X	X		X		X		X	X		X		X
24-h urine collection				X										X				X			X			
Stool collection				X					X					X				X			X			
FFQ				X										X				X			X			
7-day food record				X										X				X			X			
24-h food record				X										X				X			X			
Anthropometry				X				X	X	X	X	X	X	X				X			X			
BIA			X											X				X			X			
BOD-POD			X											X				X			X			
Indirect calorimetry			X											X				X			X			
Medication use record	X			X				X	X	X	X	X	X	X				X			X			
Blood pressure				X				X	X	X	X	X	X	X				X			X			
Heart rate				X				X	X	X	X	X	X	X				X			X			

V, visit; FFQ, food frequency questionnaire; BIA, bioelectric impedance analysis; BOD-POD, air displacement plethysmography; Pre-op, preoperative period; Post-op, postoperative period; d, days; m, months; y, years.

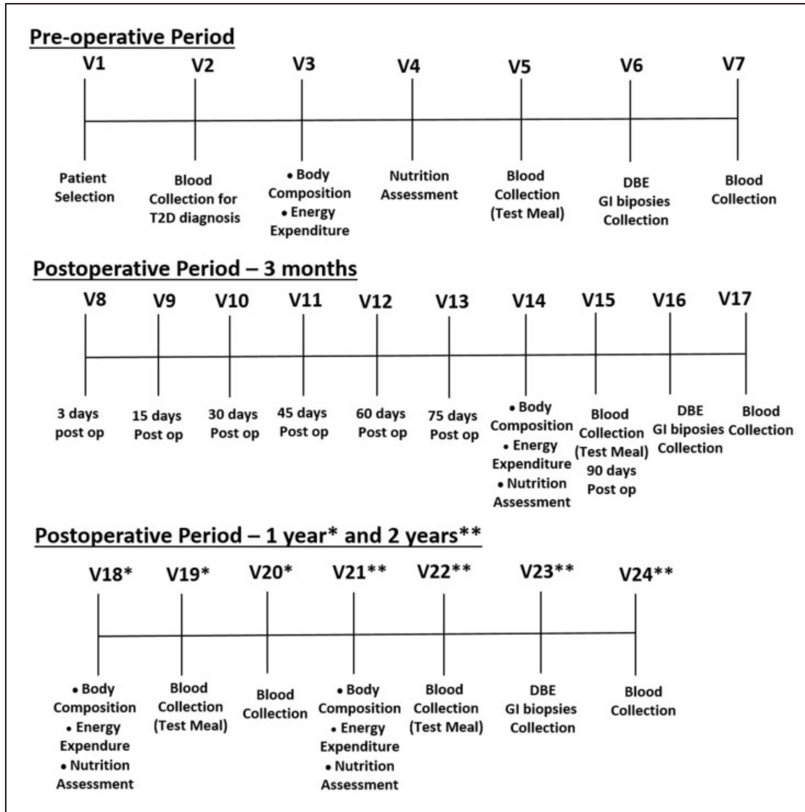

Figure 3. Schedule of study procedures.
V, visit; T2DM, type 2 diabetes mellitus; DBE, double-balloon enteroscopy; GI, gastrointestinal; post op, postoperative period.

GI "omic" evaluations. This sample was determined to provide 80% power for the detection of other general systemic effects of RYGB using parametric (one-way analysis of variance) and nonparametric (Wilcoxon signed-rank test) approaches, with an estimated alpha value of 0.05. The effect size was calculated under the assumption that GI hormone variation would be twice as great in the postoperative than the preoperative period.

Data analysis. The challenge faced in our multi-omics analyses was the integration of all data while dealing with the restrictions of a small sample. The statistical methods used to analyse multi-omics data consist of high-throughput data-generation platforms that demand high computational performance, including machine learning.[50] Before integration, we planned pre-processing of each omics dataset by procedures such as normalization or scaling, missing value imputation, variable selection, and dimension reduction.[51]

For microarray gene expression analysis, we planned to primarily use the software Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA). This tool performs a functional analysis (*in silico*), resulting in networks of gene interactions.[52]

For "omics" integrated analysis, we planned to perform initial exploratory data analyses. We then planned to apply

multivariate methods such as co-inertia analysis, PCA, or correspondence analysis to examine single datasets and explore relationships between two or among more than two datasets. We also considered the application of approaches that allow the visualization of different omics data types on pathway maps; the use of classification trees to identify variables predicting the expression of associated genes[53]; and the application of other advanced statistical methodologies that have been used in multi-omics analysis, such as the construction of artificial or Bayesian neural networks[54] and the application of dynamic control system theory. These approaches would allow for the creation of a global network of pathway interactions from genetic, proteomic, and metabolomic data.

Additional statistical analysis of each study variable and its temporal relationship, as well as analyses integrating all omics data, were also planned. The R statistical software (http://www.r-project.org), including the R Bioconductor repository, was chosen to conduct the statistical tests. For integrative analyses of diverse omics data, we planned to use Galaxy (https://usegalaxy.org) and STATegra (http://www.stategra.eu) software.

Results

Initial results

The first 20 obese patients selected for omics analysis were included in this study (demographic data are provided in Table 2); the 5 patients to be used for validation remain under selection. The 20 initial patients completed all GI tissue, blood, urine, and faecal sample collections. In addition, all omics (GI gene expression, plasma protein, plasma metabolite, plasma lipid, faecal metagenomic, and faecal metabolite), nutritional, anthropometric, body composition, and REE data were obtained preoperatively and 3 months postoperatively, and statistical analyses are underway. Urinary metabolites remain to be assessed, and data from 24 months after RYGB are under collection. The protocol steps completed and those in the process of completion for these 20 patients are shown in Figure 1.

Table 2. Descriptive data for obese female patients ($n = 20$) obtained before and 3 months after surgery.

Variable	Preoperative	Postoperative
Age (years)	46.9 ± 6.2	
Body weight (kg)	115.0 ± 16.0	94.5 ± 12.8
Body mass index (kg/m^2)	46.5 ± 5.3	38.2 ± 4.2
Fasting glucose (mg/dL)	221.1 ± 69.4	105.4 ± 23.9
Glycated haemoglobin (%)	13.0 ± 9.2	7.0 ± 6.2

Data are presented as mean $\pm$ SD.

Discussion

Ideally, research questions should be simple and involve only a few key variables. However, the multifactorial nature of some contemporary diseases has hindered the achievement of this premise. Modern researchers increasingly face the need to develop studies involving a wide range of variables to answer simple questions. Aiming to contribute to the resolution of this issue, our article describes the protocol for a pan-omics clinical study of obese patients, a useful research approach that is rarely applied because of its great complexity.

Obesity is a multifactorial disease that involves several metabolic disturbances and chronic inflammation associated with the development of a wide range of comorbidities. A major challenge of our study was to correlate a large number of GI findings with several systemic datasets to answer the main question of whether potential RYGB-induced changes in GI genes affect T2DM remission and long-term T2DM recurrence. A specific statistical approach was designed to overcome this obstacle.

Our statistical design aimed to enable correlation of a large number of variables in relation to single clinical outcomes. For this purpose, all analyses were initiated individually, considering each variable and its relationship to the study time points; significant findings will then be integrated to examine potential interrelationships among variables. This statistical integration will be conducted after concluding data collection and analysis for the time point of 2 years after RYGB. During application of the statistical integration, small adjustments in the statistical design will likely be necessary.

Challenges and limitations of our protocol encountered to date have been related to the acquisition of GI biopsy specimens. The development of invasive procedures for use in humans is a main challenge of translational clinical hypothesis testing. The local ethics committee was initially reluctant to approve the collection of GI biopsy specimens from patients for this study. Our Digestive Endoscopy Unit is a world reference centre at the forefront of DBE development that enabled us to obtain approval after intense discussions and under the condition of properly advising patients that biopsy specimen collection was specific to the study protocol, and not part of standard care, on the informed consent form.

The DBE technique has been well tolerated by patients in most studies, and biopsy specimen collection during such a procedure is quite safe. Practitioners from a German centre reported a <1% complication rate among a total of 3894 DBE interventions, including those performed in patients with mild to moderate acute pancreatitis (0.34%), organ perforation (0.20%), and small bowel bleeding (0.15%), the latter two of which were related mainly to polypectomy.[55] In our centre, no major complications have been reported for 364 DBE procedures, 112 of which included biopsy collection.[56]

The amount of GI tissue obtained from the pre- and postoperative biopsies in this study was sufficient for gene expression analysis for all enrolled patients. However, the excluded stomach portion was inaccessible for postoperative biopsy sample collection in some patients because of stenosis in the biliopancreatic limb ($n = 3$) and the difficulty of reaching this portion of the GI tract ($n = 10$). The excluded stomach may be in a ‘‘fallen’’ position because of an excess of gastric fluid in this region secondary to duodenal bile reflux, which hinders passage of the enteroscope.

Our strict patient selection criteria and requirement for consent to the performance of additional invasive procedures significantly hindered patient recruitment. In addition, to ensure the safety of the DBE intervention, the number of biopsy specimens collected was limited. We are still seeking to optimize our methods to achieve minimum tissue use during GI genetic analysis, but this limitation will probably prevent us from performing intestinal proteomic and metabolomic analyses, which could improve the interpretation of potential RYGB-induced changes in intestinal function.

One relevant point to be observed in conducting clinical studies is the potential scientific contribution and clinical application. BS has positive effects on obesity-related comorbidities and is associated with decreased mortality in obese patients.[4] Although currently recognized as the most effective available treatment for obese patients, this procedure is invasive and has potential for complications. Due to the marked burden of obesity and related comorbidities on many patients worldwide, new and effective noninvasive treatments are urgently needed.

The performance of BS, especially techniques involving malabsorptive procedures, have an enigmatic metabolic effect that manifests primarily as T2DM remission almost immediately postoperatively. Among

the several hypotheses proposed to explain this phenomenon, the effect of reduced post-operative food intake is the only proposed theory that does not involve bowel-induced metabolic factors. This hypothesis suggests that deprivation of oral ingestion in the immediate postoperative period minimally challenges pancreatic β cells, but the hypothesis is weakened when we consider that food deprivation occurs regardless of the surgical technique applied and that postoperative glycaemic control is minimal after strictly restrictive procedures.[57]

The other proposed hypotheses (which implicate intestinal gluconeogenesis, the hindgut, and the foregut) involve the potential participation of intestinal products in T2DM remission, mainly by correcting the imbalanced release of anti-diabetogenic signals (incretins and other insulinotrophic hormones) and diabetogenic signals (incompletely understood anti-incretin factors) that are apparently involved in its pathophysiology.[58,59] Taken together, these hypotheses strongly suggest that the GI tract actively participates in the metabolic effects of some BS procedures on glucose homeostasis; to date, however, these hypotheses are supported only by experimental data. The SURMetaGIT study was designed to contribute to this issue by providing clinical evidence explaining the molecular mechanisms involved in T2DM remission after RYGB, with consideration of the human gut as the major driver of these processes.

In addition to their benefits in terms of T2DM remission, RYGB-induced metabolic changes may contribute to the improvement or worsening of other obesity-related comorbidities involving metabolic factors. For instance, preoperative bone metabolism dysfunction and osteopenia are common in obese individuals and are associated in part with vitamin D deficiency. In the postoperative period, these bone disturbances may be accentuated and correlated partially with weight loss, even when vitamin D deficiency is reduced by supplementation and other bone health markers have improved.[60] In this context, although the SURMetaGIT study focuses on T2DM remission, its complex design may provide unpredictable additional answers concerning the remission or enhancement of other obesity-related conditions, such as metabolic syndrome, changes in cognitive function, and bone metabolic disorders.

In conclusion, application of our protocol allowed us to collect essential data that will contribute to the understanding of molecular mechanisms involved in the remission of T2DM and other obesity-related comorbidities. These data may also guide the design of new noninvasive treatment approaches for obesity-related metabolic disorders, particularly T2DM. The potential scientific contributions of the SURMetaGIT study are promising, and their successful manifestation will require proper statistical analysis of the data generated and the application of knowledge for larger-scale interpretation. Our partial results demonstrate the applicability of our protocol by showing reliable patient adherence and data collection over time. Effects data are not presented here because they are beyond the scope of this article, which only describes our protocol. Confirmation of T2DM remission (using the primary endpoints) at least 1 year after RYGB and the statistical integration of data after the 2-year follow-up will likely generate several articles that better detail our findings.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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