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# Gastrointestinal Transcriptomic Response of Metabolic Vitamin B12 Pathways in Roux-en-Y Gastric Bypass

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**OBJECTIVES: Vitamin B12 (B12) deficiency after Roux-en-Y gastric bypass (RYGB) is highly prevalent and may contribute to postoperative complications. Decreased production of intrinsic factor owing to gastric fundus removal is thought to have a major role, but other components of B12 metabolism may also be affected. We evaluated changes in the expression levels of multiple B12 pathway-encoding genes in gastrointestinal (GI) tissues to evaluate the potential roles in contributing to post-RYGB B12 deficiency.**
**METHODS: During double-balloon enteroscopy, serial GI biopsies were collected from 20 obese women (age, 46.9 $\pm$ 6.2 years; body mass index, 46.5 $\pm$ 5.3 kg/m$^2$) with adult-onset type 2 diabetes (fasting plasma glucose $\geq$ 126 mg/dl; hemoglobin A1c $\geq$ 6.5%) before and, at the same site, 3 months after RYGB. Gene expression levels were assessed by the Affymetrix Human GeneChip 1.0 ST microarray. Findings were validated by real-time quantitative PCR (RT–qPCR).**
**RESULTS: Gene expression levels with significant changes ($P \leq 0.05$) included: transcobalamin I (*TCN1*) in remnant (−1.914-fold) and excluded (−1.985-fold) gastric regions; gastric intrinsic factor (*GIF*) in duodenum (−0.725-fold); and cubilin (*CUBN*) in duodenum (+0.982-fold), jejunum (+1.311-fold), and ileum (+0.685-fold). Validation by RT–qPCR confirmed ($P \leq 0.05$) observed changes for *TCN1* in the remnant gastric region (−0.132-fold) and *CUBN* in jejunum (+2.833-fold).**
**CONCLUSIONS: RYGB affects multiple pathway-encoding genes that may be associated with postoperative B12 deficiency. Decreased *TCN1* levels seem to be the main contributing factor. Increased *CUBN* levels suggest an adaptive genetic reprogramming of intestinal tissue aiming to compensate for impaired intestinal B12 delivery.**
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## INTRODUCTION

Bariatric surgery is the most effective treatment for severe obesity and its comorbidities, producing sustained weight loss and reduced morbidity and mortality rates.[1] Roux-en-Y gastric bypass (RYGB) is the most widely used bariatric procedure worldwide.[2] As an invasive procedure, RYGB is not free of complications. Most patients experience some postoperative gastrointestinal (GI) side effects, including deficiencies of macronutrients and micronutrients or aggravation of previous nutritional deficits.[3] Deficiency of functional vitamin B12 (B12 or cobalamin) is particularly well reported. A recent study of 21,345 patients found an incidence of B12 deficiency after gastric bypass of 20% until 12 months postoperative.[4] B12 is a cofactor in many metabolic processes, and its deficiency is associated with neurological disorders.[5,6] Patients undergoing RYGB require B12 supplementation for the remainder of their lives.[7]

Post-RYGB B12 deficiency is associated with the restrictive and malabsorptive procedures that are applied in this technique. Anatomical rearrangement induced by gastric fundus restriction leads to early satiety, as well as decreased hydrochloric acid (HCl) and pepsin secretion. This situation leads to poor release of B12 from food and loss of food exposure to intrinsic factor (IF)-secreting cells,[8–10] resulting in B12 malabsorption.[3] Gastric restriction or partial intestinal bypass can limit the absorption of B12 when provided by the oral pathway, supporting B12 supplementation by the intramuscular route. However, intramuscular B12 supplementation can be inconvenient, leading to poor patient compliance.[7]

B12 metabolic pathways involve various GI molecular mediators that may be influenced by RYGB-induced GI rearrangement. Dietary B12 binds transcobalamin I (TCN1) in the stomach and is transported to the duodenum, where TCN1 is degraded by pancreatic enzymes. Next, B12 binds IF (also known as TCN3) and travels through the GI tract to the ileum, where the vitamin is absorbed by enterocytes through the cubam receptor complex (IF–B12 linked to the cubilin and amnioless subunities of cubam receptor). Finally, B12 is transported to plasma by TCN2.[11]

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We hypothesized that, in addition to IF, other molecules involved in the B12 metabolic pathway may contribute to its post-RYGB deficiency. Identification of such molecules may add information for developing a better clinical approach to postoperative B12 deficiency. We used transcriptomic analysis to evaluate changes in GI expression of B12 pathway-encoding genes. Mucosal biopsies from several different sections of the GI tract were obtained from obese women before and after RYGB. Expression levels of relevant genes were measured and validated by microarray and real-time quantitative PCR (RT–qPCR), respectively.

## METHODS

**Ethical statement.** This prospective study was performed according to the ethical standards of the World Medical Association's Declaration of Helsinki. The protocol was approved by the local institutional ethics board (CAPPesq 1011/09) and registered at www.ClinicalTrials.gov (NCT01251016). Written informed consent was obtained from each patient before trial participation.

**Subjects.** Twenty adult (18–60 years) women admitted for elective RYGB to the Gastrointestinal Surgery Division of the Hospital das Clínicas at the University of São Paulo Medical School were screened for eligibility. Additional inclusion criteria were a body mass index $\geq 35\,kg/m^2$, diagnosis of type 2 diabetes mellitus (fasting plasma glucose $\geq 126$ mg/dl; hemoglobin A1c $\geq 6.5\%$) and/or use of oral antidiabetic medication, and absence of *Helicobacter pylori* bacterium. Exclusion criteria were male sex, type 1 diabetes mellitus or nondiabetic, insulin use, diagnosis of thyroid or hepatic disease, candidate for bariatric surgery technique other than RYGB, refusal to participate, and current or recent participation in another interventional study protocol.

**Bariatric surgery.** All patients were submitted to standardized RYGB, without silicon rings and with biliary-pancreatic loops (50–60 cm) and feed handles (100–120 cm).

**Biopsies from each GI site.** Serial biopsies of GI mucosa (10–15 mg) were collected during double-balloon enteroscopy (DBE), performed through oral access and under deep sedation some weeks before and 3 months after RYGB. Patients fasted for 12 h and abstained from oral drug use for 3–5 days before the procedure to avoid any impact of food or oral medications on gut gene expression.

Biopsies were obtained from the stomach cardia/remnant stomach (equivalent preoperative/postoperative regions), stomach body/excluded stomach (equivalent preoperative/postoperative regions), duodenum, jejunum, and ileum. Each intestinal site of preoperative biopsy collection was highlighted with India ink (SPOT; GI Supply, Camp Hill, PA) to guide postoperative collection at the same location. Biopsy samples were immersed in liquid nitrogen after collection and stored at − 80 °C until analysis.

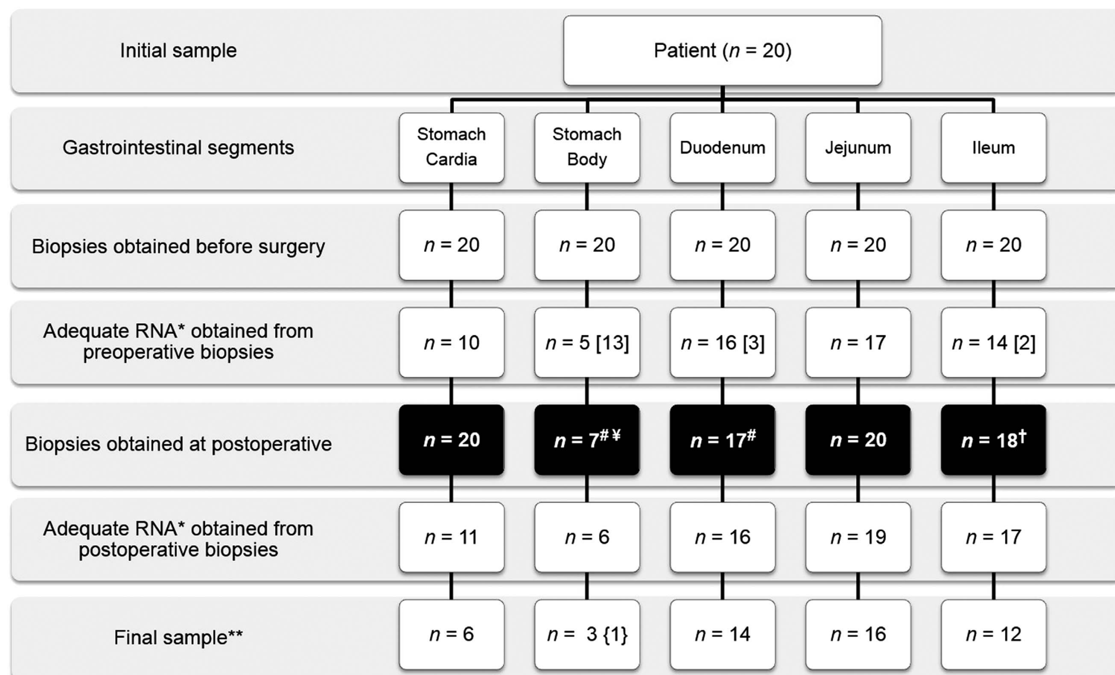


**Figure 1** Consort diagram of biopsies and adequate RNA obtained for microarray analysis. *RNA quantity > 100 ng/µl and/or RNA integrity number (RIN) ≥ 7. Thirteen samples from the stomach body, three from duodenum, and two from ileum were not subjected to RNA extraction because no postoperative sample was available from the same site. Reasons for biopsy collection failure included: #Stenosis in the biliopancreatic limb ($n = 3$), ¥inability to reach excluded stomach because of duodenal bile reflux ($n = 10$), and †stenosis in common limb ($n = 2$). **Total number of samples with adequate RNA from preoperative and postoperative matched segments, which were applied for microarray analysis. Black blocks correspond to the final number of biopsy samples subjected to real-time quantitative PCR analysis. One stomach body sample did not pass the standardization test.

**Transcriptomic analyses.** RNA was extracted from GI biopsies by the RNeasy Plus Kit (Qiagen, Germantown, MD). Expression of B12 pathway-encoding genes was assessed with the Human GeneChip 1.0 ST Array (Affymetrix, Santa Clara, CA). Only samples with adequate RNA quantity and quality ($>100$ ng/μl and RNA integrity number $\geq 7$, as suggested by the manufacturer) for both preoperative and postoperative biopsies were included in microarray analysis.

Genes of interest with altered expression levels by microarray analysis were validated by RT–qPCR (adequate RNA samples defined as those with RNA integrity number $\geq 5$).[12] Candidates for gene validation were selected based on originality: either the change in expression after RYGB was not reported previously or the gene was expressed at a different site than is normally observed. Genes expressed at multiple sites were validated at the most physiologically relevant site. RT–qPCR was developed with TaqMan gene expression assays (Thermo Fisher Scientific, Waltam, MA), using beta-actin as a reference gene. From the 20 patients selected, RT–qPCR was performed in a larger sample size than microarray technique, as described in black blocks in Figure 1. In addition to the 20 patients selected in the study, 5 other patients were selected for gene expression validation by RT–qPCR.

**Assessment of serum B12 levels.** Serum B12 levels were assessed by electrochemiluminescence immunoassay by Cobas (Roche Diagnostics, Indianapolis, IN).

**Sample size and statistical analysis.** This study comprises a secondary outcome of a larger trial that aims to analyze changes in the expression of genes encoding GI hormones related to postoperative glycemic homeostasis. A sample size of 20 patients was estimated using parametric (one-way analysis of variance) and nonparametric Wilcoxon signed-rank test based on this aim, assuming 80% power and the significance level of 5%. Effect size was calculated by considering GI hormone variations to be approximately twice in the postoperative vs. the preoperative period. For the specific analysis of postoperative changes in B12 pathway-encoding genes, considering a same power (80%) and significance level (5%), the Wilcoxon signed-rank test will detect differences with an effect size $\geq 1.35$ (i.e., 1.35 times the s.d. of the difference) with a minimum sample size of seven patients. Statistical analysis considered an $\alpha$ error (type I error) of 5% and $\beta$ error (type II error) of 20%.

**Table 1** Descriptive data for obese woman before and 3 months after bariatric surgery

| Variable | Preoperative (*n*=20) | Postoperative (*n*=20) |
|---|---|---|
| Age (years) | 46.9 ± 6.2 | NA |
| Height (cm) | 157 ± 21 | NA |
| 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 |

Abbreviation: NA, not available. Data are reported as mean ± s.d.

Array quality was checked by boxplot, correlation, and principal components analyses. Expression values were obtained by the robust multiarray average of preprocessing data.[13] The ComBat method (http://jlab.byu.edu/ComBat/Abstract.html) was applied to remove batch effects.[14] The Rank products[15] method was used to select differentially expressed genes with $P<0.05$, after correction for false discovery rate.[16] Tools listed above are available in the R Bioconductor program (www.bioconductor.org). Spearman's rank correlation was applied as a nonparametric test to identify clusters and assign *P* values to pairs of GI genes with highly similar temporal expression patterns.

Change in postoperative serum B12 levels was evaluated by paired Student's *t*-test using the software R.[17]

## RESULTS

**Patients.** All 20 enrolled patients met the selection criteria and completed the study protocol. Preoperative and postoperative demographic data are shown in Table 1.

**Success of GI biopsy collection and RNA adequacy for transcriptomic analysis.** Adequate tissue amounts to permit the analysis of gene expression were obtained from preoperative and postoperative GI biopsies of all enrolled patients. For some patients, tissue amounts were insufficient for analysis of all GI segments. DBE was unable to reach the excluded stomach in 13 patients owing to stenosis in the biliopancreatic limb (3 patients) or difficulty in reaching this portion (10 patients). Tissue was successfully collected and adequate quantities of RNA were obtained from other GI segments, including duodenum (70%), jejunum (80%), and ileum (60%), for both the periods. Total remnant and excluded stomach biopsies obtained from only six and three patients, respectively, provided adequate RNA for gene expression analysis by microarray. Figure 1 illustrates a flowchart of the sample collection, microarray, and RT–qPCR analysis processes. We also assessed preoperative and postoperative matched biopsies of stomach cardia ($n=5$), stomach body ($n=2$), duodenum ($n=4$), jejunum ($n=5$), and ileum ($n=5$) collected from five additional women who met the same selection criteria as were applied to the 20 studied women. The fold change obtained from RT–qPCR analysis was calculated from stomach cardia ($n=25$), stomach body ($n=9$), duodenum ($n=21$), jejunum ($n=25$), and ileum ($n=23$).

**Expression of B12 pathway-encoding genes.** Data on GI expression of B12 pathway-encoding genes are shown in Table 2. Before RYGB, in normal GI anatomy, microarray analysis displayed a poorly explored or previously unreported intestinal expression of IF-encoding gene (*GIF*) and gastric expression levels of cubilin-encoding gene (*CUBN*) and transcobalamin II-encoding gene (*TCN2*). After RYGB, in rearranged GI anatomy, microarray analysis revealed a significant decrease of gastrin-encoding gene (*GAST*) and transcobalamin I-encoding gene (*TCN1*) expression in the excluded gastric segment. No compensatory increase in B12 pathway-encoding gene expression was observed in

**Table 2** Differential expression levels of vitamin B12 pathway-encoding genes before and after bariatric surgery

| Gene name (abbreviation) | Gene expression by gastrointestinal segment | | | | | Main role in B12 metabolism |
|---|---|---|---|---|---|---|
| | Remnant stomach | Excluded stomach | Duodenum | Jejunum | Ileum | |
| Gastrin (*GAST*) | −0.044 (M) | **−1.515 (M)** | **+0.233 (M)** | −0.060 (M) | −0.077 (M) | Encodes hydrochloric acid to obtain B12 from ingested food |
| Pepsin C (*PGC*) | −0.069 (M) | −0.038 (M) | −0.026 (M) | −0.084 (M) | +0.115 (M) | Encodes pepsin to obtain B12 from ingested food |
| Transcobalamin I (*TCN1*) | **−1.914 (M)**<br>**−0.132 (V)** | **−1.985 (M)**<br>−0.398 (V) | **+0.115 (M)** | +0.048 (M) | +0.021 (M) | Encodes transcobalamin I to carry B12 from stomach to intestine |
| Transcobalamin II (*TCN2*) | +0.042 (M) | +0.177 (M) | +0.045 (M) | +0.194 (M) | +0.170 (M) | Encodes transcobalamin II to carry B12 from the intestine to the blood and liver |
| Gastric intrinsic factor (*GIF*) | +3.697 (V)<br>−0.271 (M)<br>−0.570 (V) | +4.079 (V)<br>+0.357 (M) | +2.637 (V)<br>**−0.725 (M)**<br>−0.813 (V) | −0.779 (V)<br>+0.112 (M) | +1.563 (V)<br>−0.103 (M) | Encodes intrinsic factor to carry B12 through gut to ileum |
| Cubilin (*CUBN*) | −0.184 (M) | +0.019 (M) | **+0.982 (M)**<br>+7.876 (V) | **+1.311 (M)**<br>**+2.833 (V)** | **+0.685 (M)**<br>+1.925 (V) | Encodes cubilin for gut absorption of B12 |

Abbreviations: M, microarray; V, validation by real-time quantitative PCR. Negative and positive values indicate decreased and increased expression, respectively. Bold values correspond to significant changes ($P \leq 0.05$).

the functional remnant segment. A significant decrease in the expression level of *TCN1* and nonsignificant decreases in the expression levels of *GAST*, pepsin C-encoding gene (*PGC*), and *GIF* were observed in remnant stomach.

Microarray analysis of intestinal biopsies revealed a compensatory increase in the expression levels of several B12 pathway-encoding genes in different segments in response to surgery. Despite a significant decrease in *GIF* expression in the duodenum, *GAST* and *TCNI* expression levels were significantly increased in the duodenum. *CUBN* expression was significantly increased in the duodenum, jejunum, and ileum. A nonsignificant increase in *TCN2* expression was found in all studied GI segments. Spearman's rank test identified many genes in statistically significant pairs, most of which were in intestinal segments (Figure 2).

Validation was performed for genes and anatomical sites of interest, including *TCN1* in remnant and excluded stomachs, *GIF* in remnant stomach and duodenum, *CUBN* in duodenum, jejunum, and ileum, and *TCN2* in all studied GI segments (Figure 3). Overall, the gene validation procedure revealed similar but nonsignificant results in almost all GI segments, except for *TCN2* in the jejunum that showed opposite results in microarray and RT–qPCR analyses (Table 2). Significant changes included decreased expression of *TCN1* in the remnant stomach (−0.132-fold) and increased expression of *CUBN* in the jejunum (+2.833-fold) ($P \leq 0.05$; Table 2).

**Serum B12 levels.** Nonsignificant changes in serum B12 levels were observed 3 months after RYGB (preoperative, 424.48 ± 142.81 pg/ml vs. postoperative 459.82 ± 211.36 pg/ml; $P = 0.534$).

## DISCUSSION

Anatomical changes of the GI tract may account for the systemic metabolic effects of RYGB.[18,19] In this study, we identified changes in the expression levels of B12 pathway-encoding genes along the GI tract as a possible mechanism contributing to post-RYGB deficiency of B12.

To our knowledge, this is the first study to assess the expression levels of B12 pathway-encoding genes at multiple GI sites after RYGB. Combined, microarray and RT–qPCR approaches are appropriate for this purpose but often result in disagreements. No additional validation tool is available for improved data interpretation.[20] Such disagreements may be avoided by employing good laboratory practices to ensure adequate RNA quantity and quality for both techniques and adequate control reference genes for RT–qPCR.[20] In addition to having adequate RNA quantity and quality for both techniques, we selected RNA extraction methods and the reference gene for RT–qPCR based on results of pilot studies testing different approaches. According to our findings, genes with expression levels within the dynamic range of the microarray qualitatively agreed with data from RT–qPCR, displaying similar changes in grade and direction in response to RYGB. However, some changes identified by RT–qPCR were nonsignificant, even providing higher fold-change values than microarray results. Therefore, we discuss our data from a physiological perspective.

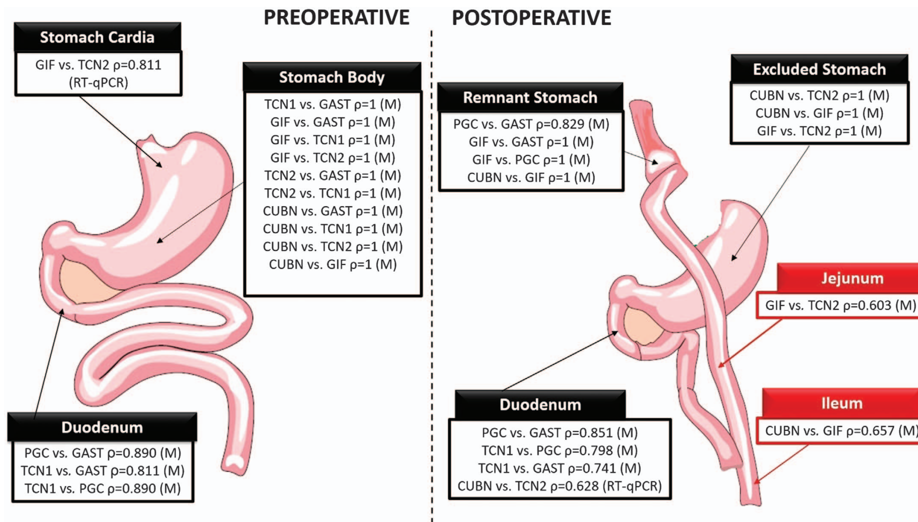


**Figure 2** Pairs of genes with significantly ($P < 0.05$) similar temporal expression patterns, according to Spearman's rank test. In the jejunum and ileum, correlated gene pairs were found only in the postoperative period (red color), suggesting intestinal genetic reprograming to increase B12 absorption. Relation between genes needs to be elucidated, but the genes probably undergo transactivation. Stomach cardia/remnant stomach (equivalent preoperative/postoperative regions), stomach body/excluded stomach (equivalent preoperative/postoperative regions); CUBN, cubilin; GAST, gastrin-encoding gene; GIF, gastric intrinsic factor; M, microarray; PGC, pepsin C-encoding gene; RT–qPCR, real-time quantitative PCR;TCN1, transcobalamin I-encoding gene; TCN2, transcobalamin II-encoding gene; $\rho$, Spearman's rho.

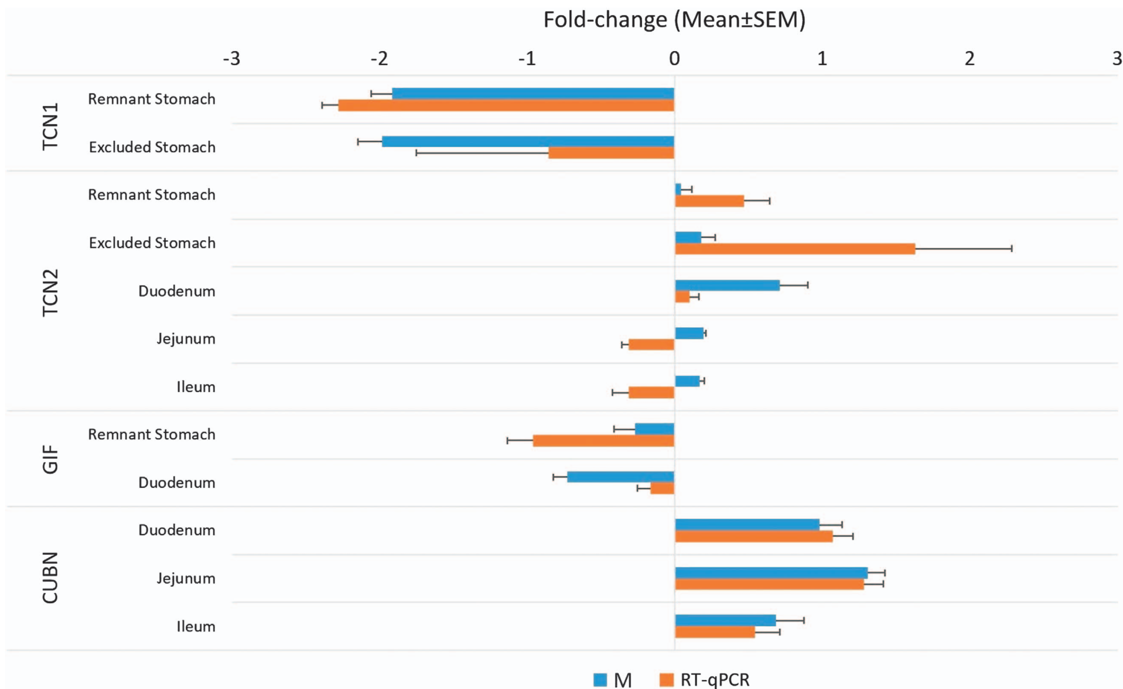


**Figure 3** Validation of expression levels of vitamin B12 pathway-encoding genes by real-time quantitative PCR (RT–qPCR). Red columns indicate that opposite results were obtained by microarray and RT–qPCR techniques. CUBN, cubilin; GIF, gastric intrinsic factor; M, microarray; RT–qPCR, real-time quantitative PCR; TCN1, transcobalamin I-encoding gene; TCN2, transcobalamin II-encoding gene.

Gastric fundus reduction by RYGB is associated with decreased gastric secretion of gastrin, pepsin, and IF by the functional remnant segment. As these changes could limit the abilities of the GI tract to obtain B12 from food and transport B12 to the intestine, they have been speculated to account for postoperative B12 deficiency.[21] Studies showed better results for patients supplemented with crystalline vs. food-associated B12 to correct postoperative deficits.[21–23] In addition to not requiring pepsin or HCl to obtain B12 from food, the crystalline form is absorbed by passive diffusion, dispensing the need for IF.[23,24]

Expression levels of *GAST*, *PGC*, and *GIF* were decreased nonsignificantly in remnant stomach 3 months after RYGB. GAST in the excluded gastric portion showed significantly decreased postoperative expression, which may represent a response of the atrophied gastric portion to reassert

functionality. The small amounts of tissue obtained from biopsies only enabled us to validate gastric expression of *GIF* as a physiologically relevant change. Although gastrin and pepsin facilitate B12 extraction, high levels of these gastric acids are not essential for digestion of some B12-food sources.[25] IF is highly specific for the attachment of B12 to cubilin,[26] and deficient production of IF is currently considered as the main contributor to postoperative B12 deficiency.[21]

RT–qPCR validation confirmed the nonsignificant decrease in *GIF* expression in the gastric remnant. RYGB-induced anatomical rearrangement may have stimulated a nonparietal expression of *GIF* in the stomach, preventing a significant decrease in its expression. Gastric parietal cells produce IF in the human stomach, but local factors can also induce nonparietal cells to produce IF.[27] For example, nonparietal cells produced IF in chronic gastritis patients with severe food-cobalamin malabsorption but not in healthy volunteers.[28]

Impaired gastric IF production may not completely explain the B12 deficit in bariatric patients. This impression is reinforced by our unexpected observation of intestinal *GIF* expression. To our knowledge, no previous study has reported a significant intestinal contribution to GIF production. Intestinal IF may compensate for gastric deficits of this B12 carrier. However, although all intestinal segments expressed *GIF*, its levels were significantly decreased in the bypassed duodenum after RYGB. This finding strongly suggests that food contact may act as a local factor to stimulate intestinal *GIF* expression. Partial lack of intestinal food contact could limit intestinal production of IF after RYGB and impair B12 transport.

In addition to impairment of B12 transport by IF, a postoperative decrease in gastric production of TCN1 may be the main gastric factor underlying impaired intestinal transport of B12. In the stomach, B12 seems to bind TCN1 preferentially instead of IF, to which B12 is transferred only at the upper small bowel. An *in vitro* study performed at a near-gastric pH of 2 found that B12 affinity was 50-fold higher for human TCN1 than for IF. Adding pancreatic proteases to the culture medium led to complete and rapid transfer of B12 to IF.[29] Furthermore, in healthy volunteers who ingested a radiolabeled liver homogenate, 66% vs. 25% of radioactive B12 bound to TCN1 in the gastric vs. jejunal aspirate.[25]

Deficient production of TCN1 (as a primary factor) and IF (as a secondary factor) may explain why postoperative B12 deficiency can only be corrected by oral supplementation when provided in very large amounts. RYGB patients supplemented with an oral multivitamin containing 140 times the recommended daily allowance of B12 showed a significant increase in serum levels and a low B12 deficiency rate (1.6%) after 12 months of supplementation.[21] Excessive B12 levels seemed to compensate for the impaired transport, and the intestine seemed to absorb B12 in an efficient manner. Consistent with this hypothesis, we found that postoperative *CUBN* expression was increased throughout all portions of the intestine, especially jejunum. *TCN2* expression was also higher in the postoperative period but not at significant levels. Increased *CUBN* expression seems to reflect an adaptive genetic reprogramming of the intestinal tissue, apparently triggered in response to decreased arrival of B12. As decreased B12 transportation till the intestine by TCN1 is a consequence of gastric resection implied in RYGB procedure, purely malabsorptive techniques (which also result in B12 deficiency) may not induce the same genetic reprogramming.[30,31]

If anatomic gastric rearrangement indirectly improves the capacity for B12 absorption after RYGB, and the absorption mechanism remains intact in the distal intestine, then what accounts for the higher rates of B12 deficiency after RYGB compared with other techniques? For instance, a recent meta-analysis[32] and a long-term cross-sectional study[33] found much greater rates of B12 deficiency among patients after RYGB compared with sleeve gastrectomy. We suggest that purely restrictive bariatric surgeries may inhibit TCN1 production, while maintaining B12 transport by intestinal IF and allowing food to continue to contact the entire intestine. This contact is a crucial factor for intestinal IF production, as evidenced by the significantly decreased expression of *GIF* in the duodenum after RYGB. After purely restrictive techniques, the B12 carrier would be less impaired than after mixed techniques, thereby resulting in a lower rate of postoperative deficiency.

At our center, all obese candidates for bariatric surgery receive micronutrient supplementation before surgery (when present with micronutrient deficits) and after surgery (regardless of micronutrient deficits). Our patients received the recommended monthly intramuscular B12 supplementation (1.000 UI) in the postoperative period.[23] This supplementation is why we could not correlate our gene expression data with systemic levels of B12 to confirm a potential deficiency in its postoperative absorption. However, given that B12 levels were maintained at normal values[34] 3 months after RYGB, changes in GI gene expression of B12 pathway-encoding genes did not seem to be influenced by altered levels of the vitamin.

Our study has some limitations that deserve a detailed discussion. The major methodological challenge was to obtain GI biopsies by DBE. The protocol was approved to be applied in a limited number of obese individuals by our Ethical Committee. The number of biopsy samples collected per patient was also limited. We obtained tissue from almost all studied segments, except for the excluded stomach. Excluded stomach may have been in a "fallen" position after surgery because of excess gastric fluid in this region (owing to duodenal bile reflux), which would hinder enteroscope entry. Some biopsies provided only small amounts of tissue and inadequate RNA for microarray analysis, and some RNA was of low quality, probably owing to the large amount of RNase and other hydrolytic enzymes in gastric tissue.[35,36] These challenges prevented us from submitting our entire sample to microarray analysis. The Rank products method scores genes based on their ranks in multiple comparisons, requiring only a few well-justified assumptions about the data. This characteristic confers to the method the ability to find genes consistently deregulated in only a subgroup of individuals. Therefore, Rank products has been shown to be particularly efficient when dealing with heterogeneous and small samples.[37–39] Relevantly, RT–qPCR, which was performed with a large number of biopsies, provided similar results to those obtained by microarray and significant results for the genes in our proposed mechanism.

To achieve a population that was as homogeneous as possible, we evaluated only women. Therefore, sex-specific gene expression could influence our results. Obese individuals can respond differently to RYGB, owing to individual genotypes and the complexity of the disease. The modest fold changes reported and the lack of significant changes in some genes during validation seem to reflect such varied response. In addition, to ensure patient safety, GI biopsies were collected from fasting patients 3 months after surgery, when the patient's body was in reasonable homeostasis. Consequently, relevant external stimuli (e.g., from surgical stress and food contact) for the activation of gene expression were weak or absent. We compared two "normal" situations, which is different from comparing normal and tumor tissues, a comparison that usually leads to larger fold changes. A majority of the informative RNAs and differentially expressed transcripts can exhibit fold changes of $<2$, as the enrichment of biologically relevant functions occurs even at very low fold changes in RNA levels.[40] One argument focuses on biological complexity: tissues often contain many different cell types whose response to a given stimulus may vary greatly. The smaller effect observed at the transcriptional level does not preclude a large biological effect.[41] Furthermore, our data showed a significant physiological coherence in explaining the postoperative deficits of B12, which should not be ignored.

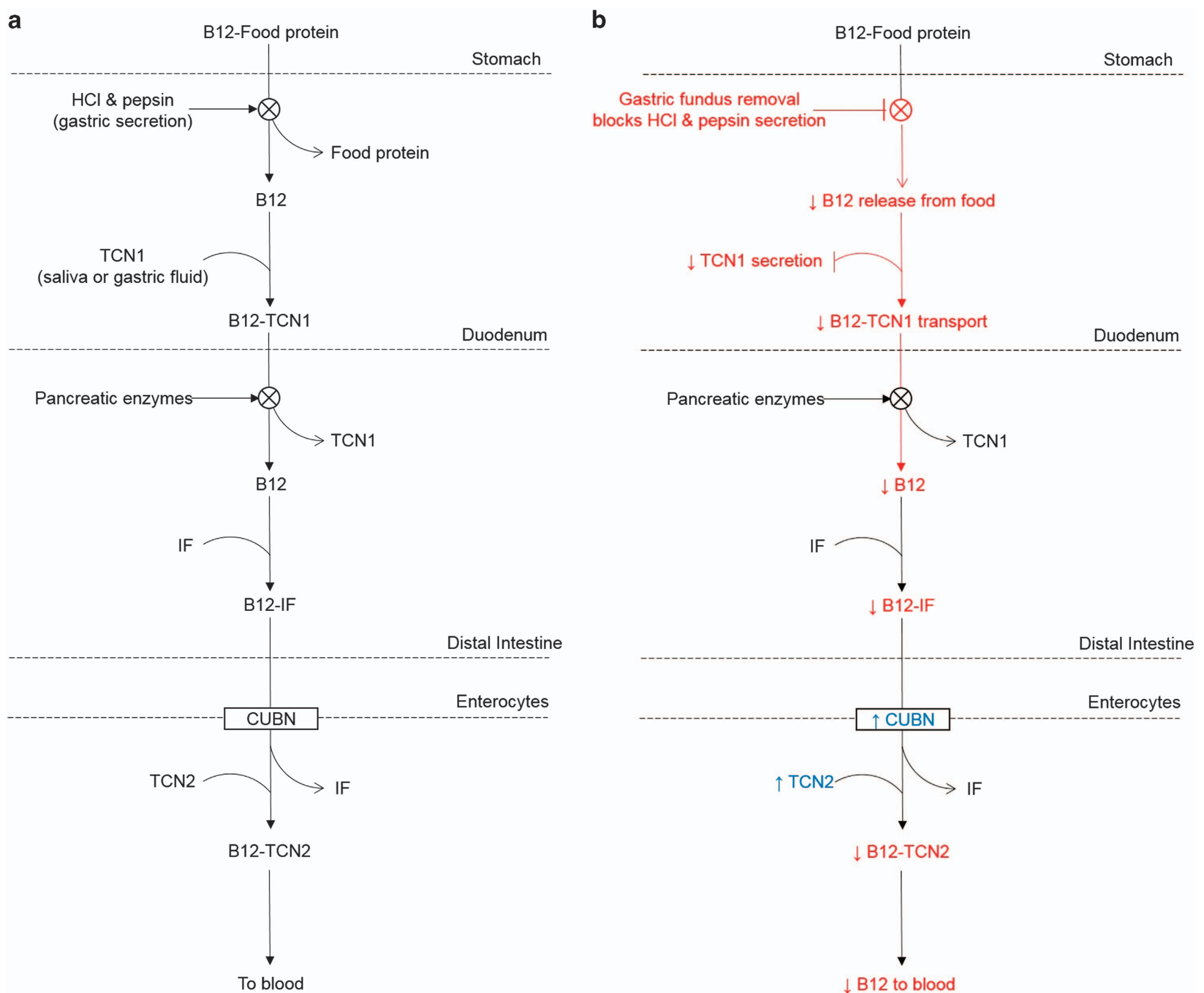


**Figure 4** Changes in gastrointestinal (GI) expression of vitamin B12 (B12) pathway–encoding genes following Roux-en-Y gastric bypass (RYGB). (**a**) Before RYGB. B12, bound to animal proteins, enters the stomach, where it is released by actions of pepsin and hydrochloric acid (HCl). B12 binds to transcobalamin I (TCN1), found in saliva or gastric fluid, and is transported to duodenum. Here TCN1 is degraded by pancreatic enzymes, and B12 is captured by intrinsic factor (IF). The B12–IF complex moves through the intestine and is absorbed by enterocytes via the cubam subunit cubilin (CUBN). Degradation of IF in enterocytes releases B12, which binds transcobalamin II (TCN2) and is transported by blood flow throughout the body. (**b**) After RYGB. Dietary B12 enters the stomach bound to animal proteins. Removal of the gastric fundus leads to impaired production of gastric and pepsin acids, which hinders B12 release from food. Decreased IF and TCN1 expression levels limit intestinal transport of B12. In response to decreased B12 levels, the intestine triggers production of CUBN and TCN2 in an (apparently unsuccessful) effort to maintain systemic B12 delivery. Red font denotes downregulated pathways, whereas blue font denotes upregulation.

Ethical aspects in performing DBE and our limited tissue sample size precluded us from performing additional controls and targeted protein expression analyses to validate our gene findings in a more physiological approach. Considering these limitations, our approach was appropriate for addressing the question of our study, especially when we consider that only preoperative and postoperative paired biopsies were analyzed.

Finally, our protocol minimized external stimuli that could interfere with our data. Most of our patients used proton pump inhibitors and other medications. When we designed our study, we took care in evaluating the half-life/effect time of each of these medications to determine an adequate washout period before GI biopsy collection. As vitamins A and D are transcription factors for molecules that compound the *CUBN* gene,[5,42] we assessed their serum levels (data not shown). Similarly to B12, serum levels of vitamins A and D remained in the normal ranges during the postoperative period. These data reinforce the notion that the observed significant changes in *CUBN* gene expression were responses to the anatomic rearrangement of GI tissues.

Our preliminary and exploratory study showed that gastric fundus removal in RYGB may lead to a significant impairment of B12 transport by decreasing TCN1 and IF levels, rather than IF levels alone, as first believed. Although the intestine is able to produce IF, partial absence of intestinal food contact may limit intestinal IF activity and contribute to postoperative B12 deficiency. Our data highlight the intestine as a highly adaptable tissue that is able to reprogram its genetic phenotype to compensate for disturbances of B12 transport in response to gastric fundus removal. This genetic reprograming, possibly trigged by reduced arrival of B12 to the intestine, was characterized by a higher intestinal expression of cubilin, suggesting an attempt to improve B12 absorption and avoid systemic deficits (Figure 4). Spearman's rank test showed that B12-pathway genes were co-regulated and mainly present in the intestine. Finally, whereas the excluded gastric segment seemed to atrophy in response to loss of function, the excluded intestinal segment (duodenum) had increased expression levels of genes related to B12 extraction from food (*GAST*), transport (*TCN1*), and absorption (*CUBN*), suggesting an attempt by the excluded portion to restore its functional role.

Our findings suggest that RYGB affects multiple B12 pathway-encoding genes that may be associated with its postoperative deficiency. Decreased *TCN1* levels, but not *GIF* levels, seem to be the main factor. Increased *CUBN* levels suggest an adaptive genetic reprogramming of intestinal tissue aiming to compensate for impaired intestinal delivery of B12 after RYGB.

## CONFLICT OF INTEREST

**Guarantor of the article**: Priscila Sala, RD

**Specific author contributions**: DLW, SH, DG-N, and PS contributed to the conception and design of the research and to data interpretation. PS, GB, and NMM equally contributed to the clinical follow-up of enrolled patients. PS and DCF developed all gene analyses. CCAP and IDCGS supervised molecular procedures and assisted in data interpretation. MAS is the expert in bariatric surgery who is responsible for the RYGB procedures. MAS also guided data interpretation concerning obesity-associated comorbidities. RKI, IFMSG, EGHM, and PS equally contributed to the collection of biopsies. PS, RSMMT, GB, GRR, and ALW contributed to data interpretation and wrote the manuscript. All authors critically revised the manuscript, gave their final approval, and agree to be accountable for all aspects of the described study, ensuring its integrity and accuracy.

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**Potential competing interests**: None.

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## Study Highlights

### WHAT IS CURRENT KNOWLEDGE

- ✓ Vitamin B12 deficiency following Roux-en-Y gastric bypass is frequent and associated with neurological complications.
- ✓ Postoperative B12 deficiency supposedly arises from decreased intrinsic factor levels after gastric fundus removal.

### WHAT IS NEW HERE

- ✓ Intestinal cells express intrinsic factor.
- ✓ Decreased transcobalamin I levels may be the main contributory factor in postoperative B12 deficiency.
- ✓ Surgery induces intestinal genetic reprograming, including an increase in the expression of B12 receptor-encoding genes.

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