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ORIGINAL CONTRIBUTIONS

No Difference in Ghrelin-Producing Cell Expression in Obese Versus Non-obese Stomach: a Prospective Histopathological Case-Control Study

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Abstract

Background To understand the role of ghrelin in the mechanism of action of laparoscopic sleeve gastrectomy (LSG), a prospective cohort case-control study to assess the expression of ghrelin-producing cells (GPC) in two groups of patients was designed.

Methods Specimens of resected stomach from 26 obese patients who underwent LSG (group A), were compared by immunohistochemistry to control stomach samples from 26 non-obese patients (group B) resected for other pathologies or during autopsy; (GIST: 6 cases, inflammatory diseases: 4 cases, post-mortem autopsy cases with stomachs from healthy persons victims of traumatic accidents: 16 cases). Immunohistochemistry investigation was performed with the use of Ventana Benchmark ultra, anti-ghrelin antibody NOVUS, mouse monoclonal 2F4, diluted at 1:100.

Results No significant difference in the expression of GPC number between group A and B was found ($p = 0.87$). No significant correlation between patients presenting a GPC number above (subgroup 1) or below (subgroup 2) the average, and EWL% changes, both at 1 and 6 years of follow-up, was recorded.

Conclusions Our study has shown that the expression of GPC is similar in the stomach of obese and non-obese controls, being mostly influenced by the inflammatory status of the gastric mucosa. A variation in the preoperative number of GPC has not influenced the weight loss in patients who underwent LSG.

Keywords Ghrelin-producing cells expression · Laparoscopic sleeve gastrectomy · Immunohistochemistry · Case-control study

Introduction

Due to the high failure rate of dietary and behavioral approaches to obesity, bariatric surgery is currently considered the only effective therapy for morbid obesity. A general improvement of life quality, accompanied by durable weight loss, together with an improved cardiovascular profile, and remission of type II diabetes (T2DM), is normally achieved [1].

In the last years, the laparoscopic sleeve gastrectomy (LSG) has become one of the preferred and most performed bariatric procedure worldwide [1]. In Italy, according to data from the Italian Society for Bariatric and Metabolic Surgery (SICOB), 7116 LSGs have been performed in 2017, making this surgery the most popular by far among the national bariatric community [2].

The LSG is an appealing procedure, and according to several authors, it achieves results similar to the standard laparoscopic Roux-en-Y gastric bypass, which is considered a more demanding surgery, presenting higher morbidity and mortality rates [3, 4].

Most studies have demonstrated the LSG being effective for weight loss, providing good results in improvement and even resolution of comorbidities like type 2 diabetes (T2DM) [5].

Ghrelin is a complex peptide consisting of 28 amino acids and presenting different biological functions. It favors the release of growth hormone and modulates stomach acid

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secretion and motility, regulating at the same time both endocrine and exocrine pancreatic secretions [6]. In particular, ghrelin enhances the GLP-1 secretory response to ingested nutrients whereas GLP-1, a gastrointestinal L-cell hormone, in turn enhances glucose-stimulated insulin secretion, favoring glucose homeostasis [7]. Ghrelin is especially known for stimulating appetite and food intake. This neuroendocrine pathway is activated by the growth hormone secretagogue receptor (GHSR), a G-protein-coupled receptor which is located in key areas of the brain, and designed to mediate specific actions of the hormone [8].

Ghrelin is secreted by the endocrine X/A-like cells of the gastric fundus mucosa. Outside from the stomach, several organs synthesize ghrelin. They are the kidney, pituitary, hypothalamus, the entire small bowel (duodenum, jejunum, ileum), colon, and pancreas.

Normally, during LSG, following the resection of the gastric fundus, where most ghrelin-synthetizing cells are localized, plasma ghrelin levels are expected to decrease; nevertheless, controversial results have been recently reported [9–11]. In this light, a clinical-histopathological prospective study has been designed to assess the expression of ghrelin-producing cells (GPC) in two groups of morbidly obese and non-obese patients.

Patients and Methods

Starting from February 2008, 445 LSGs have been performed at our institution on 289 female and 156 male patients. Mean age was 39 ± 7.8 (range 17–65) while mean preoperative BMI was 47 ± 6.6 Kg/m^2 (range 36–64). An informed consent to surgery was obtained from all patients who underwent a LSG procedure. All patients underwent preoperatively an esophago-gastro-duodenoscopy (EGDS), a *Helicobacter pylori* screening and pulmonary thromboembolism (PE) prophylaxis according to SICOB guidelines [4]. One dose of 2 g ceftriaxone was administered intravenously about 15 min before surgery for infection prophylaxis. In all cases, patients were approached by laparoscopy. A standard technique, extensively described elsewhere [12, 13], was routinely performed to model the gastric sleeve. All resected gastric specimen routinely underwent standard histopathological examination. With the aim to define the expression of ghrelin-producing cells, specimens of the resected stomach from a cohort of morbidly obese patients who underwent LSG were compared with the gastric specimen from non-obese controls. Ethical approval to this study was obtained from "Federico II" University institutional review board.

Control Group Selection

With the aim to have similar groups, stomachs from the control group were selected from patients having the same sex and age and general health status, of patients who underwent LSG. This in general limited a lot the number of controls available but provided two homogeneous groups. Being the control group made of non-obese patients, eventual obesity-related diseases as T2DM or hypertension could not be obviously matched.

Pathological Evaluation

A specific immune-histochemical evaluation was conducted at the Department of Pathology of the "Federico II" University of Naples. Specimens of resected stomach from 26 consecutive obese patients who underwent a LSG (group A) were analyzed and compared to control stomachs samples from 26 non-obese patients (group B) presenting similar demographic data, resected for other pathologies or during autopsy (GIST: 6 cases, inflammatory diseases: 4 cases, post-mortem autopsy cases with stomachs from healthy persons who are victims of traumatic accidents: 16 cases). In the latter case, as required by the Italian judicial authority, autopsy was performed within 24 h from death; all families were in any case asked to sign an informed consent allowing pathologists to use cadaveric stomachs for scientific purpose.

All samples were taken at the level of the fundus, corpus, and preantral regions of the stomach. All specimens were then formalin fixed, processed, and paraffin embedded following standard laboratory procedures. Tissue sections were cut at 4 μm on microtome and mounted on slides for different staining. Hematoxylin-eosin staining was performed for tissue analysis and for the diagnosis of a possible gastritis (see Fig. 1a); Giemsa staining was performed for assessment of infection by *Helicobacter pylori*; immunohistochemistry investigation was performed with the use of Ventana Benchmark ultra, anti-ghrelin antibody NOVUS, mouse monoclonal 2F4, diluted at 1:100 (see Fig. 1b, c). The rat stomach, which is known to express ghrelin, was used as a positive control for the experiment, and the negative control was a specimen that received phosphate buffer solution (PBS) instead of primary antibody. The positive result was indicated by the appearance of brown color in the antibody binding site. For each specimen, the histological evaluation of the expression of ghrelin-secreting cells was performed by two experienced pathologists on slides representative of all gastric parts (fundus, corpus, and preantral zone), using a LEICA DM1000 microscope. Five consecutive selected fields containing the full mucosa thickness at × 400 magnification that correspond at about 1 mmq of tissue were studied for the count of ghrelin-secreting cells in each slide. Positive cells in the epithelial layer showed perinuclear signal and were counted in each field. The total number for five fields was evaluated for all gastric parts studied and the average number for the field of ghrelin-secreting cells was recorded.

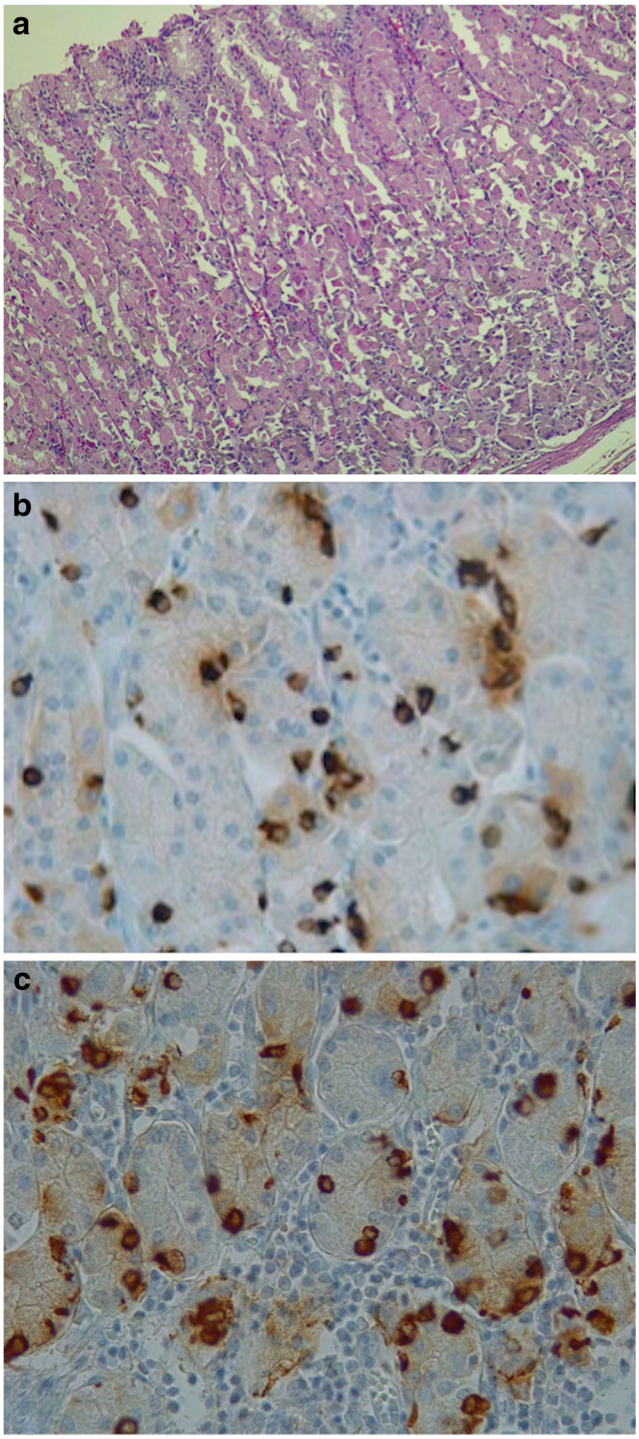

Fig. 1 **a** Hematoxylin eosin, × 20 magnification. Gastric oxyntic mucosa of LSG. Note that in all analyzed specimens from LSG, only oxyntic mucosa was present. **b** Immunohistochemistry antibody antighrelin, × 400 magnification. This picture shows perinuclear positivity for antighrelin in a morbid obese patient. At this magnification the count was made. **c** Immunohistochemistry antibody antighrelin, × 400 magnification. This picture shows perinuclear positivity for antighrelin in a control patient with gastritis. At this magnification the count was made

Statistical Analysis

Statistical analysis was performed with the Statistical Package for Social Sciences (Version 20.0 for Windows; SPSS Inc., Chicago, Ill, USA). The Yates corrected χ^2 test was used as a means of evaluating differences in categoric variables, and the Mann-Whitney *U* test was used for continuous variables. The average number of GPC was then compared between group A and group B.

Moreover, among patients who underwent LSG (group A), the relation between stomachs presenting a number of GPC above (subgroup 1) or below (subgroup 2) the average, and excess weight loss rate (EWL%) at 1 and 6 years follow-up was evaluated with the Mann-Whitney *U* test. The BMI changes following surgery were studied as well. A p value of < 0.05 was considered statistically significant.

Results

In general, the main histopathological findings of the 445 resected stomach samples were: 165 cases of mild/moderate chronic gastritis (37.0%), 23 cases of moderate/severe chronic gastritis (5.2%), 10 cases of intestinal metaplasia (2.3%), and 5 cases of gastric fundus polyps (1.1%). Preoperative demographic data did not present any significant difference in terms of gender, age, or drug assumption (at eventual death) between the two groups. All 26 obese patients included in our study who underwent LSG were allowed a liquid oral diet on postoperative day (POD) 3, and they were routinely discharged from the hospital on POD 5. No early or late surgical complications were recorded.

On 26 resected LSG stomachs from obese patients (group A), immunohistochemical analysis showed an average number of ghrelin-producing cells of 22.7 ± 5.7 (range 6.2–29.2) per slide. The same immunohistochemical test performed on 26 stomach specimens of non-obese controls (group B) showed a mean number of GPC of 22.8 ± 3.5 (range 15–27) per slide. No significant difference in the expression of ghrelin-producing cell number between the obese and non-obese groups was found ($p = 0.87$) (see Fig. 2).

In group A, there were three gastric specimens from patients affected by moderate/severe gastritis. The average number of ghrelin-producing cells in these cases was 9.0 ± 2.7 (range 6.2–12.8) per slide; the same finding was also observed in four stomach samples from group B. The average number of ghrelin-producing cells in these specimens was 16.3 ± 0.7 (range 15.0–17.0) per slide. In both groups, a significant decrease ranging from 40 to 70% in ghrelin-producing cell expression was detected.

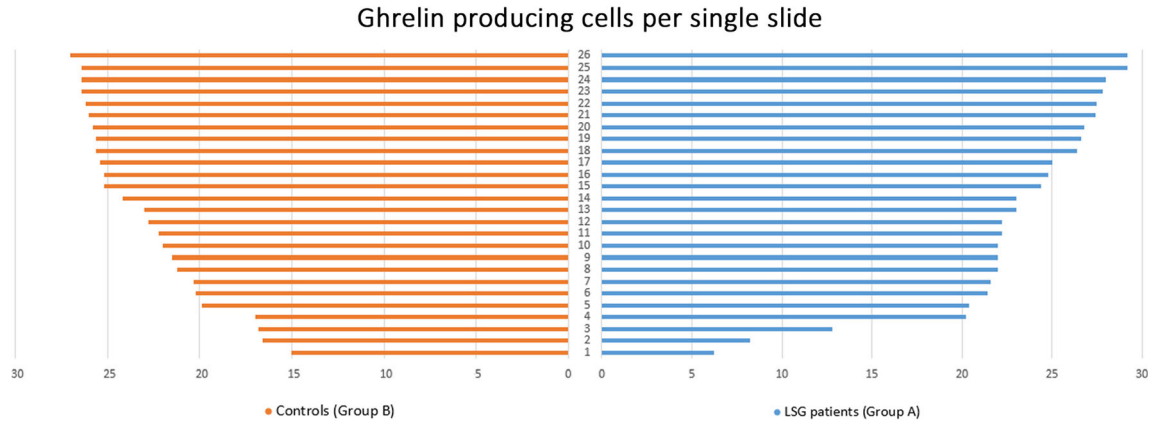

Fig. 2 Linear expression of ghrelin-producing cells distribution in groups A and B. The axis of the ordinates reports the number of patients investigated. The axis of the abscissas reports the average number for the field of ghrelin-secreting cells (see the "Patients and methods" section)

Follow-Up

Relating to 26 patients who underwent LSG (group A), data about weight loss at 1-year follow-up were collected (in 26/26; 100%) and for 12/26 (46.1%) at 6 years from surgery.

A significant weight loss was recorded by comparing pre-operative BMI with both 1 year (43.43 ± 4.20 vs 34.46 ± 6.19, $p = 0.001$) and 6 years (43.43 ± 4.20 vs 35.31 ± 7.02, $p < 0.001$) follow-up data. Furthermore, the Mann-Whitney test revealed no significant correlation between patients presenting an expression of GPC above (subgroup 1) or below (subgroup 2) the average and EWL% changes, both at 1 and 6 years of follow-up (see Table 1 and Fig. 3a, b).

Discussion

According to a recent report, the laparoscopic sleeve gastrectomy (LSG) has become the preferred surgical approach to morbid obesity worldwide [14]. There are several reasons for this result. LSG remains an appealing procedure, providing satisfying results in the treatment of morbid obesity. It is rather easy to perform, offering results very similar to the standard Roux-en-Y gastric bypass, which is conversely considered a more challenging procedure under a technical standpoint [5]. LSG presents at the same time other advantages; a quicker gastric emptying, the lack of implanted foreign bodies, or the absence of intestinal shortcuts involving some malabsorption are in fact frequently underlined. Furthermore, in the case of weight regain or intractable reflux, LSG may be easily converted into a LRYGBP, a BPD-DS [15], or a MGB-OAGB [16]. This has led many authors to suggest LSG as an effective single-step procedure [17].

Despite recent warnings regarding the risk of postoperative leaks [18], the potential risk of favoring micronutrient deficiency [19], the reported onset of de novo gastro-esophageal reflux disease (GERD) [20] or even Barrett's lesions in the long term [21], the results observed suggest that LSG can lead to substantial and long-lasting excess weight loss and significant improvement in obesity-related co-morbidities [22, 23]. During the last years, several authors have investigated the mechanism of weight loss-promoting action of LSG. The results have been however controversial. LSG has been defined either a simple restrictive procedure [10, 24] or, alternatively, a food-limiting surgery involving complex hormonal responses [11, 25, 26].

Considering that the highest number of ghrelin-producing cells is located in the proximal stomach, especially at fundus level [27], an area that is completely resected during LSG, a key role of changes in the circulating level of ghrelin has been proposed. Since the biological functions of ghrelin include the stimulation of appetite and food intake, the decrease in circulating hormone level has been indeed pointed as the cornerstone of the efficacy of LSG, since this surgery has been suggested as a stand-alone procedure [3, 28, 29]. Nevertheless, several studies

Table 1 EWL% variations between patients who underwent LSG. Patients presenting a number of ghrelin-producing cells (GPC) above the average (subgroup 1) were compared to patients presenting a number of ghrelin-producing cells (GPC) below the average (subgroup 2) at 1 and 6 years follow-up

	Subgroup 1	Subgroup 2	*p* value
Patients	14/26 (53.8%)	12/26 (46.2%)	
EWL% (1y FU)	43.14 ± 23.72	41.20 ± 24.30	0.837
EWL% (6y FU)	47.12 ± 22.76	41.50 ± 21.80	0.465

Data are expressed as mean ± standard deviation

EWL excess weight loss, *FU* follow-up

Subgroup 1 = patients presenting a number of ghrelin-producing cells (GPC) above the average

Subgroup 2 = patients presenting a number of ghrelin-producing cells (GPC) below the average

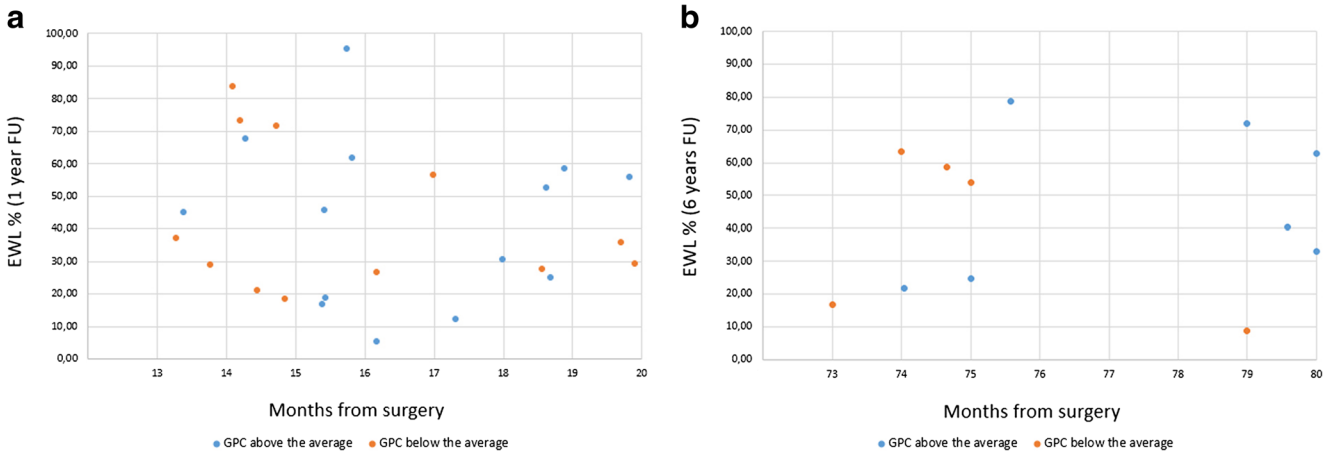

Fig. 3 Scatter plot of correlation between patients with ghrelin-producing cells (GPC) number above or below the average and excess weight loss rate (EWL%) at 1 (**a**) and 6 (**b**) years of follow-up

from different authors have failed to show a univocal decrease of circulating ghrelin levels after LSG. While Karamanakos [30] in a prospective trial reported a marked decrease in postoperative ghrelin levels following LSG, Terra [9] in a more recent study showed an increase of the circulating levels for this hormone. These controversial results have been later confirmed in a prospective study by Braghetto [10], which concluded there is no relation between ghrelin postoperative levels and weight loss, 1 year following LSG.

The reasons for these unclear results regarding post-LSG circulating ghrelin levels, in our opinion, have been exhaustively explained in a large review by Tymitz [31].

The first cause for the difficulty in interpreting studies attempting to correlate changes in ghrelin and BMI following bariatric surgery may be found in the different laboratory procedures, given that collection methods, sample storage, and analyses are all potential sources of variation. Another confounding factor is represented by the differences in follow-up period. A short-term follow-up is in fact usually associated with steep reductions in plasma ghrelin levels [32]. Again, ghrelin must be measured when the patient is weight-stable. A patient in a negative energy mode, as such occurring when dieting, anorexic, or otherwise losing weight, normally presents increased ghrelin levels [33]. A last bias is finally represented by eventual differences in surgical techniques. Variation in the size or shape of the gastric sleeve may in fact affect ghrelin circulating levels.

Following these observations, with the aim to define a possible hormonal determinant for the LSG, we therefore considered more interesting to study the expression of ghrelin-producing cells between two different cohorts of patients. Contrary to circulating ghrelin levels, the expression of GPC can be indeed assessed in a more standardized form, being less prone to the previously identified specific biases [34].

Our results, showing a similar expression of GPCs between obese and non-obese control patients lead to a number of observations. The first one is that, if on one side ghrelin expression is surely involved in the regulation of food intake and the modulation of gastric acid secretion and motility, and is modulated by weight loss, this role does not appear to be strictly related to the reduced food intake induced by bariatric surgery. This is confirmed in previous studies by Zigman [6] and by Tschop [35]. Thus, bariatric surgery appears to play a marginal role in the ghrelin homeostasis, which is instead more influenced by the moment in which ghrelin circulating levels are assessed. The second observation is that our study confirms mild chronic gastritis to be a very common finding in obese patients undergoing LSG [36]. The ghrelin-producing cell expression in both groups herein investigated may be biased by the inflammatory condition of the gastric mucosa, or by the eventual *Helicobacter pylori* infection [34, 37], more than by morbid obesity per se. Our study confirms therefore the expression of GPC being unrelated to the results achieved by laparoscopic sleeve gastrectomy, and this is confirmed by experimental studies, showing the effects of LSG being independent from ghrelin circulating levels [38]. To support these data, pointing mainly toward a restrictive role for LSG, Fahmy has recently observed how weight regain was strictly related to postoperative dimensions of the sleeved stomach [39].

The main limitations of our study are represented by both the small sample size and by the heterogeneity of the two cohorts; matching of control group was in fact not based on multiple variables. Nevertheless, in support of our inclusion of autoptic cases in group B, it should be underlined that the immunohistochemical distribution of peptide-producing cells in organs obtained from human autopsy has been previously reported [40, 41]. Also, the distribution of GPC at the level of the gastric mucosa should be considered as a partial surrogate of ghrelin circulating levels. Again, it should be considered that the same number of ghrelin-producing cells might present a different activity and therefore different levels of hormone secretion. Finally, because of the controversial results reported [31], ghrelin circulating levels were not assessed.

In conclusion, our study has shown that the expression of GPC is similar in the stomach of obese and non-obese controls, being mostly influenced by the inflammatory status of the gastric mucosa.

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Authors' Contribution Authors MM, MDA, GG, GP, and FPDA gave their substantial contributions to the conception or design of the work, while authors FDC, NV, AB, KDL, and SC dedicated to the acquisition, analysis, or interpretation of data for the work.

All authors:

• Were involved in drafting the work or revising it critically for important intellectual content;

• Gave final approval of the version to be published;

• Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Compliance with Ethical Standards

All LSG procedures performed by authors were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical approval to this study was obtained from "Federico II" University institutional review board.

Conflict of Interest The authors declare that they have no conflicts of interest.

Informed Consent An informed consent to surgery was obtained from all patients who underwent a LSG procedure performed by anyone of the authors of this study during these years.

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