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ARTICLE

Molecular Biology

# Gastric ghrelin cells in obese patients are hyperactive

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## Abstract

**Background/objectives** Distribution and activity of ghrelin cells in the stomach of obese subjects are controversial.

**Subjects/methods** We examined samples from stomachs removed by sleeve gastrectomy in 49 obese subjects (normoglycemic, hyperglycemic and diabetic) and quantified the density of ghrelin/chromogranin endocrine cells by immunohistochemistry. Data were compared with those from 13 lean subjects evaluated by gastroscopy. In 44 cases (11 controls and 33 obese patients) a gene expression analysis of ghrelin and its activating enzyme ghrelin O-acyl transferase (GOAT) was performed. In 21 cases (4 controls and 17 obese patients) the protein levels of unacylated and acylated-ghrelin were measured by ELISA tests. In 18 cases (4 controls and 14 obese patients) the morphology of ghrelin-producing cells was evaluated by electron microscopy.

**Results** The obese group, either considered as total population or divided into subgroups, did not show any significant difference in ghrelin cell density when compared with control subjects. Inter-glandular smooth muscle fibres were increased in obese patients. In line with a positive trend of the desacylated form found by ELISA, Ghrelin and GOAT mRNA expression in obese patients was significantly increased. The unique ghrelin cell ultrastructure was maintained in all obese groups. In the hyperglycemic obese patients, the higher ghrelin expression matched with ultrastructural signs of endocrine hyperactivity, including expanded rough endoplasmic reticulum and reduced density, size and electron-density of endocrine granules. A positive correlation between ghrelin gene expression and glycemic values, body mass index and GOAT was also found. All obese patients with type 2 diabetes recovered from diabetes at follow-up after 5 months with a 16.5% of weight loss.

**Conclusions** Given the known inhibitory role on insulin secretion of ghrelin, these results suggest a possible role for gastric ghrelin overproduction in the complex architecture that takes part in the pathogenesis of type 2 diabetes.

## Introduction

Ghrelin is a powerful orexigenic hormone mainly produced and secreted by endocrine cells of the gastric glands of the stomach fundus and acting in the brain to regulate food intake [1–5]. In addition to its orexigenic role, ghrelin also plays a role in carbohydrate metabolism and in gastrointestinal motility [2, 6–8]. Indeed, it has been shown that acylated ghrelin, the active form of the hormone acylated by the enzyme ghrelin O-acyltransferase [GOAT; also known as membrane-bound O-acyltransferase 4 (MBOAT4)] has an inhibitory action on insulin secretion in pancreatic beta-cells [6, 9, 10]. Though obese patients, with or without diabetes, generally display reduced ghrelin levels and a blunted fluctuation of the hormone throughout the day [11–13], accumulating evidence points to a significant role of ghrelin in the pathogenesis of hyperglycemia and insulin resistance

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accompanying obesity [14, 15]. The vast majority of subjects suffering from severe obesity, undergoing bariatric surgery, are mainly treated by sleeve gastrectomy [16]. This technique removes a large part of the stomach, including the entire area of the fundus where most ghrelin-producing cells (GPCs) are located [3]. A high percentage of obese patients are also insulin-resistant, have high circulating levels of insulin, hyperglycemia or have frank diabetes [17, 18]. Of those subjects treated with bariatric surgery using sleeve gastrectomy, 60–70% have an improvement in glucose metabolism, up to the complete disappearance of diabetes in a very short time after surgery, even before a significant loss of body weight [19]. The available data on the anatomy and function of ghrelin-secreting cells in the stomach of obese subjects are somewhat discordant because some reports are in favour of an increased number of GPCs [20–22], while others found no differences with controls [23, 24]. On these bases, we developed a quantitative analysis of these cells and studied the functional activity of gastric ghrelin cells by gene expression analysis of the hormone and its activator enzyme (GOAT). Furthermore, we analysed the ultrastructure of organelles in gastric ghrelin cells by electron microscopy. Considering the well-known activity of ghrelin on gastric motility [7, 8], we also analysed the mucosal smooth muscle fibres and different types of nerve fibres. Finally, we correlated the anatomical and functional data with the glycemic metabolism of the patients examined.

Our results showed that the absolute number of ghrelin cells or their density in the endocrine population of the stomach of obese patients was not different from controls, either considering the total population of obese patients or the obese subgroups: normoglycemic (Ob NG), hyperglycemic (Ob HyG) or diabetic (Ob Dia). Smooth muscle fibres of the inter-glandular mucosae were increased in obese patients, but no evidences of altered innervation were found. The hormonal secretory activity of ghrelin cells was significantly increased, especially in the hyperglycemic subgroup, in line with the ultrastructural morphology of these cells. A positive correlation was found between ghrelin expression and GOAT, glycemic values and body mass index. All diabetic patients recovered from diabetes in the early period post-surgery.

## Patients, materials and methods

### Patients

A total of 49 obese patients (11 males and 38 females), scheduled to undergo laparoscopic sleeve gastrectomy, and 13 controls (Ctrls, 7 males and 6 females) were consecutively included in the study (Supplementary Table 1 and 2). In the entire population, the samples for all the analyses consisted of the gastric fundic mucosa. Of the 49 obese subjects, 12 patients had diabetes mellitus (24.5%). In terms of additional disease, 14 (28.57%) of the 49 obese patients had concurrent hypertension. Lean controls comprised 13 healthy patients who underwent endoscopy of the upper gastrointestinal tract for dyspeptic diseases and resulted in normal histology. In the obese patients, average age was 42.7 ± 1.4 years, ranging from 26 to 61 years and average BMI was 43.96 ± 0.6 kg/m$^2$, ranging from 35 to 57.26 kg/m$^2$. In the control group, average age was 37.7 ± 2.6 years, ranging from 25 to 52 years (difference vs obese patients not significant, $p = 0.132$) and average BMI was 23.7 ± 0.6 kg/m$^2$, ranging from 19.4 to 27.3 kg/m$^2$ (difference vs obese patients $p = 0.0001$).

Exclusion criteria included previous bariatric surgery, chronic liver or renal disease, heart failure, current drug or alcohol abuse, uncontrolled severe psychiatric illness. Further exclusion criteria included histological parameters such as erosion and/or ulceration in the mucosal surface epithelium, neutrophilic infiltration in parietal cells, severe lymphocytic infiltration, as well as sections with transversally cut fundic gland.

All patients who were scheduled to undergo bariatric surgery were required to have routine labs, electrocardiogram, gastroscopy, pulmonary function studies and a medical evaluation. The fasting period for both bariatric and control patients was the same (12–15 h). Information such as diabetic medication use, fasting glucose, insulin, and glycated haemoglobin levels were documented for each obese diabetic patient preoperatively and in the postoperative periods (Supplementary Table 2).

The study protocol was approved by the local Research Ethics Committee of Garibaldi Hospital (Catania) (Protocol number 528/CE, 26 July 2016) and all patients gave written informed consent before study entry in accordance with the Helsinki Declaration.

### Surgical procedure

Sleeve gastrectomy was performed as a laparoscopic procedure, following the procedure described by other authors [25]. Morphological and molecular analyses were performed in the fundus mucosae.

### Light microscopy and immunohistochemistry

Stomach specimens resected during laparoscopic sleeve gastrectomy and mucosal biopsies were thoroughly washed in saline solution immediately after extraction to remove debris, fixed with 4% paraformaldehyde for 24 h and embedded in paraffin. Sections 4 µm thick, perpendicular to the mucosal layer, were cut on a sliding microtome (Leica RM 2135, Leica Microsystem, Milan, Italy) and stained

**Table 1** Primary antibodies used in the study.

| Antibodies | Host[a] | Diluition | Incubation time | Source | Antigen retrival |
|---|---|---|---|---|---|
| Anti-Calcitonin gene-related peptide (CGRP) | R | 1:1000 | 20 min | Abcam, Cambridge, England, ab91007 | Pt in buffer pH 6 |
| Anti-CD56 | M | 1:100 | 30 min | Roche Diagnostics GmbH Mannheim, Germany, 790-4465 | Pt in buffer pH 6 |
| Anti-Chromogranin | M | 1:1000 | 20 min | Roche Diagnostics GmbH Mannheim, Germany, 760-2519 | Pt in buffer pH 6 |
| Anti-Ghrelin | R | 1:400 | 10 min | Abcam, Cambridge, England, ab129383 | Pt in buffer pH 6 |
| Anti-Neuron-specific enolase (NSE) | M | Ready to use | 20 min | Roche Diagnostics GmbH Mannheim, Germany, 760-4786 | Pt in buffer pH 9 |
| Anti-Substance P (SP) | M | 1:1000 | 30 min | Abcam, Cambridge, England, ab14184 | Pt in buffer pH 6 |
| Anti-Tyrosine hydroxylase (TH) | S | 1:200 | 20 min | Abcam, Cambridge, England, ab1542 | Pt in buffer pH 7,5 |
| Anti-Vesicular acetylcholine transporter (VAChT) | S | 1:1000 | 30 min | Abcam, Cambridge, England, ab31544 | Pt in buffer pH 6 |

[a]*R* rabbit, *M* mouse, *S* sheep, *Pt* pretreatment.

with haematoxylin and eosin for routine use. Immunohistochemical staining was performed on paraffin sections by the Bond Polymer Refine Detection system (Leica Microsystem, Milan, Italy) following manufacturer's instructions, and using the Bond-Max Autostainer (Leica Microsystems, Milan, Italy). All steps were performed at room temperature, unless otherwise specified. Briefly, for antigen retrieval the sections were subjected to pretreatment in acid or basic buffer (depending on the used marker) at high temperature (70 °C). The tissue sections were treated for 5 min in 3% hydrogen peroxide solution to block endogenous peroxidase activity and then incubated with the primary antibody (Table 1). Specific staining was visualised by using 3,3′-diaminobenzidine as chromogen. The sections were counterstained in Mayer's haematoxylin for 7 min and coverslipped. Negative controls were treated identically without the primary antibody. Positive controls, as suggested by data sheets, were performed for each type of antibody unless there was a presence of internal positive controls.

The mucosal specimens of obese patients and lean controls were also stained, immunohistochemically, with anti-NSE and anti-CD56 antibodies. Elongated structures resembling smooth muscle fibres were immunoreactive for the specific antigens NSE and CD56. These structures were measured by two different operators. For quantitative morphometric analysis, proportions of NSE and CD56 expression in the sections were evaluated with an image-analysis system, consisting of a light microscope, a digital camera, and an imaging workstation personal computer with a software for image acquisition and analysis (AxioVision Release 4.8.2-SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). Three random areas of each section were scanned by the microscope and all images captured under ×20 magnification. The percentage area of smooth muscle fibres stained by NSE and CD56 antibodies was measured in the images. In order to derive the tissue proportions of NSE and CD56 (% NSE and % CD56) within each section, the mean of the percentage area measurements of the images from each section was calculated.

Further immunohistochemical analyses included antibodies against nerve fibres in order to evaluate the innervation supply of the mucosae. These analyses were performed by TH (noradrenergic fibres), VAchT (cholinergic fibres), SP and CGRP (afferent fibres) antibodies.

## Light microscopy and morphometric analysis

The epithelial tissues of obese patients and controls were stained immunohistochemically with anti-ghrelin and anti-chromogranin antibodies. An Axioskop 40 microscope (Carl Zeiss GmbH, Jena, Germany) with a digital camera attached and AxioVision Rel 4.8 microscopic imaging software were used. Ten fields of mucosal tissues, from each stained slide, were randomly chosen and captured at ×20 magnification. Two different observers blinded to the patient group performed the analysis by counting chromogranin-producing cells (CPCs) and GPCs in the entire set of photographs for each slide. Only the nucleated cells with brown chromogen in the cytoplasm were considered positive for counting. The total number of CPCs and GPCs was recorded and density of GPCs with respect to CPCs was calculated.

## RNA extraction and quantitative real-time-PCR

Total RNA was extracted from 44 samples: 11 healthy patients and 33 obese, further subdivided into 16 Ob NG, 7 Ob HyG, and 10 Ob Dia using an RNeasy Mini Kit, according to the manufacturer's instructions. RNA concentration and quality was determined using an ND-1000

spectrophotometer (NanoDrop, Thermo Scientific, USA). Single-stranded cDNA from total RNA (4 µg) was synthesised using SuperScriptTM III First-Strand Synthesis SuperMix (Life Technologies Waltham, Massachusetts, USA).

Quantitative real-time RT-PCR (qRT-PCR) experiments were performed using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer pairs were designed using the NCBI primers designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) for the following transcripts:

Ghrelin transcript variant-1 (GHRL, accession number: NM_016362), amplified region between exon one and exon two; hGHRL 1 F: 5′ CTT CCT GAG CCC TGA ACA CC 3′; hGHRL 2R: 5′ CTC TGC CCC TTC TGC TTG AC 3′; 130 bp size product.

Human GOAT (or MBOAT4, accession number: NM_001100916), amplified region between exon one and junction exon one-two; GOAT 1F: 5′ GGC TTT GGC TGT TCT TTC TCC 3′; GOAT 1-2R: 5′ AGA GGT ACC TGG CAC GAG T 3′.

The human housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, accession number: NM_002046) was used as the endogenous control, amplified region between exon three and junction exon four-five; 158 bp size product; GAPDH 3F: 5′ GTCAAGGCTGAG AACGGGAA 3′; GAPDH45R: 5′ AAATGAGCCCCA GCCTTCTC 3′.

Each PCR reaction (10 µL final volume) was carried out using a QuantiFast SYBR Green PCR kit (Qiagen Hilden, Germania), 1 µM of primers and 3 µl of diluted cDNA (25 ng/µl). Standard conditions were used for PCR amplification (95 °C for 5 min followed by 45 cycles at 95 °C for 10 s, 60 °C for 30 s). As a negative control, we performed reactions without cDNA (no template control, NTC). All reactions were performed in triplicate and a melting curve was included for assay validation for each pair of primers. The relative mRNA abundance of the gene of interest was deduced from the cycle number at which fluorescence increased above background level (Ct) in the exponential phase of the PCR reaction, and calculated using the $2^{-\Delta\Delta Ct}$ as previously described [26]. Qualitative PCR was performed for all analysed samples and solved by electrophoresis agarose gel (1.8%) and SYBR Safe DNA Gel Staining (data not shown).

## Electron microscopy and morphometric analysis

In 18 selected samples, based on gene expression and GPCs' density data (4 CTRLs and 4 Ob NG, 3 Ob HyG and 7 Ob Dia patients), electron microscopy was also performed. Small tissue samples of about 1 $mm^3$ were immersed in a fixative composed by 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L phosphate buffer pH 7.4, for 4 h. They were then washed with PB, fixed in 1% of $OsO_4$ for 60 min at 4 °C, dehydrated in acetone and included in a mixture of Epon-Araldite. Thin sections were obtained with an MTX ultramicrotome (RMC, Tucson, AZ, USA), stained with lead citrate and examined with a Philips CM 10 transmission electron microscope (Philips, Eindhoven, Netherlands). Two µm resin sections (semithin sections) stained with toluidine blue were used to select the thin sections (60 nm). For each patient, at least three different sections of gastric mucosae samples were studied in semithin sections, and a number of thin sections from min 2 to max 5 were performed for each patient in order to allow the analysis of an average number of about ten different ghrelin-like endocrine cells per patient. Morphometric analysis was performed on cells with electron microscopic features corresponding to those described for human GPCs [35] and selected among all endocrine-like cells photographed in the gastric mucosa of controls and obese patients studied by electron microscopy at original magnification of 7.500–13.000x. All selected images were analysed with ImageJ morphometric program (RRID:SCR_003070). In the control group 6 ghrelin-like cells (GLCs) from 3 patients were studied. In the obese subgroups 6 GLCs for the Ob NG (from 1 patient), 15 GLCs for the Ob HyG (from 2 patients) and 20 GLCs for the Ob Dia (from 6 patients) were studied. For each GLC, the whole cell, nucleus, rough endoplasmic reticulum, Golgi complex, secretory granules, microfilaments and lipofuscin areas were measured. Furthermore, secretory granule mean diameter was also calculated from the average area. Results are given as the density mean value ± standard error (SEM) of each organelle area per 1000 µm² of cytoplasm. Differences between groups were evaluated by one-way ANOVA (GraphPad Prism software) and were considered significant at $p < 0.05$.

## Enzyme immunometric assay for ghrelin peptide

The level of the unacylated and acylated 28-amino acids ghrelin peptide was measured by an enzyme immunometric assay based on a double antibody sandwich technique, using reagents provided by Bertin Pharma (human unacylated ghrelin immunoassay kit, cat. number A05319, human acylated ghrelin immunoassay kit, cat number A05106, SPI-BIO, France). Mucosal samples (100 mg) were homogenised (1:10 weight/volume) in enzyme immune assay buffer (cat. Number A07106, SPI-BIO, France) with the addition of aprotinin (at final concentration of 10 µg/ml) by a teflon/glass homogeniser in ice, submitted to three cycles of freezing at −80 °C /thawing at room temperature, and centrifuged at 1500 g for 10 min. Supernatants were collected and used for assays following protocols provided by Bertin Pharma.

## Statistical analysis

Data are presented as mean value ± standard error (SEM). Non-parametric Mann−Whitney and Kruskal−Wallis tests were used to compare GPC density in the stomach of lean and obese patients and the gene expression of Ghrelin and GOAT and protein levels of Ghrelin One-way ANOVA was used to compare the density of various organelles among the patients groups. Finally, the Spearman correlation coefficient (r) and its significance (p) were calculated between variables. $p < 0.05$ was considered statistically significant.

## Results

In gastric specimens from patients undergoing sleeve gastrectomy the absolute number of GPCs measured with a method similar to that used by other authors [20, 23] was not different from that found in control individuals. The number of gastric endocrine cells can vary in single individuals, and the density of a specific endocrine cell in the general endocrine population (expressing chromogranin; CPGs) has been considered as the more reliable method of analysis [27]. Even when measuring the GPC density (GPCs/CPGs) we did not observe any difference between obese and lean patients (Fig. 1A). We did not find any significant difference even when the obese subgroups (Ob NG, Ob HyG, Ob Dia) were compared with lean controls (Fig. 1A and B).

Light microscopy of haematoxylin and eosin sections showed, together with a slight lymphocytic infiltration, an apparent increase of elongated structures in the interglandular area of the mucosae. These structures, initially interpreted as nerve fibres, were immunoreactive for NSE and CD56, general markers for nerves that can also be found in smooth muscle fibres [28, 29]. Indeed, electron microscopy revealed their unequivocal smooth muscle fibre nature (data not shown). A quantitative assessment of these structures confirmed their increment in obese patients (Supplementary Fig. 1).

Considering the pleiotropic actions of ghrelin [4, 30, 31], we also checked whether there was an increment of mucosae nerve fibres by testing biopsies with TH (noradrenergic fibres), VAChT (cholinergic fibres) and SP and CGRP (afferent fibres) antibodies by immunohistochemistry. We did not find any relevant visual difference in the mucosal nerve distribution compared with controls (data not shown).

In rats, two different cytotypes of GPCs have been described, “open” and “closed”, depending on which they are, or are not, in contact with the lumen [32]. In our study, only the closed type of GPCs was found among a total of about 1000 fundic gastric glands examined by two independent operators in accordance with other studies performed in human tissues [33].

Gene expression data from about 71% of cases representative of all patients’ categories showed a significant

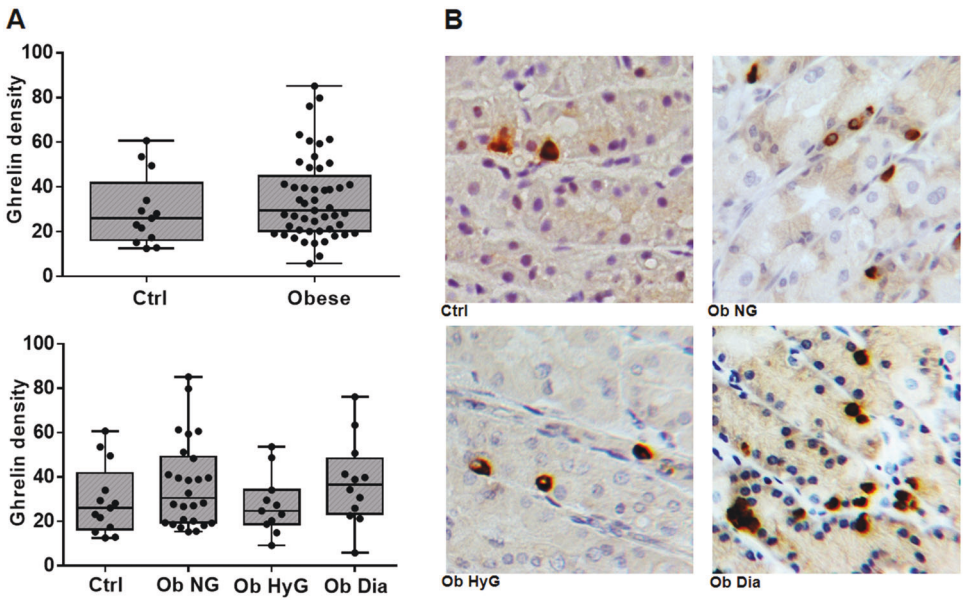


**Fig. 1 Density of ghrelin-producing cells (GPCs) in human stomach. a** Density of gastric ghrelin-producing cells (GPCs) vs gastric chromogranin-producing cells in lean and obese patients (upper panel). GPC density is not different from that of lean patients even in the different metabolic obese subgroups (lower panel). (Ctrl: lean, Ob NG: normoglycaemic obese, Ob HyG: hyperglycaemic obese, Ob Dia: diabetic obese). Statistical analysis for obese patients vs controls were performed by Mann–Whitney test, while multiple comparisons for subgroups Ob NG, Ob HyG and Ob Dia were performed using Kruskal–Wallis test. **b** Representative immunohistochemistry with anti-ghrelin antibodies of gastric mucosae of studied groups. Bar: 20 μm in all panels.

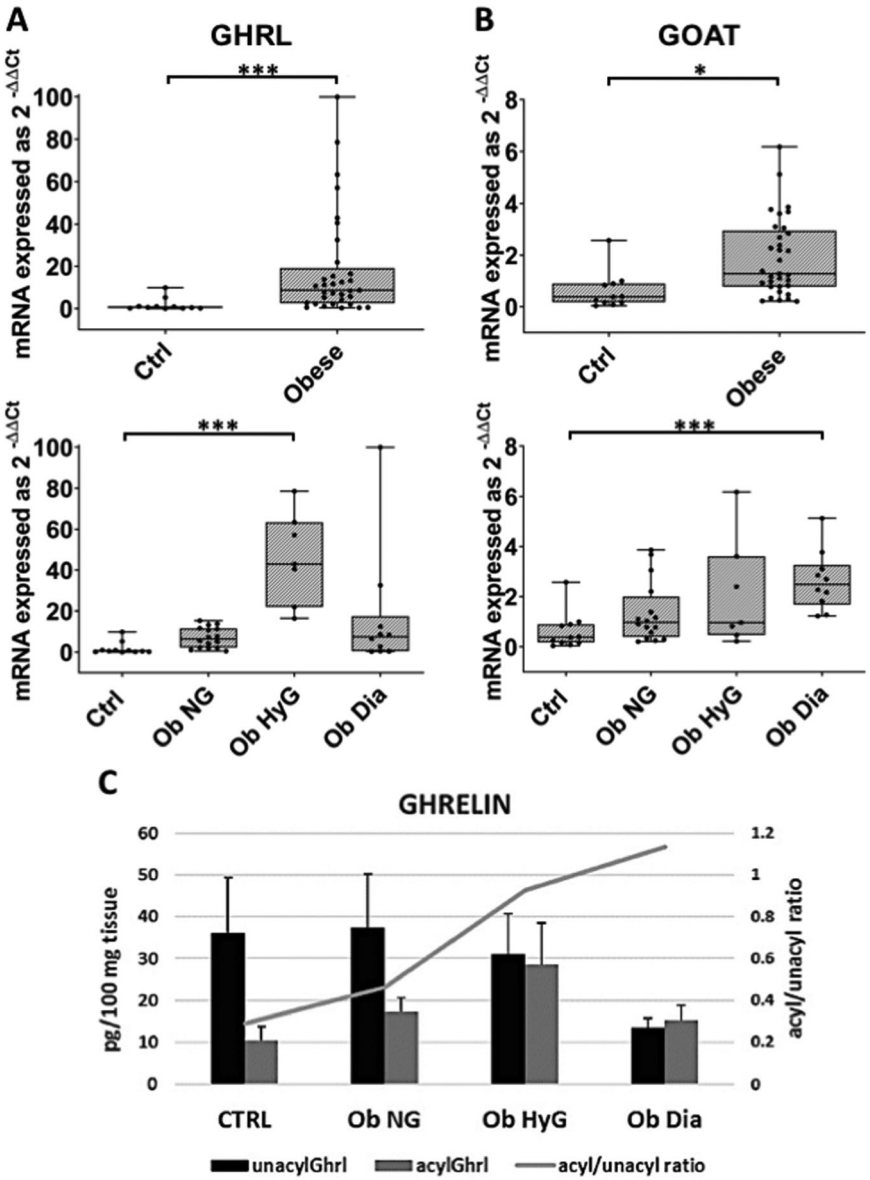


**Fig. 2 Gene expression of ghrelin and its activator ghrelin O-acyltransferase in human stomach.** Ghrelin (**a**) and ghrelin O-acyltransferase (GOAT) (**b**) gene expression in lean and obese patients. Expression of both genes is higher in obese patients, with the highest expression of ghrelin in hyperglycaemic patients. Normalised values vs GAPDH were expressed as $2^{-\Delta\Delta Ct}$. ***$p < 0.001$ vs Ctrl and *$p < 0.05$ vs Ctrl. **c** Protein levels of unacylated and acylated-ghrelin measured by ELISA tests. A reciprocal tendency of unacylated and acylated ghrelin content of Ghrelin in obese subjects compared with controls is evident. The acyl/unacylated ratio is reported in grey line. Statistical analysis comparing obese patients vs controls were performed by Mann–Whitney test, while multiple comparisons for subgroups Ob NG, Ob HyG and Ob Dia were performed using Kruskal–Wallis test. ***$p < 0.001$ vs Ctrl and *$p < 0.05$ vs Ctrl. Measures were performed by triplicate.

increase of ghrelin mRNA expression in obese patients (about fourfold in Ob NG, about 26 times in Ob HyG and about 9 times in Ob Dia patients), and a progressive parallel increase of GOAT gene expression (about 2 times in Ob NG, about 3 times in Ob HyG and about 4 times in Ob Dia patients) (Fig. 2). Considering the obese subgroups, ghrelin gene expression was particularly high in the Ob HyG subgroup of patients compared with CTRL (Fig. 2).

The measure of the levels of unacylated and acylated ghrelin protein in our specimens did not show significant differences among groups. However, in comparison with control and Ob NG subjects, Ob HyG and Ob Dia patients showed a tendency to a reduction of unacylated ghrelin content (Fig. 2), in accordance with the low levels of this peptide found in the blood of obese patients [34]. In contrast, acylated ghrelin showed a tendency to be more expressed in the Ob HyG patients (Fig. 2), mirroring the increased mRNA expression of the GOAT enzyme in the stomach of these subjects.

At electron microscopy human GPCs display peculiar features, allowing their easy recognition among all other endocrine cells of the stomach [35]. The study of normal mucosae confirmed these features (Supplementary Fig. 2). In brief, typical and unique characteristics (considered together) were perinuclear microfilaments; large lipofuscins; and typical small (about 168 ± 5.7 nm in diameter) and dense endocrine-like granules. Electron microscopy showed that endocrine cells with the above described unique features of gastric GPCs (ghrelin-like cells) maintained their typical ultrastructure in all subgroups of obese patients. However, often and only in obese patients, ghrelin-like cells particularly rich in microfilaments were observed (Fig. 3). Of note, ghrelin-like cells in the OB HyG

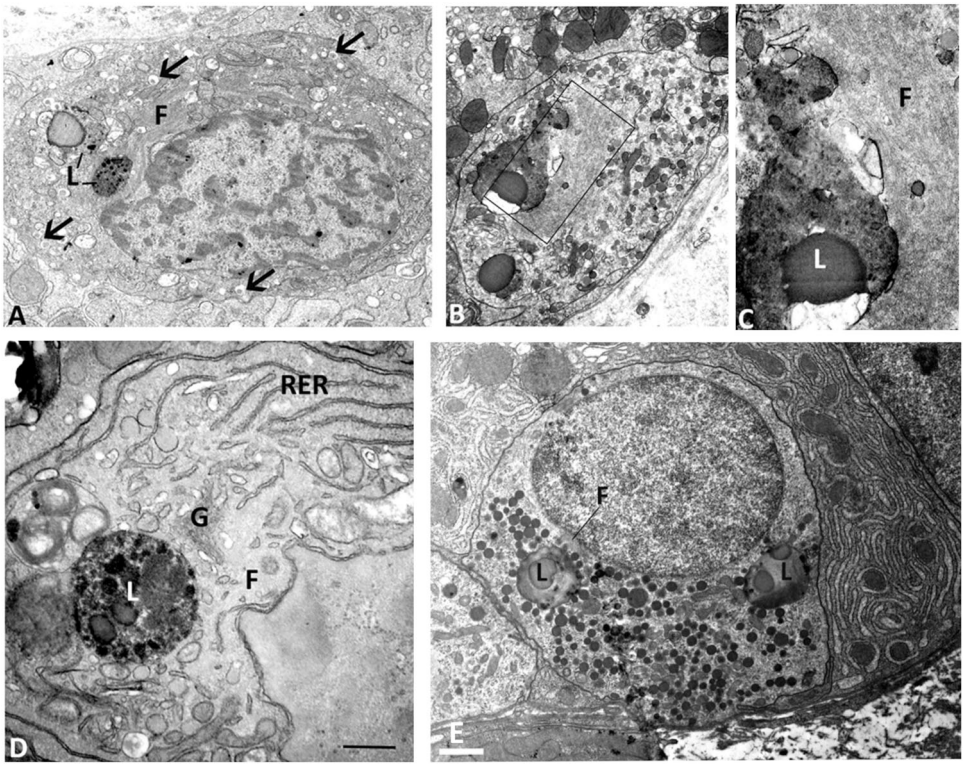


**Fig. 3 Representative electron microscope images showing ghrelin-like cells of obese and control patients.** The unique features found in these cells of lean patients are also present in obese patients: perinuclear microfilaments (F), large lipofuscin bodies (L) and endocrine granules (arrows, some indicated). **a** and **b** Two different obese diabetic patients. **c** Enlargement of squared area in (**b**) showing the abundance of microfilaments (F). **d** Hyperglycaemic obese patient. Note the hypertrophic organelles related to synthesis (stacked rough endoplasmic reticulum: RER) and hormone secretion (Golgi complex: G). **e** Representative electron microscopy of classic gastric ghrelin cell found in a control patient. L: lipofuscins, F: perinuclear microfilaments. Black Bar: 1 µm in (**a**) and (**b**), 370 nm in (**c**) and 300 nm in (**d**). White Bar: 1 µm in (**e**).

subgroups of patients (with the highest level of ghrelin gene expression) showed abundant organelles related to protein synthesis and secretion: i.e. rough endoplasmic reticulum in stacked cisternae and often hypertrophic Golgi complex (Fig. 3). Interestingly, the electron-density of endocrine granules was evidently reduced in ghrelin-like cells of all obese subgroups of patients (Fig. 3). These ultrastructural aspects prompted us to perform morphometric measurements (Fig. 4). Whereas the Golgi complex area did not change among the different groups, the cytoplasmic area occupied by the rough endoplasmic reticulum was significantly higher in the Ob HyG group than in control subjects. In addition, both granule size and the cytoplasmic area occupied by them were significantly reduced in the Ob HyG and Ob Dia groups in comparison with the controls. Finally, whereas an increased albeit not significant content of lipofuscins was found in all obese groups, a very significant increase of microfilaments was found in the Ob Dia subgroup in comparison with the controls.

Considering the inhibitory effect of acylated ghrelin on insulin secretion of the pancreas [4, 6], we checked whether any correlation was present between the levels of ghrelin gene expression and GOAT expression, glucose, insulin and glycated haemoglobin (HBA1c) blood levels and body mass index in obese patients. We found positive correlations between ghrelin expression and fasting glycemic values, body mass index and GOAT. Furthermore a positive correlation between BMI and GOAT gene expression was also found. Notably, the correlation between ghrelin and glycemic values was even more significant when only Ob NG and Ob HyG patients were taken into consideration (Fig. 5).

A clinical follow-up analysis was performed in 10 of the 12 Ob Dia patients with a classical glucose tolerance test about 5 months post-bariatric surgery, when an average of $16.5 \pm 2.1\%$ of total weight loss ($42 \pm 4.8\%$ of excess weight loss) was reached. Data showed that all Ob Dia patients recovered from diabetes (Supplementary Table 2).

## Discussion

Bariatric surgery is, to date, the only therapy of obesity with long-lasting results [36]. Sleeve gastrectomy is the most used technique for bariatric surgery [37]. This implies

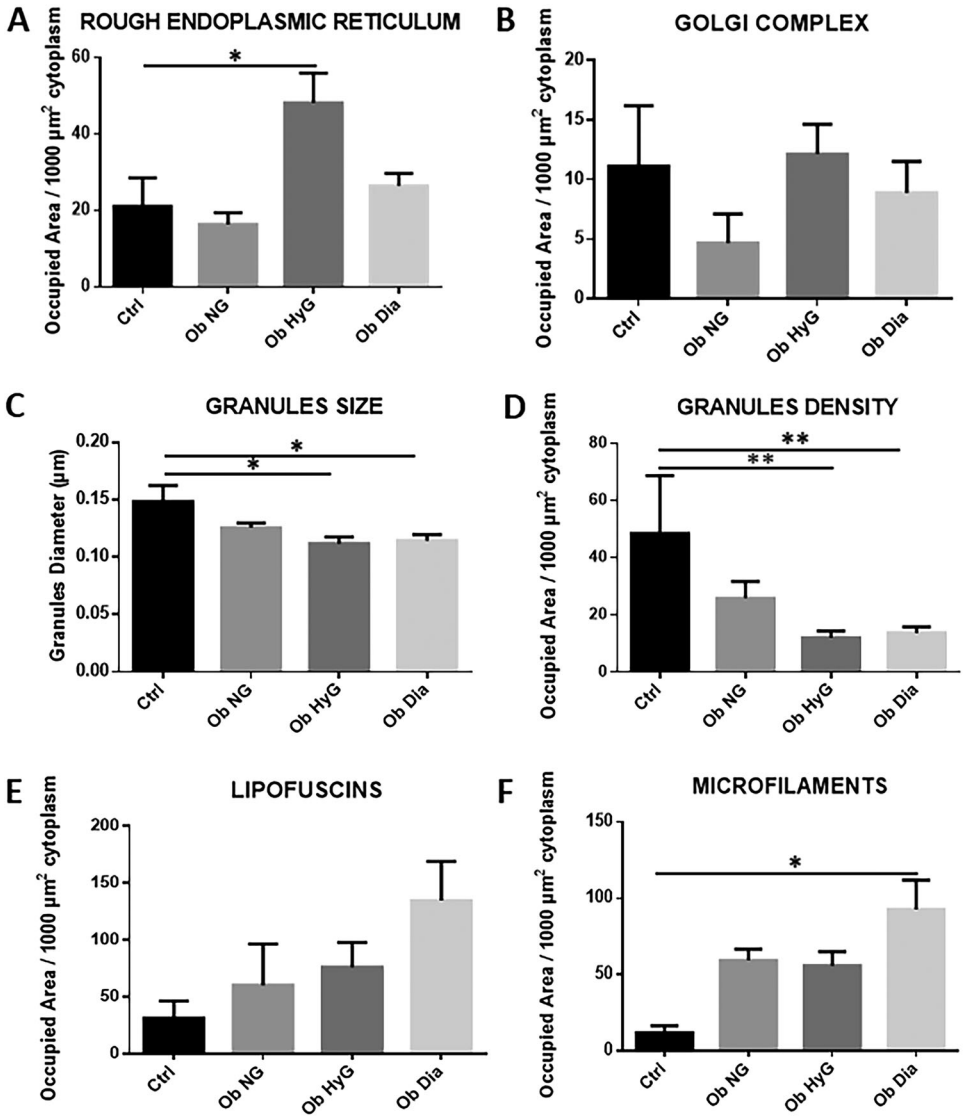


**Fig. 4 Morphometric analysis performed on electron microscope images of ghrelin cells in obese and control groups.** Cytoplasmic area occupied by (**a**) Rough endoplasmic reticulum, (**b**) Golgi Complex, (**d**) Granules, (**e**) Lipofuscins and (**f**) Microfilaments in Control, Ob NG, Ob HyG and Ob Dia patients. **c** Granules size. A significant decrease in size was found in Ob HyG and Ob Dia subjects compared with the control group. Statistical analysis between groups were performed by one-way ANOVA. Data are mean ± SEM, $*p < 0.05$ compared to Ctrl group.

removal of a large part of the stomach, including the principal anatomical site for GPCs [4]. Gastric ghrelin plays a pivotal role in glucose metabolism [6, 10] and the well-known recovery from type 2 diabetes in post-bariatric obese patients has multi-faced aspects, most of which are deserving of further studies. The published literature regarding these arguments is quite controversial [20–24].

Our data support the idea that gastric GPCs do not increase their number or density in obese patients, in line with recent data observed by other authors [23, 24]. Other studies have shown an increased number of GPCs in obese patients [20–22, 38]. Comparing the methods, these studies analysed a smaller amount of gastric tissue, which could explain the different results. The high number of patient population enroled in our study could also explain the difference, as well as the fact that a quite high variability of data among patients was found in all similar studies. Furthermore, both morphometric techniques for quantification of GPCs—absolute number [20, 21, 23] or density—used in the literature [27] gave the same result. Ghrelin impacts on glucose metabolism [6, 10], but none of the studies performed by other groups showed a detailed analysis comparing obese patients with different glycemic status. In our study, no significant quantitative modification was found, even when the subgroups of obese patients were taken into consideration (Ob NG, Ob HyG and Ob Dia). Other authors [38, 39] found a difference in the number of GPCs between genders, but our data did not confirm these results; moreover, no difference was found when comparing patients of different ages (data not shown).

The absence of any visual sign of changes in innervation is in line with the anatomical preservation of gastric ghrelin cells and data observed by other authors in humans and mice [40–42]. The reduced innervation in type 1 diabetic patients, found in another study [43], could depend on the shorter period of diabetes conditions in our type 2 diabetes obese patients.

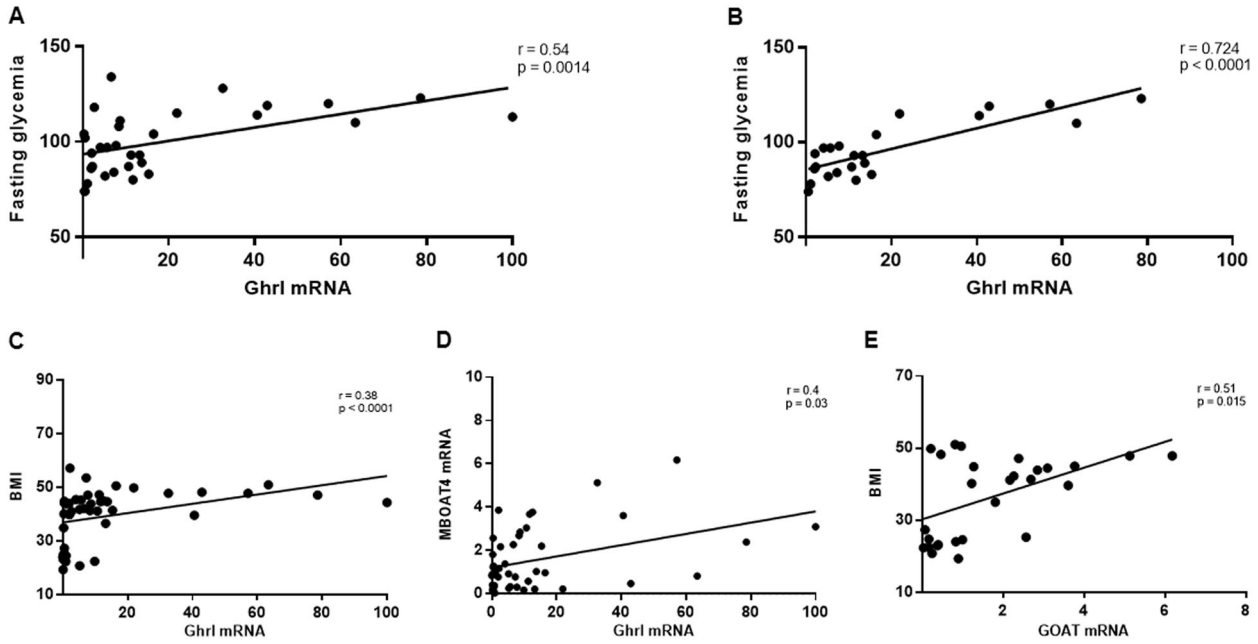


**Fig. 5 Correlation between glycaemic values and ghrelin gene expression in obese patients. a** All obese patient groups. **b** Without obese diabetic subgroup. **c** Correlations between ghrelin gene expression and BMI, (**d**) ghrelin and mBOAT4 gene expression, (**e**) GOAT gene expression and BMI. The Spearman correlation coefficient was calculated and it was evaluated statistically significant at $p < 0.05$.

Our data suggest inter-glandular smooth muscle hyperplasia, never described in the stomach of obese patients, which could be related to gastric hypermotility, a well-known effect of ghrelin [44], or to a direct trophic effect of the hormone [45, 46].

Our gene expression data clearly showed that GPCs are more active in the production of the acylated form of the hormone, especially in the Ob HyG subgroup of patients. Interestingly, electron microscopy showed that the general characteristics of ghrelin cells, allowing their easy recognition (perinuclear microfilaments, large lipofuscins and endocrine granules), were maintained in the stomach of obese patients. However, GPCs from obese patients showed a reduced electron-density of the endocrine granules associated with a reduction in granule size and cytoplasmic area occupied by them that was especially evident in the Ob HyG and Ob Dia subgroups. These aspects are likely due to a reduced amount of stored protein in the organelle, in line with their increased secretory activity [47]. Of note, the significant increase of the cytoplasmic area occupied by the rough endoplasmic reticulum in Ob HyG patients is in agreement with the gene expression data indicating very high hormonal hyperactivity in this patient subgroup. The functional significance of increased microfilaments and lipofuscins content in GPCs of obese subjects remains unknown and deserves further studies, although a cytoskeleton adaptation to specific distension of stomach wall in obese patients could be in line with a mechanical role in the functionality of these cells [48].

In agreement with these data, we found a positive correlation between ghrelin gene expression and GOAT, glycemic values and body mass index in the obese patients. Considering the well-known anti-insulin action of acylated ghrelin [4, 6] our data, taken together, suggest that overproduction of ghrelin by stomach may be involved in the pathogenesis of type 2 diabetes in obese patients [6, 49, 50]. Though published papers generally report a reduction of ghrelin blood levels during obesity [11–13], our results showing GPCs hyperactivity especially in the Ob HyG subgroup suggest that ghrelin may distinctively be involved in the pathogenesis of type 2 diabetes when patients are still hyperglycemic but not yet diabetic. Thus, specific clinical studies aimed at measuring ghrelin levels and role in this clinically silent and still underestimated period of obesity progression should be undertaken to test this hypothesis.

Our follow-up data showed a complete recovery of diabetes in all Ob Dia patients studied, with only 16.5% of weight reduction. Bariatric surgery is used as a metabolic treatment [19] and fat inflammation is a widely accepted link between obesity and metabolic dysfunction [51]. In a previous study, we found a significant reduction of subcutaneous fat inflammation after 1-year post-bariatric surgery, with an average 30% of weight loss [52]; however, a previously reported dietary treatment with a weight loss of about 10% did not show an improvement in fat histopathology [53]. Interestingly, Nannipieri et al. found an improvement of beta-cell sensitivity to glucose as early as

15 days after surgery, when the weight loss was on average only 3 kg [43, 54].

Overall, our data, together with the well-known reduced levels of plasma ghrelin in early post-bariatric period [54] indicate that the metabolic improvement obtained in our type 2 diabetic patients could be related to the sleeve gastrectomy per se, even if further confirmatory studies are needed.

## Conclusions

The main result of this study is the evidence that the total number and density of gastric GPCs do not change in obese patients even if the different subgroups of patients are considered. However, gene expression data, together with electron microscopy findings, suggest that GPC activity is increased and correlates with glycemic levels. Taken together, these data suggest that GPCs could play a role in the pathogenesis of type 2 diabetes in obese patients.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.



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