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Ghrelin gastric tissue expression in patients with morbid obesity and type 2 diabetes submitted to laparoscopic sleeve gastrectomy: immunohistochemical and biochemical study

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Abstract

Introduction. Obesity and type 2 diabetes mellitus (T2DM) are leading causes of morbidity and mortality worldwide. Ghrelin is implicated in the pathophysiology of both disease states. Laparoscopic sleeve gastrectomy is an emerging safe therapeutic technique for patients with morbid obesity. Since the removal of ghrelin-secreting cells by sleeve gastrectomy may be associated with diminished hunger sensation the aim of the study was to: (i) compare body weight and body mass index (BMI) in both obese non-diabetic and obese diabetic patient groups, (ii) determine the ghrelin expression in the resected gastric tissue in both groups, (iii) evaluate relationships between ghrelin cell expression and pre- and post-operative serum ghrelin concentration and glucose levels, and (iv) assess the influence of sleeve gastrectomy on serum glycaemic parameters in this patient population.

Material and methods. Twenty morbidly obese female patients from Saudi Arabia, of whom ten suffered from T2DM participated in the study. All subjects underwent laparoscopic sleeve gastrectomy. The removed fundus, body and antrum were biopsied and underwent immunohistochemical staining to detect ghrelin cell expression. Serum samples were assayed for ghrelin concentration and indicators of glycaemic status at the baseline and three months after sleeve gastrectomy.

Results. BMI ($p < 0.05$) and body weight ($p < 0.001$) were significantly lower in non-diabetic obese patients compared with diabetic patients before and 3 months after the surgery. Also, pre-operative serum ghrelin level was higher in non-diabetic patients compared with diabetic patients group, and postoperative plasma ghrelin level was reduced in diabetic patients ($p < 0.001$) compared with non-diabetic patients. Gastric fundic mucosa of the diabetic patients exhibited lower number of ghrelin-positive cells ($p < 0.05$) compared with non-diabetic patients. There were significant negative correlations between pre- and post-operative ghrelin serum level and blood glucose ($r = –0.736$, $p = 0.0002$ and $r = –0.656$, $p = 0.0007$, respectively) in all patient populations.

Conclusions. The results of this study suggest that the diabetic status of obese female patients may affect the incidence of ghrelin cells in three major stomach's regions and this novel observation warrants further studies.
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Key words: morbid obesity; type 2 diabetes mellitus; sleeve gastrectomy; ghrelin expression; morphometry; serum ghrelin; IHC

Introduction

Obesity is a global public health epidemic impacting approximately one eighth of the planet's population. Its presence is correlated with significant morbidity and premature death, especially amongst individuals

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in their second, third and fourth decades. To categorise the extent of the condition, body mass index (BMI) is used. A BMI of 35–50 kg/m^2, together with one or more related morbidities, is required for a diagnosis of morbid obesity [1].

There is a close association between obesity and diabetes [2]; in patients with type 2 diabetes mellitus (T2DM), BMI is usually high. T2DM is a metabolic disorder that associated with hypergylcaemia, impaired insulin production and insulin resistance (IR). The latter is a commonly recognised finding that exacerbates elevated serum glucose measurements in patients with both diabetes and obesity [3]. The combination of these morbid conditions often has a negative impact on the achievement of either a normal weight or normoglycaemia, despite long-term behavioural changes towards a healthier life style [4].

The enteroendocrine cells of the stomach produce the circulating hormone, ghrelin. Ghrelin is 28-amino acid peptide which plays an important role in glucose homeostasis, insulin sensitivity, hunger control and energy metabolism. It has orexigenic and adipogenic effects, increasing calorie consumption and body mass. There are two major circulating molecular forms of ghrelin: (i) acyl-ghrelin (AG), which physiological action depends on acylation at amino acid serine 3 by the enzyme ghrelin-O-acyl transferase (GOAT) [5], and (ii) des-acyl ghrelin (DAG). The action of AG includes ability to bind to the growth hormone secretagogue receptor, GSH-R, while DAG is implicated in apoptosis, feeding habits, energy control and glucose pathways regulation [5]. There are many studies in the literature describing the contribution of ghrelin to glucose homeostasis [6]. The hormone has been associated with insulin sensitivity, release and IR, particularly in patients with higher BMI [3]. AG and DAG have opposite metabolic effects; the former has a hyperglycaemic action, whereas the activity of DAG inhibits the glucose-releasing stimulatory effect of AG [7].

Insulin resistance appears to be enhanced by AG, whereas both molecular forms of ghrelin increase insulin sensitivity [7]. A relationship between IR and ghrelin together with an altered ghrelin profile has been reported in obese subjects. The association of these factors is complex. A negative correlation between ghrelin levels and IR was described by Gauna *et al.* [7] in individuals with raised BMI, whereas a further study evaluating the relationship between ghrelin and IR in patients with obesity and T2DM demonstrated a positive correlation [7].

Bariatric surgery is a therapeutic intervention designed to aid patients with weight loss, not only by making physical changes to the gastrointestinal tract but also by influencing various metabolic pathways to increase basal metabolic rate. Indications for such procedures have been broadened to include T2DM patients with uncontrolled blood sugars [8, 9]. Over the last twenty years, laparoscopic sleeve gastrectomy (SG) has become a routine and sought-after bariatric procedure for the treatment of morbid obesity. In contrast to the more traditional open surgeries performed, *e.g.* gastric bypass (GPB) or biliopancreatic diversion (BPD), the non-invasive laparoscopic route has ensured that SG is a technically less complex and shorter operation, consequently with a lower surgical risk [10, 11]. Together with its clinical efficacy, these attributes have facilitated the rise of SG as a procedure of choice.

In this study, female obese non-diabetic and obese patients with T2DM underwent laparoscopic SG. Research objectives were: (i) to identify body weight (BW) and body mass index (BMI) in both obese non-diabetic and obese diabetic patients post and pre SG surgery; (ii) to determine ghrelin cellular expression in different parts of the resected stomach (fundus, body and antrum); (iii) to check the correlations between ghrelin cellular expressions and pre-operative serum ghrelin concentration with serum glucose levels, and (iv) to assess the effect of SG on serum glycaemic parameters and ghrelin levels in both groups of patients three months after the surgery.

Material and methods

Patients and study design. This research was conducted in the Department of General Surgery, Najran University Hospital, Najran, Saudi Arabia, between January 2018 and January 2019. Ethical approval was obtained from the institution's Bioethics and Research Committee under the reference number RE:10-08-01-2020 EC. All subjects signed to conduct the SG surgery and to agree to use the resected part of the stomach for the shortly described scientific study.

Twenty female patients with morbid obesity who underwent laparoscopic SG were divided in two groups as the following:

Group I: ten obese non-diabetic patients (controls); the inclusion criteria were: BMI between 35 and 40 kg/m^2, age range 18–60 years, they did not receive any medications.

Group II: ten obese T2DM patients with BMI between 35 and 40 kg/m^2, age range from 18 to 60 years. Therapy received by the patients of the latter group included varying combinations of hypoglycaemic drugs such as metformin, gliclazide, and insulin (7 patients were on insulin). Exclusion criteria included pregnancy, lactation, the presence of significant concomitant acute or chronic co-morbidities, and use of any medication that might bias weight assessment such as steroids.

Assessment of obesity parameters. Body weight and height were measured. A specially assigned nurse practiced in using apparatus made the anthropometric measurements of the participants. All measurements were taken according to the recommended techniques described by Borse *et al*. [12] BMI was calculated using weight in kilograms divided by the square of the height in meters. BMI = Weight (kg)/ /Height (m^2) [1].

Laparoscopic sleeve gastrectomy. Laparoscopic SG was performed under general anaesthesia and started with division of the greater curvature blood supply. The Gagner's [13] technique was used. This was followed by resection of the fundus and greater curvature from 6 cm from the pylorus until the angle of His using an EndoGIA stapler green cartridge (4.8/60 mm) at the prepyloric area (higher thickness) and a gold cartridge (3.8/60 mm) at the body and fundus (less thickness), along with a bougie (36 Fr). Prolene sutures were used to reinforce the staple line; methylene blue was injected intraoperatively to check for any leakage. Postoperative gastrografin study was carried out on all patients. The patients started eating on the seventh day following the operation.

Blood biochemistry. Patients should be in the fasting state, and the venipuncture performed in the morning (08:00–11:00 a.m.) for blood samples' collection. Plasma was divided into aliquots and stored at –20° C until analysed. The following parameters and laboratory biochemical analysis were assessed.

Serum glucose concentration was measured by using Flex® reagent cartridge on the Dimension Vista® SIEMENS system. Glucose level was determined using a bichromatic (at absorbance 340 and 383 nm) endpoint technique, and was expressed as mmol/L [14].

Serum insulin concentration was measured using RayBio® human insulin enzyme-linked immunosorbent assay (ELISA). Glycated HbA_{1c} (HA_{1C}) levels were determined using Bio Vision® Human HbA_{1c} ELISA kit and expressed in ng/ml.

Homeostatic insulin resistance index (HOMA-IR) was calculated according to the method of Seo *et al.* [15] by the following equation: HOMA-IR = glucose concentration (mmol/L) × insulin concentration (μIU/L)/22.5. Plasma ghrelin concentration was measured with the RayBio® ghrelin Enzyme Immunoassay (EIA) Kit, based on the competitive enzyme immunoassay principle. The sensitivity limit of total ghrelin was 0.1 ng/mL and intra- and inter-assay coefficients of variability of the assay were < 10% and < 15%, respectively. A standard curve of known concentration of ghrelin peptide can be established and the concentration of ghrelin peptide in the samples can be calculated accordingly. Ghrelin level were presented in pmol/L [16].

Histological and immunohistochemical methods. After SG surgery, tissue samples from each part of the stomach were dissected (fundus, body and antrum), then immersed in 4% paraformaldehyde (PFA) for 24 h in labelled cassettes, and afterwards placed in phosphate-buffered saline (PBS) until used for standard histological tissue processing, followed by embedding in paraffin.

Glandular stomach sections of 5 μm thickness were deparaffinised, rehydrated, washed with PBS and then incubated with 10% normal goat serum in PBS for 60 min at room temperature (RT). Immunochemistry of ghrelin was performed by using standard streptavidin-peroxidase method. Shortly, sections were incubated overnight in a humid chamber at 4°C with a polyclonal antihuman ghrelin antibody (amino acids 13–28; dilution 1:500; Phoenix Pharmaceuticals, Burlingame, CA, USA). On the next day slides were incubated with biotinylated goat anti-human IgG (Thermo Fisher Scientific, Waltham, MA, USA) for 60 min at RT, and thereafter with a streptavidin–biotin––horseradish peroxidase complex (Neomarker, Fremont, CA, USA) for another 60 min. The immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) chromogen. The sections were counterstained with haematoxylin. The negative control sections were obtained by excluding the primary antibodies. The positive controls for the antibodies were healthy gastric mucosa [17].

Morphometry of ghrelin-immunoreactive cells in three gastric mucosa regions. Nine slides from the resected stomach of each patient were taken as the following: 3 slides from fundus, 3 slides from body and 3 slides from antrum. Ten microscopic fields were randomly chosen from each slide for counting the ghrelin positive cells. Morphometric results were evaluated using Leica Qwin 500 image analysis computer system (Leica Microsystems Ltd, Cambridge, UK) at the magnification of 20×.

The computer connected to the microscope and the images were controlled by Leica Qwin 500 image analysis software. The software is tracking the stained cells (immunoreactive cells); the mean value of ghrelin expression of each region of each group was calculated.

Statistical analysis. All biochemical, anthropometric measurements outcomes, and the ghrelin cell density were expressed as mean ± standard error (mean ± S.E.). The values were analysed by paired Student's *t*-tests. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Pearson's correlation coefficient was used to test the correlation among the indicators. $p < 0.05$ was considered statistically significant.

Results

Patients' characteristics

Prior to SG, BW ($p < 0.05$) and BMI ($p < 0.001$) of group I subjects was significantly lower than in the group II, diabetic cohort (Table 1). Patients in both

Table 1. Preoperative and postoperative obesity parameters, glycaemic parameters, and plasma ghrelin level in all subjects (obese non-diabetics and obese diabetics)

Groups	Before sleeve gastrectomy		Three months after sleeve gastrectomy	
	Group I: obese non-diabetic patients (n = 10) Mean ± SE	Group II: obese diabetic patients (n = 10) Mean ± SE	Group I: obese non-diabetic patients (n = 10) Mean ± SE	Group II: obese diabetic patients (n = 10) Mean ± SE
BW [kg]	106 ± 4.1$^{\&}$	120.7 ± 2.5$^{\&}$	86.4 ± 3$^{\$}$	96.6 ± 3.9$^{\$}$
BMI [kg/m^2]	36.3 ± 0.7$^{\&}$	41.9 ± 0.8$^{\&}$	29.3 ± 0.5$^{\$}$	29.2 ± 0.5$^{\$}$
Glucose [mmol/L]	4.4 ± 0.3$^{\&\&}$	16.6 ± 0.9$^{\&\&}$	4.2 ± 0.2	5.5 ± 0.2
Insulin [μIU/mL]	17.5 ± 3.7$^{\&\&}$	56.7 ± 5.6$^{\&\&}$	14.1 ± 1.7	14.5 ± 2.6
HbA_{1C}	4.8 ± 0.3$^{\&\&}$	9.3 ± 0.5$^{\&\&}$	3.6 ± 0.5	5.5 ± 0.3
HOMA-IR	4.6 ± 0.9$^{\&\&}$	44.6 ± 4.9$^{\&\&}$	3 ± 0.6$^{\%}$	33.2 ± 1.6$^{\%}$
Ghrelin [pmol/L]	14.5 ± 0.6$^{\&\&}$	10.1 ± 0.5$^{\&\&}$	9.5 ± 0.3$^{\%}$	6.1 ± 0.3$^{\%}$

Data are presented as mean ± standard error (SE). Student's *t*-test was applied to show significant differences. $^{\&}$group I *vs.* group II before surgery: ($^{\&}p < 0.05$, $^{\&\&}p < 0.001$) $^{\%}$group I *vs.* group II after surgery, $p < 0.001$; $^{\$}$group II before *vs.* after surgery $p < 0.001$; $^{\#}$group I before *vs.* after surgery $p < 0.001$.

groups exhibited lower BW and BMI after surgery. The decrease in BMI was significantly greater ($p < 0.05$) in the non-diabetic group compared with group II. Following the procedure, in group II patients BW and BMI were significantly lower ($p < 0.001$ and $p < 0.001$, respectively) than the BW and BMI before the procedure.

Glycaemic parameters

Baseline concentrations measurements of serum glucose, insulin and glycosylated haemoglobin (HbA_{1C}) were significantly lower ($p < 0.001$) in group I, compared with group II. These parameters were all reduced ($p < 0.001$) after the surgery in the diabetic patient group. In non-diabetic subjects (group I), serum glucose, insulin and homeostatic model assessment of insulin resistance (HOMA –IR) showed a non-significant fall ($p > 0.05$) following SG. However, the reduction in these parameters, together with HbA_{1C}, reached significance ($p < 0.001$) in the group with T2DM (Table 1).

Ghrelin plasma level

Serum ghrelin concentrations decreased in group I ($p < 0.001$) and group II ($p < 0.001$) following laparoscopic SG, although baseline levels were higher in non-diabetic patients compared with the diabetic subjects. Post-procedure, ghrelin concentration was lower ($p < 0.001$) in the diabetic cohort than in the non-diabetic group (Table 1).

Morphology and morphometry of ghrelin-immunoreactive cells in gastric mucosa

Gastric mucosa demonstrated uniform histology throughout the organ, with the gastric pits evident in the superficial layer with transected coiled glands visible in the deep layer. The fundus was recognised by its typical glandular region pattern.

Immunohistochemical staining with anti-ghrelin antibody was performed in samples of the indicated stomach's regions from both patient groups.

Fundus of the stomach

We observed the presence of multiple cells that reacted positively to the anti-ghrelin antibody (Fig. 1: Fundus A). Fewer positively staining cells were noted in the biopsy samples from group II subjects (Fig. 1: Fundus D) what was confirmed by morphometric analysis (Fig. 2).

Body of the stomach

Ghrelin immunoreactive (ir) cells were distributed throughout the gastric mucosa in the stomach body in the control group (Fig. 1: Body B). A marked reduction in the number of ghrelin-ir cells was observed in diabetic patients (Fig. 1: Body E and Fig. 2). Moreover, some areas of the mucosae did not contain ghrelin-ir cells. These observations were confirmed by morphometric analysis (Fig. 2).

Gastric antrum

High densities of ghrelin-ir cells were evident in the antral mucosa of patients from group I (Fig. 1: Antrum C). However, in the group II (T2DM patients) significantly lower populations of these cells were identified; a distinct absence of ghrelin-ir cells was noted in certain regions (Fig. 1: Antrum F, Fig. 2). These observations were confirmed by morphometric analysis (Fig. 2).

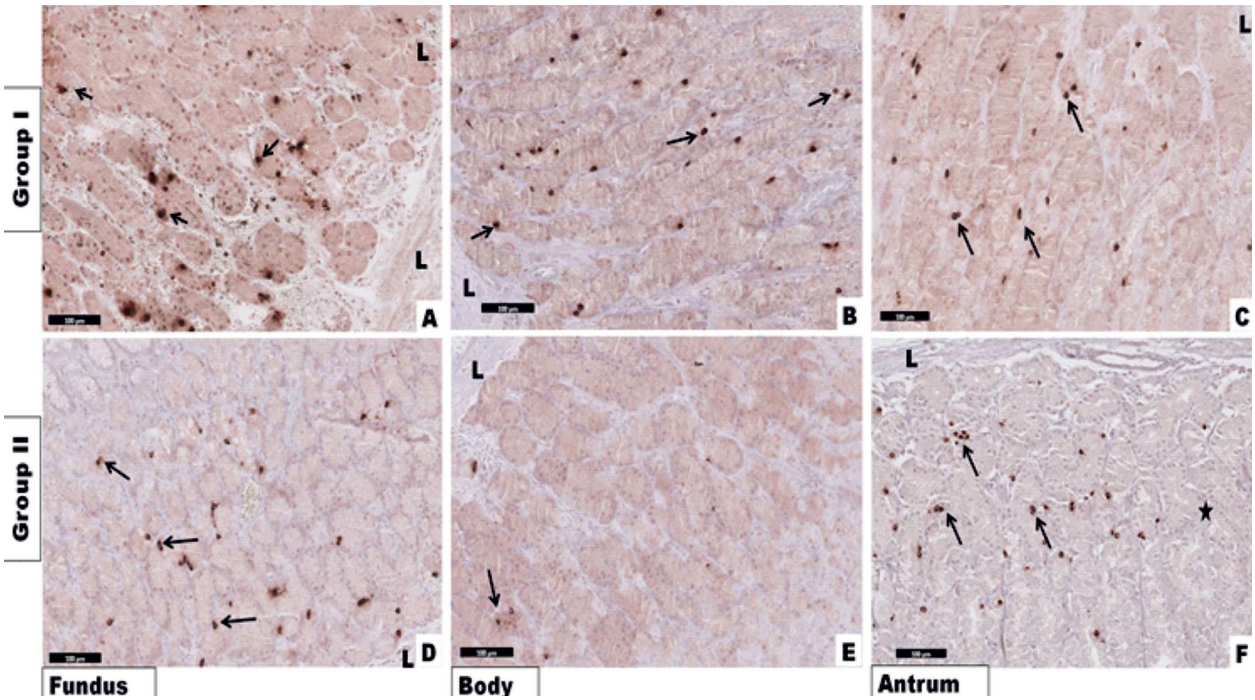

Figure 1. Microphotographs of immunopositive ghrelin cells (arrows) in stomach's fundus, body, and antrum in non-diabetic obese patients (group I) and patients with the type 2 diabetes (group II). L — lamina propria. Scale bar 100 μm.

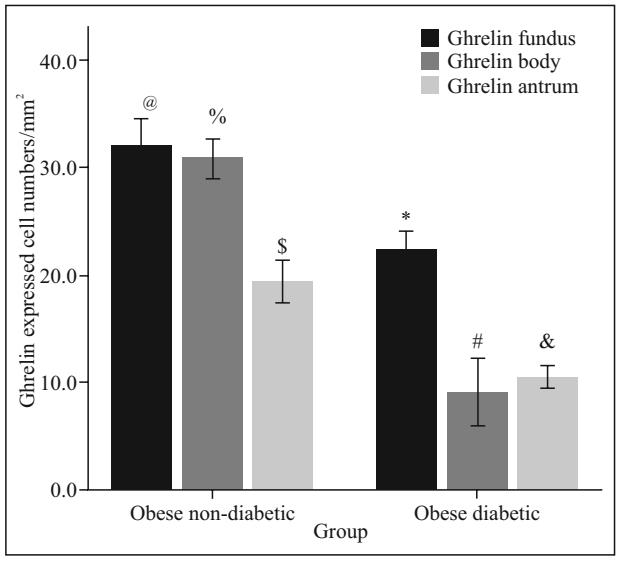

Figure 2. Densities of ghrelin-immunoreactive cells (cells/ /mm^2) in the gastric mucosa in obese non-diabetes patients (group I) and obese patients with type 2 diabetes (group II) in three stomach's regions (fundus, body, and antrum). *p < 0.05 group II *vs.* group I, fundus mucosa; #p < 0.05 group 2 *vs.* group I, body mucosa; &p < 0.05 group 2 *vs.* group I, antrum mucosa. $p < 0.05 antrum *vs* body in group I; @p < 0.05 antrum mucosa *vs* fundus mucosa group I. %p < 0.5 body mucosa *vs.* fundus mucosa group I.

In summary, the morphometric analysis provided a convincing evidence of the lower number of ghrelin-immunoreactive cells in the mucosa of the studied stomach's regions in patients with type 2 diabetes as compared with non-diabetic obese patients ($p < 0.001$) (Fig. 2).

Correlations of the obesity and glycaemic parameters before and after sleeve gastrectomy

Results are presented for both groups combined in this section before the SG surgery. Pearson's correlation coefficient was positive for the correlations of baseline BW and BMI with serum glucose, insulin and HbA_{1C} levels. Glycaemic parameters also correlated positively with HOMA-IR index; however, no correlation was observed between HOMA-IR and BW (Table 2).

The correlations between obesity and glycaemic parameters after SG surgery are presented in (Table 3). Significant positive correlations were identified between the HOMA-IR index and serum glucose and HbA_{1C} levels, and also between serum glucose, HbA_{1C} and insulin concentrations. BMI demonstrated a negative correlation with glucose and HOMA-IR (Table 3).

Table 2. Pearson's correlation between preoperative obesity parameters, and glycaemic parameters in all subjects (obese non-diabetics and obese diabetics, n = 20), Pearson correlation coefficient = r

Preoperative correlations						
	BMI	**BW**	**Glucose**	**Insulin**	**HA_{1C}**	**HOMA-IR**
BMI						
r	1	0.42	0.77**	0.59**	0.67**	0.65**
Sig. (2-tailed)		0.064	0.000	0.007	0.001	0.002
BW						
r	0.42	1	0.48*	0.49*	0.50*	0.41
Sig. (2-tailed)	0.064		0.031	0.027	0.024	0.073
Glucose						
r	0.77**	0.48*	1	0.76**	0.85**	0.89**
Sig. (2-tailed)	0.000	0.031		0.000	0.000	0.000
Insulin						
r	0.59**	0.49*	0.77**	1	0.59**	0.86**
Sig. (2-tailed)	0.007	0.027	0.000		0.005	0.000
HA_{1C}						
r	0.67**	0.50*	0.85**	0.59**	1	0.70**
Sig. (2-tailed)	0.001	0.024	0.000	0.005		0.001
HOMA-IR						
r	0.65**	0.41	0.89**	0.86**	0.70**	1
Sig. (2-tailed)	0.002	0.073	0.000	0.000	0.001	

*p < 0.05, **p < 0.01

Correlations between pre and post-operative serum ghrelin levels with obesity and glycaemic parameters

In Figure 3 we present Pearson's correlations between preoperative baseline serum ghrelin concentrations and BW, BMI, serum glucose, insulin, HA_{1C} and HOMA-IR index. Figure 3A shows that the negative correlation of ghrelin serum level with BW was insignificant (r = –0.435, p = 0.055). However, preoperative ghrelin serum level showed significant negative correlations with BMI (r = –0.629, p = 0.003), blood glucose level (r = –0.736, p = 0.0002), serum insulin levels (r = –0.790, p < 0.0001), HA_{1C} (r = –0.705, p = 0.0005) and HOMA-IR (r = –0.794, p < 0.0001) (Fig. 3; B, C, D, E and F).

After laparoscopic SG, the correlations between ghrelin serum level and BW, BMI, serum glucose, insulin, HA_{1C} and HOMA-IR index were determined and are presented in Figure 4. There were insignificant negative correlations with BW (r = –0.202, p = 0.393), BMI (r = –0.033, p = 0.892), serum insulin levels (r = –0.304, p = 0.192) (Fig. 4A, B, D). However, statistically significant negative correlations with blood glucose level (r = –0.656, p = 0.0007), HA_{1C} (r = –0.593, p = 0.005) and HOMA-IR (r = –0.891, p < 0.0001) were established (Fig. 4C, E, F).

We also determined Pearson's correlations between the cell density of ghrelin-immunoreactive cells in gastric tissues and serum ghrelin level. We found significant positive correlations with ghrelin cell density in the fundus (r = 0.644, p = 0.002), body (r = 0.691, p = 0.0007) and antrum (r = 0.748, p = 0.0002) (Fig. 5).

Discussion

The safety profile and efficacy of laparoscopic SG means that it is rapidly becoming a preferred procedure for the management of patients with obesity and its associated morbidities [18]. It is well-documented that the ghrelin plays a role in glucose homeostasis and can be used to evaluate insulin resistance in patients with significantly raised BMI [19, 20]. SG is associated with a post-operative decrease in ghrelin concentration; this contributes to weight loss and is associated with prompt improvement in glycaemic parameters [21, 22].

In this prospective patient series, the distribution of gastric mucosal ghrelin cells in varying parts of the stomach was examined by an immunohistochemical method in obese female patients with and without

Table 3. Pearson's correlation between postoperative obesity parameters, and glycaemic parameters in all subjects (obese non-diabetics and obese diabetics n = 20), Pearson correlation coefficient = r

Postoperative correlations						
	BMI	**BW**	**Glucose**	**Insulin**	**HA_{1C}**	**HOMA-IR**
BMI						
r	1	–0.34	–0.23	0.16	0.23	–0.02
Sig. (2-tailed)		0.142	0.321	0.501	0.323	0.934
BW						
r	–0.34	1	0.36	–0.24	0.11	0.36
Sig. (2-tailed)	0.142		0.120	0.312	0.641	0.114
Glucose						
r	–0.23	0.36	1	–0.14	0.40	0.76**
Sig. (2-tailed)	0.321	0.120		0.553	0.078	0.000
Insulin						
r	0.16	–0.24	–0.14	1	0.45*	0.02
Sig. (2-tailed)	0.501	0.312	0.553		0.048	0.938
HA_{1C}						
r	0.23	0.11	0.40	0.45*	1	0.58**
Sig. (2-tailed)	0.323	0.641	0.078	0.048		0.007
HOMA-IR						
r	–0.02	0.36	0.76**	0.02	0.58**	1
Sig. (2-tailed)	0.934	0.114	0.000	0.938	0.007	

*$p < 0.05$, **$p < 0.01$

diabetes. Pearson's correlation coefficient was used to compare data. In addition, baseline serum ghrelin concentrations were evaluated against markers of obesity and glucose metabolism. The influence of SG on ghrelin levels and measurements of glucose homeostasis was also investigated in both patient groups.

Female subjects were selected for this research, since obesity is generally more common in females than in men, regardless of age [23]. It was noted that both BW and BMI, indicators of obesity, were reduced from baseline following SG in all patients three months after the surgery.

Decreased BW and BMI after SG were reported by many authors. *E.g.* Mahmoud *et al.* [1] described loss of mean body mass of approximately 30 kg following SG whereas BMI was also diminished after intervention, falling to 40.8 ± 7.2 from a pre-operative value of 52.6 ± 12.8 kg/m^2. They also described improvements in obesity-related co-morbidities including diabetes and hypertension in patients after SG surgery [1]. Another study reported similar results, again noting weight loss, mitigation of obesity-associated complications and improved general health [18]. A study that was performed on 7 males and 11 female with T2DM who underwent SG, revealed that there was an improvement of T2DM following 12 months of the surgery [24]. SG was an effective treatment for Japanese obese patients with T2DM, as it conserved the secretion of insulin a year after the surgery [25].

We observed improved control of glucose metabolism following SG in the diabetic patients. Similar results have been demonstrated by Nosso *et al.* [11] (age 45 ± 9 years, BMI 48 ± 8 kg/m^2, M ± SD) who found that obese patients with T2DM not only could sustain the significant weight loss and improvements in glucose metabolism parameters seen after SG, but also oral hypoglycaemic medications could be reduced over a period of 9–15 months of the follow-up.

It is not fully understood why parameters of glucose homeostasis improve after laparoscopic SG in patients with T2DM. One theory postulated is that there is an improvement in insulin sensitivity [26]. Approximately 70% of patients with T2DM who underwent bariatric SG showed resolution of T2DM over a mean clinical observation period of 3 years. HOMA-IR returned to practically normal values when assayed *via* euglycaemic clamping [27]. This finding aligned with the current study, HOMA-IR

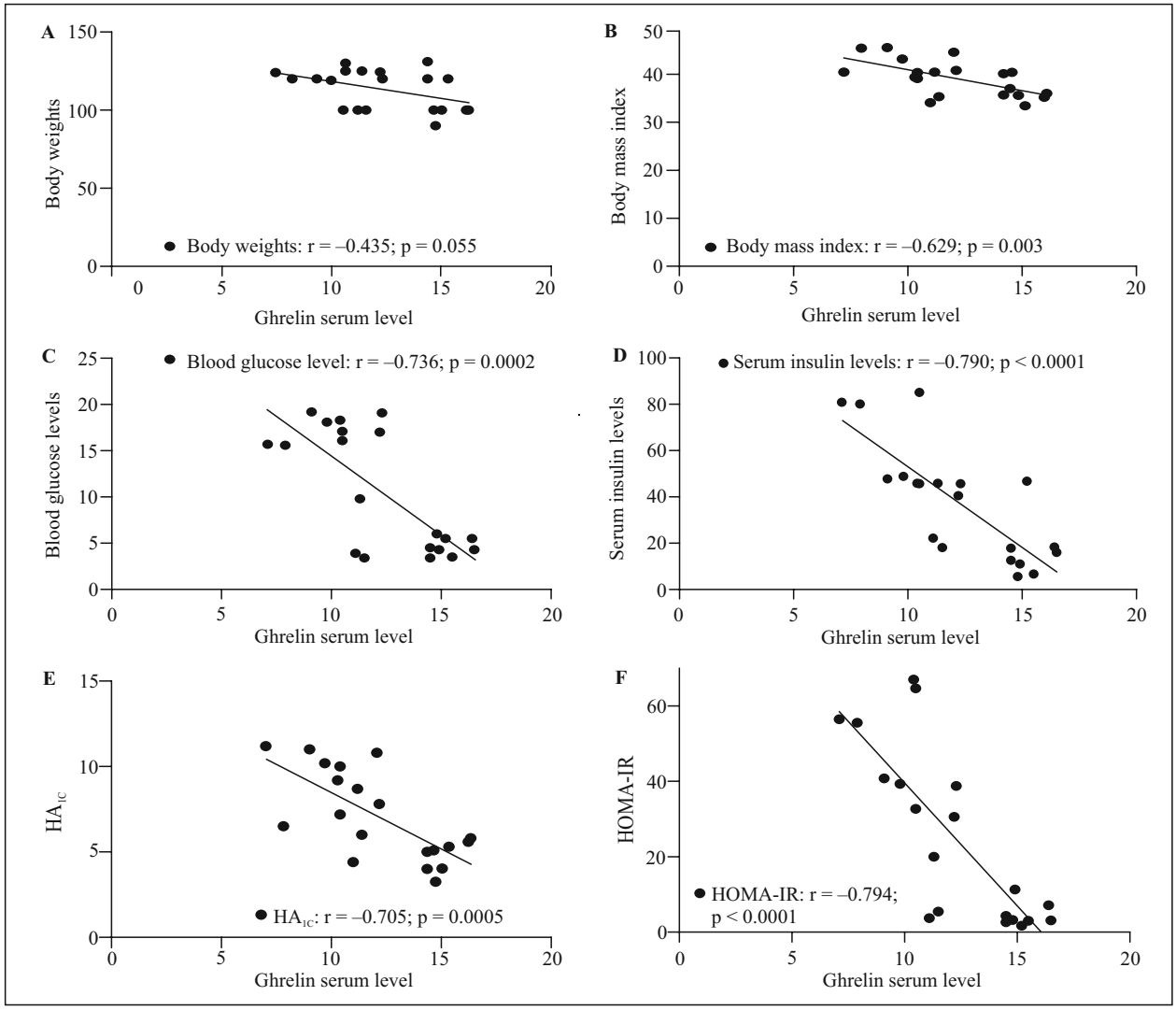

Figure 3. Pearson's correlations between preoperative ghrelin blood concentration and obesity parameters, and glycaemic parameters in all subjects (obese non-diabetics and obese diabetics).

levels were diminished post-operatively in the series of obese diabetic patients. Nosso *et al.* [11] age (45 ± 9 years, BMI 48 ± 8 kg/m^2, M ± SD) suggested that the improved sensitivity to insulin may be due to a combination of factors, including reduced BMI and lower food (calorie) intake, although some additional factors unrelated to weight loss may contribute to this effect.

The identification of an inverse relationship between serum ghrelin and insulin concentrations was the first evidence to suggest that ghrelin had a role in glucose metabolism [26, 27]. Consistent with findings in the literature, all patients undergoing fundectomy by SG demonstrated reduced serum ghrelin levels after the intervention [28, 29]. However, there are studies in which the reduction in ghrelin concentration was found [30]; in fact in some cases an augmentation of serum peptide levels was demonstrated after SG [31, 32]. Resolution of T2DM was noted within a year of the RYGB (Roux-en-Y-gastric-bypass) procedure in one study [21]. These patients exhibited marked weight loss associated with enhanced insulin sensitivity and changes in both glucagon and ghrelin levels, which the authors attributed to the anatomical consequences of the procedure, changes in neural pathways and metabolic consequences of the reduction in BMI.

Ghrelin was discovered in 1999 as the endogenous ligand for growth hormone secretagogue-receptor (GHS-R) which is predominantly present in the previously uncharacterized X/A-like cells of the stomach [33]. Ghrelin-expressing cells can be classified, similarly to other enteroendocrine cells, into closed- and open-type cells. The open-type cells are in contact

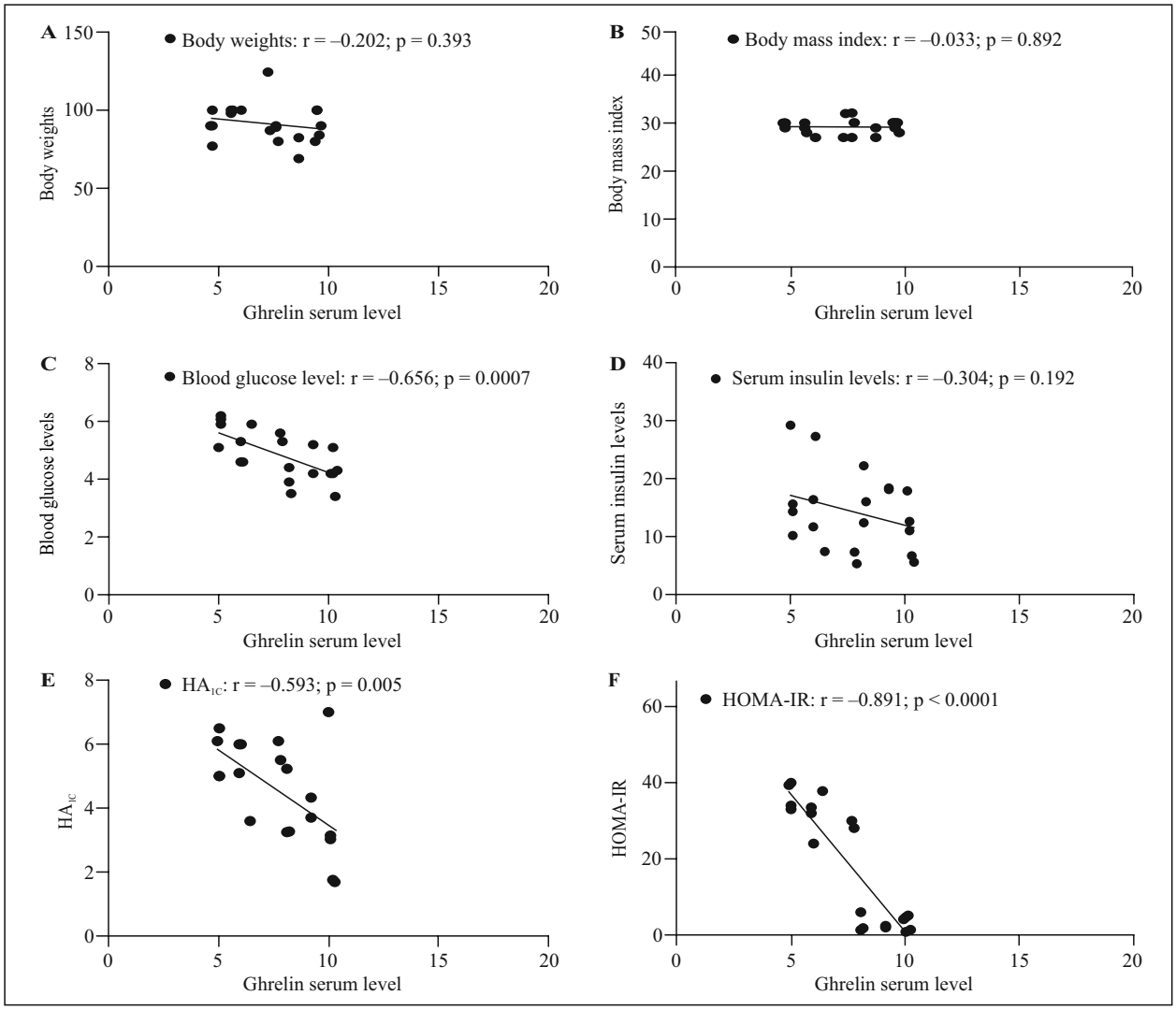

Figure 4. Pearson's correlations between postoperative ghrelin blood level with obesity parameters, and glycaemic parameters in all subjects (obese non-diabetics and obese diabetics).

with the lumen of the gastrointestinal tract and most widely spread in the stomach fundus. Ultrastructure of ghrelin cells resent in gastric mucosa reveals cytoplasmic process at the apical cell membrane and circular secretory granules in the cytoplasmic compartment [34]. Nesfatin-1 can be co-localized with ghrelin in gastric mucosa cells [33]. The function of ghrelin cells is varying from controlling food intake and BW according to the amount of ghrelin released. In the condition of obesity there was an increase in nesfatin-1 leading to increase BMI [33]. The measurements of ghrelin mRNA in various stomach's regions of morbidly obese patients revealed that the fundus has its highest level compared with body and pre-antral region [33], what is in line with the prevalence of ghrelin-immunoreactive cells in fundic mucosa found in the present study.

The results of our study are in accordance with the report which revealed that in the stomach of morbidly obese patients ghrelin cells count was higher in fundus comparing with body and pre-antral region (60 ± 40, 45 ± 20 and 39 ± 13 ghrelin-ir cells/high power field, mean ± SE, respectively) [34]. The presence of excessive body mass seems to have no effect on the cell density in gastric mucosal regions because Musella *et al.* [35] found no differences in the distribution patterns of ghrelin immunoreactive cells in fundus, body, and pre-antral regions of the stomach of 26 obese and 26 non-obese patients [35]. However, a study on 33 morbidly obese patients (24

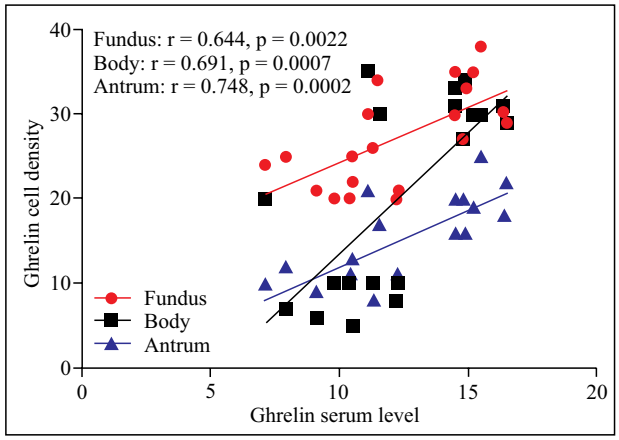

Figure 5. Pearson's correlations between preoperative ghrelin blood level with the density of immunoreactive ghrelin cells in different regions of the stomach in all subjects (obese non-diabetic and obese diabetic patients).

female, 9 male), and 8 non-obese controls showed that gastric mucosa of females presented higher number of ghrelin-immunopositive cells compared with males ($p = 0.007$) [36].

In this study we used anti-ghrelin antibody to quantify the expression of these cells in females in non-diabetic obese patients and obese diabetic patients. Interestingly, in group II, *i.e.* the obese patients with T2DM, a lower population of cells positive for anti-ghrelin antibody staining was seen in gastric mucosa specimens from all three gastric territories when compared with non-diabetic patients. The statistical significance of these findings was confirmed by morphometric analysis, and this novel finding warrants further investigations. Of note, in patients with increased BMI, Di Cristofano [22] found variations in the distribution of ghrelin positive cells in stomach fundus mucosa (37%) compared with the body (32%) and antrum (31%). Similarly as in our study, the cell density of ghrelin-immunoreactive cells was greatly reduced in diabetic patients [22].

Positive correlations of the histological findings of ghrelin cells and baseline serum ghrelin levels were identified [37]. It was suggested that the ghrelin cell proliferation occurred in response to the lower serum ghrelin levels seen in patients with diabetes. Interestingly, in a study by Miyazaki *et al.* [38] there was a slight correlation between gastric mucosal ghrelin-positive cells and serum ghrelin concentrations in morbidly obese patients ($R2 = 0.19$, $p = 0.04$).

However, we found that there was a positive correlation between the number of ghrelin cells and ghrelin serum level. A positive correlation between the number of ghrelin positive cells in the gastric mucosa and serum ghrelin levels has the potential for clinical benefit. It would suggest that, in principle, the greater the number of ghrelin positive cells removed by SG, the more successful the procedure for the improvement of patients' energy homeostasis.

Negative correlations were identified between serum ghrelin concentrations and the parameters of glucose homeostasis pre- and post-SG in all subjects. Insulin resistance has been previously shown to have a negative relationship with ghrelin titres in obese children and teenagers [39], and adults [40, 41]. In our study serum ghrelin levels showed a positive correlation with baseline BMI measurement in all the patients, but no relationship was seen with obesity parameters after SG. This may be due to the fact that only 3 month follow-up data was presented [42], a longer period of observation may be required to demonstrate change. However, a study by Mahmoud *et al.* also failed to demonstrate any correlation between BMI or BW and serum ghrelin concentration six months postoperatively [1]. It is possible that differences in study methodology, patient follow-up periods, assay techniques and diurnal variations may contribute to these inconsistent findings [29, 39].

In summary, the results of this prospective clinical patient study have demonstrated that obese patients with T2DM are more likely to demonstrate lower serum ghrelin levels after SG. Additionally, serum ghrelin concentration is positively correlated with the number of ghrelin-IR cells in gastric mucosa; the latter were noted to be more numerous populations in stomach fundus than in the body or antrum. Restoration of parameters of glucose homeostasis towards normal values confirmed the value of laparoscopic SG as a therapy for the improvement of T2DM in subjects with morbid obesity.

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Conflicts of interest

Authors declared they have no conflicts of interest.

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