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Correlation between leptin and ghrelin expression in adipose visceral tissue and clinical-biological features in malignant obesity

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Abstract

Morbid obesity is a metabolic disease characterized by an excessive accumulation of adipose tissue (≥40%). This disorder is commonly associated with cardiovascular disease, arteriosclerosis, type 2 diabetes, hypothyroidism and some types of cancer. The most common metabolic signals associated with the disease are leptin, ghrelin, with antagonic effects. Our study aimed at highlighting leptin and ghrelin expression levels, as well as establishing correlations between them and clinical-biological parameters in obese patients. The biological material was taken intraoperatively from the visceral adipose tissue. Expression of genes of interest was performed after total RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR) and amplification with TaqMan specific primers. The results of the study showed significant differences in the expression of leptin mRNA between obese patients and the control group as well as the gender of the subjects. Ghrelin levels correlated positively with obesity, but not with gender. There were no significant correlations between the expression of the genes of interest and the parameters studied (age, body mass index – BMI, cholesterol, triglycerides, glycemia, diabetes, hypothyroidism and hypertension). The results of the study suggest that the evaluation of leptin levels can be used clinically in assessing the metabolic status of the patient with malignant obesity.

***Keywords*:** malignant obesity, leptin, ghrelin, RT-PCR.

Introduction

Excess weight represents a main medical problem of the last 50 years. *World Health Organization* (WHO) appreciates that globally there are more than one billion adults being overweight out of which 300 million are obese [1, 2] and that child morbidity is 10% [3]. Furthermore, obesity has a tendency to reach epidemic proportions globally [4]. The *Romanian Association for the Study of Obesity* reports a prevalence of obesity of 21.3% for the year 2016, considered to be the lowest at European level. WHO defines IIIrd class obesity by a body mass index (BMI) ≥40 kg/m^2. Surgical literature divides this category in morbid obesity (BMI ≥40–44.9 kg/m^2) and super obesity or malignant obesity (BMI ≥45 kg/m^2). The disease falls into metabolic syndrome, along with hyperinsulinemia, insulin resistance, dyslipidemia, glucose intolerance and it is often associated with cardiovascular diseases, type 2 diabetes, hypothyroidism, osteoarthritis, asthma, gastro-esophageal reflux and some types of cancer. Recent studies assess that obesity represents the sixth risk factor in terms of lowering life expectancy [3] and that it should be regarded as a chronically pathological state, resulting from the interaction of a cumulus of factors: cultural, physiological and genetic [5].

The adjustment of body fat reserves is controlled by a complex group of genes, orexigenic and antiorexigenic with ghrelin and leptin being part of this group. Ghrelin secretion is realized mainly by P/D1 cells located in the stomach and by epsilon cells from the pancreas. It stimulates appetite through an increase in gastro-intestinal motility and decreased insulin secretion [6, 7]. High levels of circulating ghrelin were observed in diet-induced negative energy balance [8]. Leptin is synthesized mainly by adipocytes and acts on specific receptors in the hypothalamus in two ways: it inhibits appetite by counteracting the effects of stimulators (Y neuropeptide and anandamides) and stimulates the synthesis of the inhibitor, alpha-melanocyte-stimulating hormone (α-MSH) [6, 8–10]. Leptin inhibits appetite long-term compared to the inhibition induced by cholecystokinin and with the slow suppression of PYY3-36 (peptide tyrosine–tyrosine). The absence of leptin leads to uncontrolled food intake and ultimately to obesity.

The aim of our study was to detect expression levels of ghrelin and leptin in adipocytes from visceral fat tissue and to establish correlations between gene expression and clinical/paraclinical parameters of patients with malignant obesity.

Patients, Materials and Methods

Biological samples

Visceral adipose tissue biopsies were obtained from patients hospitalized for bariatric surgery (n=40). Malignant

and morbid obese patients (BMI ≥40 kg/m^2) were selected. The control group (n=10) was represented by adipose visceral tissue samples from non-obese patients, obtained during abdominal surgical interventions (umbilical hernia, colic diverticulitis, cholecystitis, appendicitis). The control group included only patients who did not show any other pathology than the one which was the subject of the surgical act. The biological parameters were within normal limits. BMI within the control group was between 20 and 28 kg/m^2. Clinical and biological parameters were acquired from the medical documents of patients. All patients were admitted in 2nd Department of Surgery, at the Emergency County Hospital, Timişoara, Romania, between 2014 and 2016, and all subjects provided written informed consent before the study. The Ethical Committee of "Victor Babeş" University of Medicine and Pharmacy, Timişoara, approved the research.

Real-time reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was purified from 100 mg adipose tissue using the PureLink® RNA Mini Kit (Invitrogen, Carlsbad, USA). Total RNA quality and quantity was assessed using a NanoDrop spectrophotometer. Thirty ng RNA was reverse-transcribed and amplified using Applied Biosystems® TaqMan® RNA-to-Ct™ 1-Step Kit (4392938) and TaqMan Assay specific primers. Relative quantification of the leptin and ghrelin was realized using the 7900 HT Fast Real-Time PCR System. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene to normalized gene expression values. The relative amounts of the gene were expressed as ΔCT.

Statistical analysis

Data are presented as means±SD (standard deviation) or as medians and interquartile range (IQR) for variables with skewed distribution. Differences between groups were analyzed with Student's t-test or Mann–Whitney U-test as appropriate. Univariable regression analyses were carried out to evaluate the significance of the relation between continuous variables. P-values for all hypothesis tests were two-sided, and statistical significance was set at p<0.05. All analyses were conducted with Stata 9.2 (Statacorp, Texas, USA).

Histological and immunohistochemical study

There were performed samplings of adipose tissue and gastric wall in pieces of surgical exeresis in order to determine the obesity histological type, as well as other changes of the gastric wall in the patients with morbid obesity. The harvested biological material was fixed in 10% neutral formalin and included in paraffin, according to the histological protocol. There were performed microtome sections with a 4-µm thickness that were stained with Hematoxylin–Eosin (HE) and green light trichrome, the Goldner–Szekely (GS) technique. For the immunohistochemical study, there were used the following antibodies: anti-chromogranin (clone DAK-A3, 1/100 dilution, Dako), anti-S100 protein (polyclonal, 1/100 dilution, Dako) and anti-synaptophysin (clone SY38, 1/150 dilution, Dako), for highlighting some neuroendocrine cells in the gastric mucosa.

Results

The study considered 40 morbidly obese patients, 75% of which were women and 25% men. Surgical procedure performed was LSG (laparoscopic sleeve gastrectomy) (Figures 1–3).

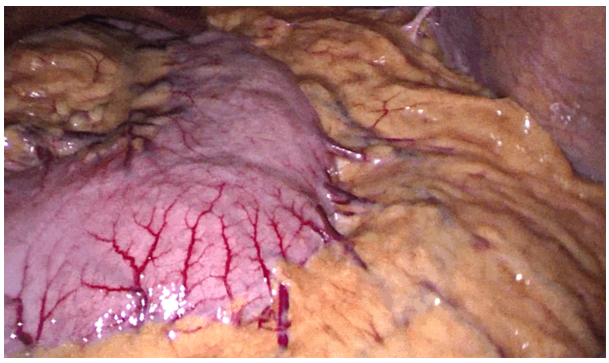

Figure 1 – ***LSG: initial aspect of the stomach.***

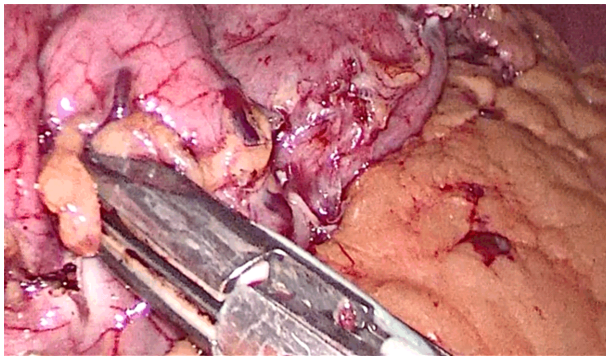

Figure 2 – ***LSG: harvesting the specimen.***

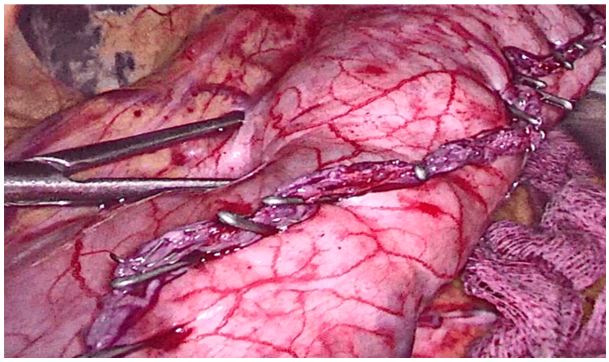

Figure 3 – ***LSG: final aspect of the resection.***

The clinical evaluation of the study group revealed multiple comorbidities associated with obesity (Figure 4). Out of these comorbidities, our study wanted to evaluate the correlation between leptin and ghrelin with the most commonly associated pathology. We assessed the correlation between the expression of the two genes of interest from adipose tissue with clinical and biological parameters that could influence the hormonal and metabolic status. The clinical and biological parameters of studied patients were: age average 39.87±11.53 years, BMI 48.56±10.35 kg/m^2, glycemia 92 mg/dL (88–105 mg/dL), cholesterol 196 mg/dL (162–204.5 mg/dL) and triglycerides 126 mg/dL (95.5–146 mg/dL). Twenty-two (55%) of patients presented high blood pressure, nine (22.5%) had type 2 diabetes and eight (20%) were diagnosed with hypothyroidism. The values presented are expressed as mean±SD and median IQR (Table 1).

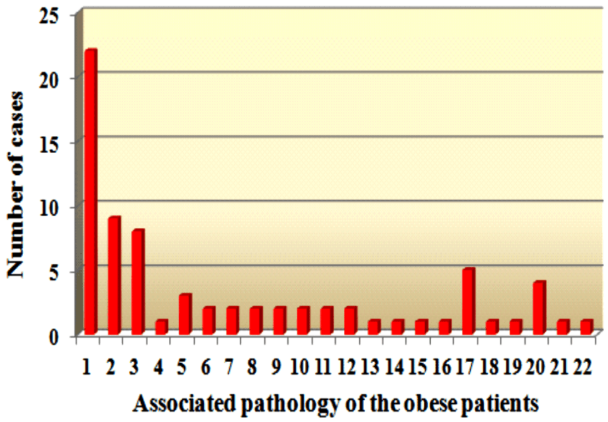

Figure 4 – ***Associated pathology of the obese patients: 1: Hypertension; 2: Type 2 diabetes; 3: Hypothyroidism; 4: Sinusal tachycardia; 5: Ischemic cardiopathy; 6: Cardiomegaly; 7: Heart failure; 8: Ventricular atrial fibrillation; 9: Tricuspid regurgitation; 10: Left ventricular hypertrophy; 11: Peripheral chronic venous insufficiency; 12: Sleep apnea; 13: Chronic obstructive pulmonary disease; 14: Mild ventilator dysfunction; 15: Pulmonary hypertension; 16: Chronic bronchitis; 17: Gastroesophageal reflux; 18: Hiatal hernia; 19: Biliary lithiasis; 20: Hepatic steatosis; 21: Mixed dyslipidemia; 22: Chronic renal failure.***

Table 1 – ***Clinical and biological data of the studied patients (n=40)***

Parameter	Values
Age [years], mean±SD	39.87±11.53
Females, No. of cases [%]	30 (75%)
Hypertension, No. of cases [%]	22 (55%)
Type 2 diabetes, No. of cases [%]	9 (22.5%)
Hypothyroidism, No. of cases [%]	8 (20%)
Leptin (arbitrary units), median (IQR)	2.62 (1.62–3.7)
Ghrelin (arbitrary units), mean±SD	9.74±1.27
BMI (kg/m^2), mean±SD	48.56±10.35
Glycemia (fasting) [mg/dL], median (IQR)	92 (88–105)
Cholesterol [mg/dL], median (IQR)	194 (162–204)
Triglycerides [mg/dL], median (IQR)	126 (95.5–146)

SD: Standard deviation; BMI: Body mass index; IQR: Interquartile range.

In order to assess the role of leptin and ghrelin in the adipocytes of morbidly obese patients we performed the relative quantification of mRNA expression of leptin and ghrelin in visceral adipose tissue (Figure 5).

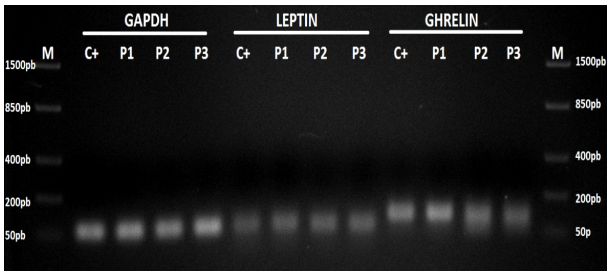

Figure 5 – ***mRNA leptin and ghrelin amplicon control in agarose gel electrophoresis. Housekeeping GAPDH gene; C+: Control; M: Molecular mass marker; P1–P3: Patients.***

After normalization with GAPDH, gene expression was detected in visceral fat. There was a statistically significant difference between cases and controls regarding leptin (p=0.0015) and ghrelin (p=0.0042) values (Figures 6 and 7). We compared then expression levels between females and males. Among cases, there was a statistically significant difference (p=0.0423) between leptin values in male and female subjects: 1.62 (1.41–2.7) *versus* 3.04 (2.21–4.44) (Figure 8). However, when ghrelin levels were assessed, no significant differences could be found between male and female patients (p=0.3149) (Figure 9).

Univariable regression analysis for leptin showed no correlation with age (p=0.2655), glycemia (p=0.9918), cholesterol (p=0.1479), triglycerides (p=0.6288) and BMI (p=0.8592). Also, there was no significant correlation between ghrelin and clinical and biological parameters: age (p=0.9228), glycemia (p=0.7441), cholesterol (p=0.112), triglycerides (p=0.4948) and BMI (p=0.3917) (Table 2).

Our study tried to establish correlations between expression levels of leptin and ghrelin with the most frequently associated pathologies of studied cases: hypertension, type 2 diabetes and hypothyroidism. Comparisons of both genes with the pathology of interest showed no significant differences between leptin (Table 3) and ghrelin (Table 4) expression levels.

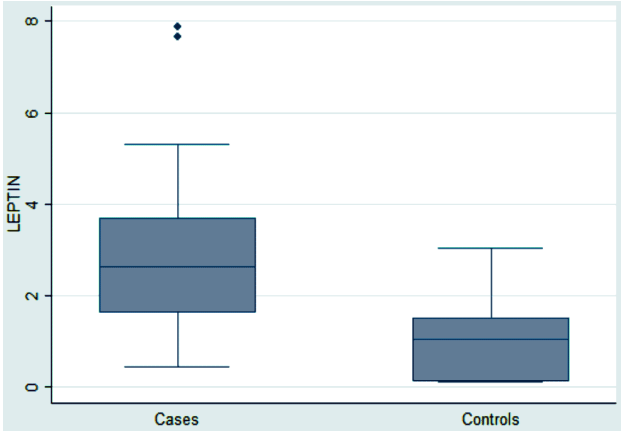

Figure 6 – ***Comparison between leptin mRNA in visceral adipocytes for the study and control groups.***

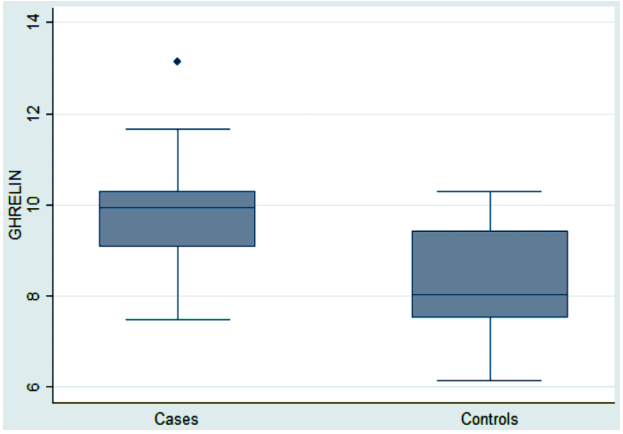

Figure 7 – ***Comparison between ghrelin mRNA in visceral adipocytes for the study and control groups.***

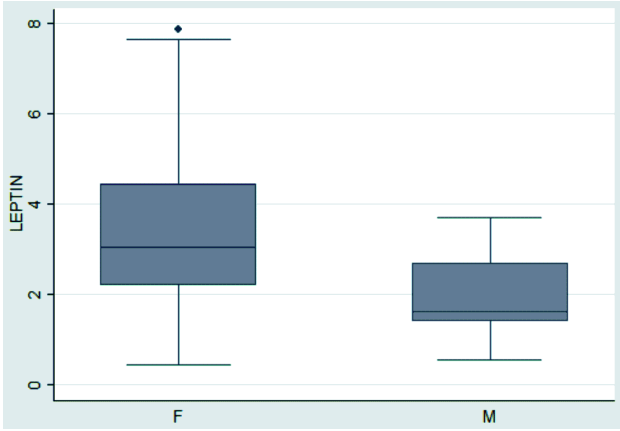

Figure 8 – ***Leptin expression based on gender. F: Female; M: Male.***

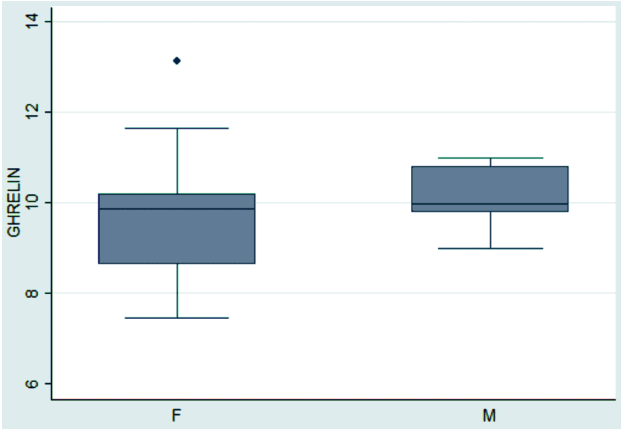

Figure 9 – ***Ghrelin expression based on gender. F: Female; M: Male.***

Table 2 – ***Univariable regression analysis for leptin and ghrelin***

Parameter	Variable	R^2	β Coefficient	P
Leptin	Age	0.0325	0.0276	0.2655
	Glycemia	0.0001	-0.0001	0.9918
	Cholesterol	0.0543	0.0116	0.1479
	Triglycerides	0.0062	0.0029	0.6288
	BMI	0.0008	0.0049	0.8592
Ghrelin	Age	0.0003	-0.0017	0.9228
	Glycemia	0.0028	0.002	0.7441
	Cholesterol	0.0651	-0.0091	0.112
	Triglycerides	0.0123	0.0029	0.4948
	BMI	0.0194	0.017	0.3917

BMI: Body mass index.

Table 3 – ***Leptin expression and associated pathology***

Pathology	Gene	Leptin
Hypertension	-	2.613 (2.206–3.704)
	+	2.682 (1.412–2.682)
Type 2 diabetes	-	2.543 (1.522–3.246)
	+	2.696 (1.648–4.82)
Hypothyroidism	-	2.619 (1.724–3.696)
	+	1.946 (0.564–3.823)

Data is presented as median and interquartile intervals (IQR).

Table 4 – ***Ghrelin expression and associated pathology***

Pathology	Gene	Ghrelin
Hypertension	-	10.023±1.369
	+	9.507±1.165
Type 2 diabetes	-	9.805±1.276
	+	9.509±1.303
Hypothyroidism	-	9.658±1.098
	+	10.064±1.874

Data is presented as mean values and standard deviation.

The histological study highlighted the presence of very large adipocytes in the adipose tissue, with a diameter over 250–300 μm (hypertrophic obesity) (Figure 10), and at stomach level, the presence of quite a thickened gastric mucosa, with large fundic glands and numerous oxyphil cells (Figure 11). There were frequently identified areas of gastric congestion of the gastric mucosa and small hemorrhages (Figure 12). The immunohistochemical examination showed an increase of the number of positive neuroendocrine cells to S100 protein (Figure 13), chromogranin (Figure 14), and synaptophysin (Figure 15) present both in the fundic and the antral area of gastric mucosa. Most neuroendocrine cells were positive at chromogranin and S100 protein.

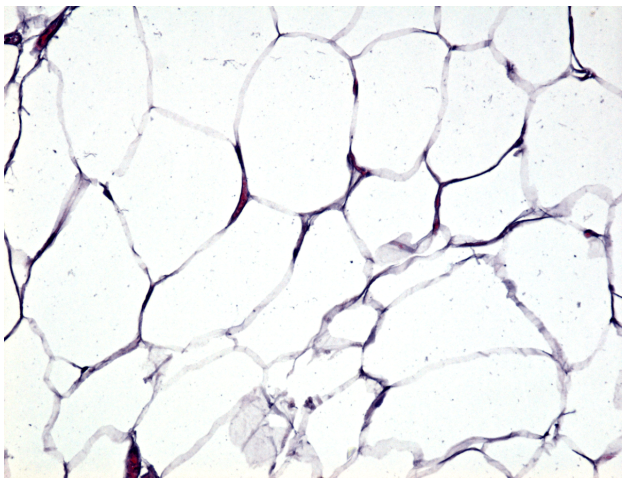

Figure 10 – ***Large adipocytes, identified in the perigastric tissue. GS trichrome staining, ×200.***

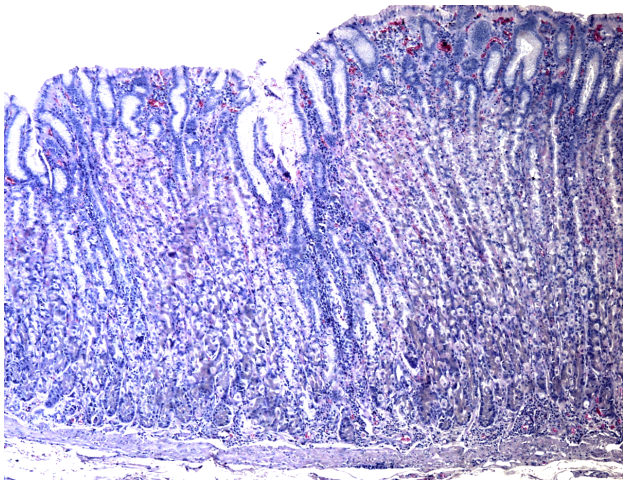

Figure 11 – ***Fundic, hypertrophied gastric mucosa, with a high number of oxyphil cells. HE staining, ×40.***

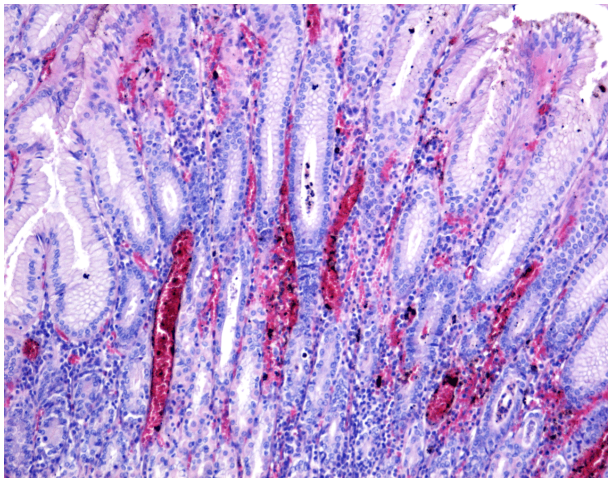

Figure 12 – ***Vascular congestion in the antral mucosa. HE staining, ×100.***

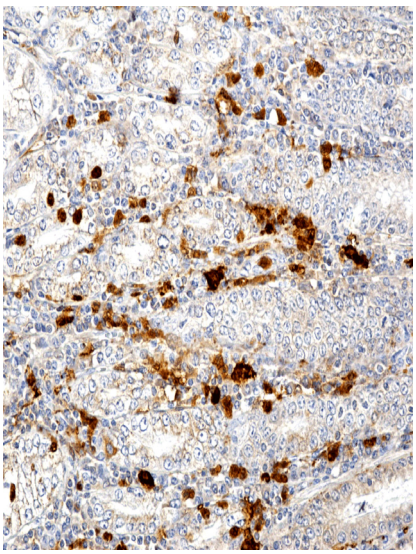

Figure 13 – ***Antral mucosa with high number of neuroendocrine cells. S100 antibody immunomarking, ×200.***

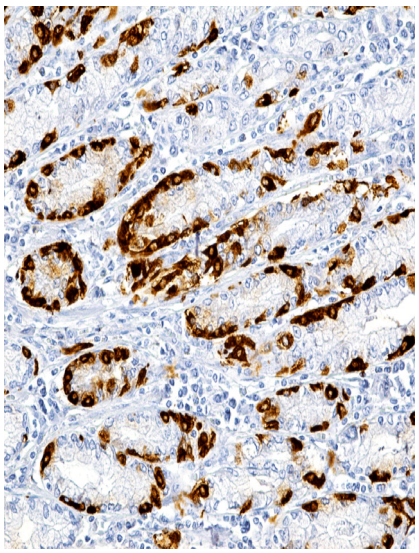

Figure 14 – ***Fundic glands with a high number of neuroendocrine cells, intensely positive to chromogranin. Anti-chromogranin antibody immunomarking, ×200.***

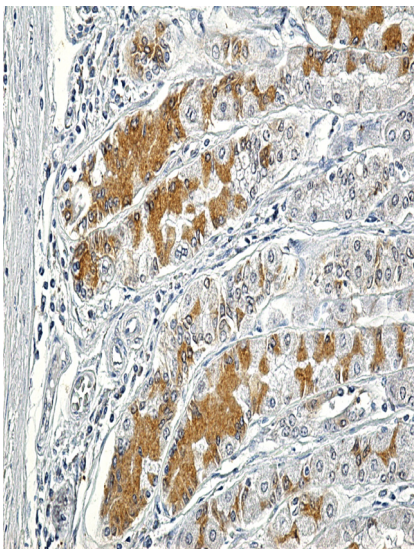

Figure 15 – ***Positive neuroendocrine cells to synaptophysin, localized at the basis of fundic cells. Anti-synaptophysin antibody immunomarking, ×200.***

Discussion

Leptin is a key molecule in the development of obesity codified by an obesity gene localized on the *q* arm of chromosome 7 in position 7*q*31. Leptin is synthesized mainly by adipose cells but also by other cell types in small quantities. Its main role is to adjust body weight through a feedback mechanism between the adipose tissue and the satiety centre in the hypothalamus [11].

Leptin has a proinflammatory and proatherogenic activity. Increased plasmatic levels correlate with the increase of adipose tissue although there are significant differences in leptin levels in patients with similar body weight [12].

In this study, we have confirmed the previous report indicating that leptin in human visceral adipose tissue is positively correlated with body fat mass. However, our results regarding leptin levels in visceral fat did not correlate with BMI although other studies found a positive correlation [13]. These contradictory results can be explained by Shah and Braverman's hypothesis. Their study shows that BMI underestimates obesity prevalence especially in female patients with increased levels of leptin [14].

Leptin and ghrelin have opposite effects in the regulation of the energy balance: leptin decreases and ghrelin increases food intake. Ghrelin has orexigenic, adipogenic and somatotrophic effects. It determines hyperphagia, increased body fat and has anabolic effects [15, 16]. When comparing ghrelin levels with BMI our study found significant differences between morbidly obese patients and healthy subjects. In the literature, there are few studies with contradictory results regarding ghrelin levels and BMI. Knerr *et al.* reported no significant differences in ghrelin levels between both obese and normal weight subjects [13]. Another study reports that ghrelin levels negatively correlate with BMI and fat mass [17]. These differences could be due to genetic polymorphisms between ethnic groups and posttranscriptional mechanisms.

Leptin synthesis is inhibited by testosterone and

stimulated by ovary hormones. As a result of sexual hormones activity, leptin levels are higher in women compared to men. This would show the influence of gender in disease development and pathology [18, 19]. Our results also show a significant difference in leptin levels between women and men. Another study reports higher leptin levels in subcutaneous fat compared to visceral fat in women and also higher levels in female compared to male subjects [20].

No differences could be observed when ghrelin levels were compared between sexes in our study. In another study, elevated ghrelin levels were observed, but significant results existed only in males, which could be explained by the onset of ghrelin resistance as described for leptin in obese subjects [21].

In our study, no statistically significant differences were observed between the age of the patients and leptin and ghrelin expression levels. The study group consisted of patients with morbid obesity who submitted themselves in the clinic for surgical treatment by bariatric surgery. Addressability for bariatric surgery in our country is the attribute of young age groups. This can be explained by the socio-economical and cultural level of the population. Malignant obesity is a major handicap for the young adult, leading to the impossibility of social integration and the labor market.

It is already known that obesity is a risk factor in the installation of type 2 diabetes. In our study, only nine of the 40 (22.5%) patients were diagnosed with type 2 diabetes. We did not detect statistically significant differences between leptin levels and blood glucose or type 2 diabetes.

In some studies, increased leptin are associated with elevated risk of type 2 diabetes. Leptin is involved in diabetes pathogenesis thought leptin resistance in β pancreatic cells, followed by hyperinsulinemia and decreasing of anti-apoptotic effect [22]. In obese patients sensitive to insulin, positive correlations were reported between BMI and insulin resistance, and also between waist circumference and leptin. There is evidence to suggest that ghrelin can contribute to metabolic syndrome; low glandular concentrations are present in pathological metabolic states such as type 2 diabetes. Ghrelin showed negative correlations with insulin resistance [23]. Our study did not show statistically significant differences between the levels of ghrelin expression and diabetes. This can be explained by the low percentage of diabetic patients and the young age of all subjects.

Hypertension is another component of the metabolic syndrome. Also, leptin expression positively correlates in both types of fat with systolic blood pressure and with diastolic blood pressure in visceral fat. This is also confirmed in other studies conducted by Baile *et al.* [24] and Uzun *et al.* [25]. Physiologically, adipokines modulate vascular homeostasis, through endocrine and paracrine pathways. Obesity determines changes in adipokines gene expression and synthesis and implicit in their action on blood vessels [26]. Silvani *et al.* reported in a study conducted on animal experimental models that in the absence of leptin, an increase in body mass can determine an increase in arterial blood pressure [27]. Fifty-five percent of the patients considered in this study were diagnosed with hypertension similar to other studies [28, 29]. The statistical analysis did not show significant differences between mRNA and hypertension.

Since ghrelin, leptin and thyroid hormones are involved in the regulation of energy metabolism and body weight control, we have sought to highlight possible correlations between the levels of expression of these genes and thyroid dysfunction. Studies on this subject on experimental animal models and on obese human subjects have controversial results. Thus, some studies associate hypothyroidism with elevated levels of leptin [30–32], others report low levels of leptin [30, 33, 34] and other unchanged levels of leptin [30, 35, 36]. The same controversial results are reported in the literature on the association between ghrelin expression and hypothyroidism [37]. Although our studied group comprised eight cases of hypothyroidism associated with malignant obesity, the statistical analysis does not show significant correlations between mRNA levels of leptin and ghrelin and thyroid dysfunction.

The histological and immunohistochemical study brings into discussion the importance of neuroendocrine cells from gastric mucosa in the onset of obesity [38]. It is a well-known fact that neuroendocrine cells are involved in the normal physiological processes at gastric mucosa level, and also in the conditions affecting the digestive tract [39].

The gastric mucosa contains at least six distinct neuroendocrine cell types that account for about 1% of the volume of the gastric mucosa [40]. Gastrointestinal hormones synthesized by neuroendocrine cells strongly influence food intake through their actions on central nervous system circuits and nerves.

Conclusions

Our study shows that mRNA levels for leptin and ghrelin are significantly comparable with the control in visceral adipose tissue. The fact that BMI and clinical and biological parameters studied did not correlate could be explained by the young age of patients considered, which limited the number of cases with associated pathology. Whereas multiple studies point out that BMI is inconclusive for the prediction of body fat based on the age and gender of patients, we consider that the association of leptin levels could improve the orientation of the surgical act. The clinical utility of leptin and its validation as a marker in morbid obesity requires further studies on a larger number of patients to highlight both genetic variation in population and circulating levels of leptin.

Conflict of interests

The authors declare that they have no conflict of interests.

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