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ORIGINAL SCIENTIFIC REPORT

Proteomic Identification of Biomarkers Associated with Eating Control and Bariatric Surgery Outcomes in Patients with Morbid Obesity

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Abstract

Background The current therapeutics of morbid obesity could be significantly improved after the identification of novel biomarkers associated with the food addiction endophenotype of obesity and with bariatric surgery outcomes.

Methods We applied differential expression proteomics and enzyme-linked immunosorbent confirmatory assays to identify (a) proteins that varied according to loss of control over eating in morbidly obese patients and (b) proteins that varied between normoweight controls and patients before and 1 year after bariatric surgery.

Results Clusterin was the only protein that consistently varied according to eating control in patients. Patients showed increased levels of serum amyloid P protein, apolipoprotein A4, serotransferrin, complement factors B and C3 and haptoglobin with respect to controls; the levels of all these proteins tended to return to control values 1 year after surgery. In contrast, apolipoprotein A1 and transthyretin were initially downregulated in patients and were scarcely changed by surgery. Leucine-rich alpha-2-glycoprotein was markedly increased in patients only after surgery.

Conclusions Clusterin could be of interest as a putative biomarker for food addiction diagnosis in people with morbid obesity. In addition, postsurgical normalization of the proteins initially dysregulated in obese subjects might help monitor clinical improvements after surgery, while lasting or newly detected alterations (i.e., those affecting transthyretin and leucine-rich alpha-2-glycoprotein) could reflect partial refractoriness and/or contribute to the early prediction of clinical problems.

Introduction

Food addiction has been claimed to be one particular phenotype of obesity [1, 2]. People with obesity and food addiction could exhibit pathological deficiencies in their brain reward systems and increased risk of "addiction transfer" after bariatric surgery [3]. The latter is an important issue since the proportion of new-onset substance abusers among bariatric patients after surgery has been estimated to range from 34.3 to 89.5% [4]. The availability of biomarkers that complement psychiatric evaluations could contribute to diagnosing food addiction and help prevent postsurgical complications; accordingly, we have applied a proteomic approach to identify plasma proteins that correlate with loss of eating control in people with morbid obesity. We focused on this particular variable since (a) it represents a key component of food craving in morbid obesity [5], which markedly resembles the loss of control over drug use among drug addicts, and (b) it is a single factor that is much more convenient to phenotyping

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patients of a small sample than multidimensional entities with higher intrinsic variability.

Novel biomarkers are also of interest in terms of increasing the accuracy of the evaluation of the therapeutic response to bariatric surgery, and to detect subtle changes that may not translate into major clinical signs in the short term but could contribute to delayed therapeutic failures. One year after bariatric surgery, people with morbid obesity usually experience very marked weight reduction, as well as extensive clinical improvements [6]. These outcomes were also observed in our own cohort of patients [7]. By that time, however, comorbidities persist in many subjects [8] and new unwanted effects of surgery could be developing (i.e., decreases in bone mineral density [9]). At longer intervals, weight regain [10] and other important problems such as the previously noted "addiction transfer" may lead to therapeutic failures resulting from aggravation of previously undetected disturbances and/or to the emergence of new ones. It is therefore important to identify sensitive biomarkers of therapeutic response that could predict the evolution of the patients beyond clinical signs. This objective can be accomplished by applying proteomic procedures that follow the postsurgical evolution of as many plasma proteins as possible.

Materials and methods

Subjects

The study included 23 patients with morbid obesity and a matched control group of 23 healthy normoweight participants selected from a population of healthcare personnel and medical students paired by age and sex with patients (Table 1). Inclusion criteria were 5 years of maintained obesity and body mass index (BMI) > 40 kg/m^2 for patients, BMI < 25 for controls, and an age of 18–60 years and psychological stability for all participants. Exclusion criteria were obesity secondary to endocrinopathies or drug treatments, major psychiatric disease, mental retardation, severe eating disorders and alcohol/drug abuse. At the beginning of the study, the patients completed the Spanish version of the State and Trait Food Cravings Questionnaire—Trait scale (FCQ-T), an instrument with excellent consistency in bariatric surgery-seeking people with morbid obesity [11]. We captured "loss of control" (LC) from the answers to six selected questions that were scored from 1 ("never") to 6 ("always"), and then summed them to calculate a final LC score for each subject. FCQ-T tests were not considered for controls since the nature of food cravings has been shown to be different in people with obesity than in other subjects [11]. Thus, direct comparisons between patients and controls regarding LC would be incorrect. Serum samples were obtained at the beginning of the study and 1 year after bariatric surgery and were frozen (− 80 °C) until the day of analysis. The study was conducted in accordance with the Declaration of Helsinki, and all procedures were carried out with the written consent of the participants.

Table 1 Subjects included in the study (means and SEM are used). The rate of the most frequent patient comorbidities (higher than 10%) is included

	Patients	Controls	*p* value
Males: females	7:16	7:16	
Age (years)	44 ± 3	39 ± 3	0.198
Height (cm)	166 ± 2	164 ± 1	0.329
Weight (kg)	131 ± 3	61 ± 2	< 0.001
BMI (kg/m^2)	47.8 ± 1.2	22.6 ± 0.4	< 0.001
Reflux esophagitis (%)	13.0		
DDD/arthritis (%)	21.7		
DM-2 (%)	26.1		
Sleep apnea (%)	26.1		
Hypertension (%)	39.1		

BMI body mass index, *DM-2* diabetes mellitus type 2, *DVT* deep vein thrombosis, *DDD* degenerative disk disease

Surgery

Laparoscopic gastric bypass was performed using five ports and involved a 30–50-cm biliopancreatic limb and a 100-cm antecolic, antegastric, alimentary limb. The surgeon constructed the jejunojejunal anastomosis side-to-side with a firing of a 45-mm linear endostapler and hand-sewed the defect. The gastric pouch was small and vertically oriented, and the gastrojejunal anastomosis was constructed with a 30-mm linear stapler and was also hand-sewed.

Identification of proteins associated with eating control in patients

Presurgical samples from patients were unfrozen, and the most abundant serum proteins were removed by using Pierce Top 12 Abundant Protein Depletion Spin Columns (Thermo Scientific, Waltham, MA, USA). The remaining serum proteins were separated with a two-dimensional electrophoresis protocol routinely used in our laboratory [12]. The gels were then stained in a Dodeca Stainer with the "Silver Stain" kit (Bio-Rad) and scanned using the densitometer GS-800 (Bio-Rad). Spots were detected, quantified and matched automatically with the PDQuest v8

software (Bio-Rad). Figure 1 shows a representative image of the stained gels.

The study of the proteins potentially associated with eating control was performed in two steps. First, the median LC score of the entire group of patients was used to subdivide the gels into two subgroups, comparing the optical densities of the spots of these subgroups with the PDQuest v8 software using *t* tests. This procedure was adopted to reduce the number of protein identifications to those spots showing statistical differences in this subgroup comparison, which were considered the most relevant. Only two spots achieved this criterion and were identified by cutting them out of the gels to be analyzed by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry at the Proteomics Facility UCM-PCM of Madrid, Spain (a member of ProteoRed network). Lastly, we checked the relationship between these proteins and eating control by studying the correlations between spot densities and LC scores in the entire group of patients (Spearman's rho).

Identification of proteins associated with obesity and surgery outcome

Once the presurgical samples of patients were processed, postsurgical samples and samples from normoweight controls were also processed using exactly the same procedure. The spots showing different optical densities between the three groups were detected and compared with PDQuest v8 software, and the corresponding proteins were identified by mass spectrometry as described above. Possible correlations between optical densities of differentially expressed proteins and % total weight loss were studied (Spearman's rho).

Enzyme-linked immunosorbent assays

Five proteins previously identified as potential biomarkers were further quantified by enzyme-linked immunosorbent assays (ELISA) to confirm proteomics. The following ELISA kits were used: E-EL-H0038 and E-EL-H0848 (Elabscience, Wuhan, Hubei, China) for clusterin and complement factor B, respectively; KA1841 (Abnova, Taoyuan, Taiwan) for serum amyloid P protein; OKIA00064 (Aviva Systems Biology, San Diego, CA, USA) for haptoglobin; and CSB-E08665h (Cusabio, Houston, TX, USA) for complement factor C3. We also quantified ghrelin and leptin with the Human Ghrelin ELISA kit and the EZHL-80SK Human Leptin "Dual Range" ELISA kit (Millipore, Billerica, MA, USA).

As with the proteomics, Spearman's rho was used to examine the possible correlations between LC scores with clusterin and serum amyloid P protein levels (and also with leptin and ghrelin), and protein concentrations were compared between controls and patients before and after surgery by using *t* tests.

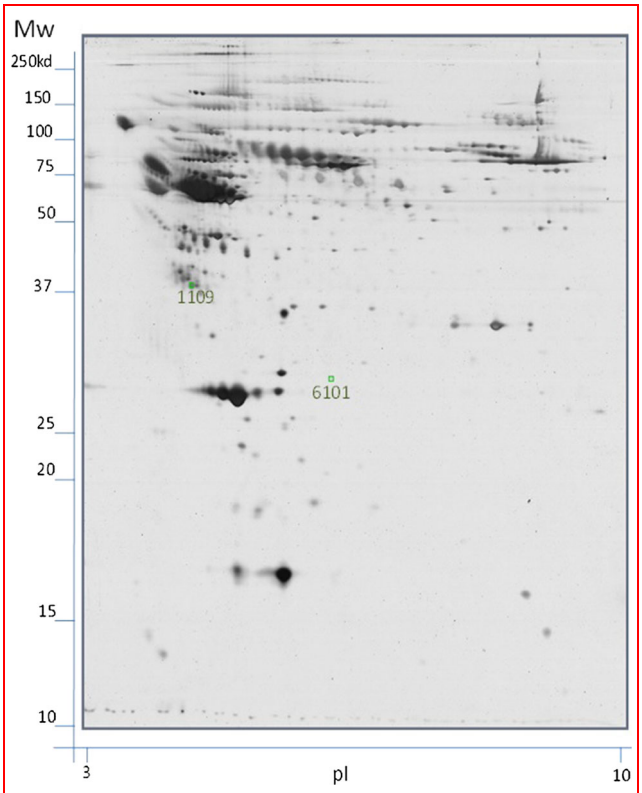

Fig. 1 Representative gel of the serum proteome. Spots labeled with numbers show statistical differences in optical density according to LC values. *Mw*, molecular weight; *pI*, isoelectric point

Results

The preliminary comparison of the serum proteomes of patients with LC scores above and below the median value detected two spots showing significant differences ($p < 0.05$) in optical density (spot 1109: 2439 ± 704 arbitrary units vs. 1450 ± 458; spot 6101: 148 ± 32 vs. 57 ± 24). Mass spectrometry identified these proteins as clusterin and serum amyloid P protein, respectively. Subsequent data analysis revealed that the optical densities significantly correlated to LC scores in the case of clusterin, but not in the case of serum amyloid P protein, which was confirmed by ELISA (Table 2); the latter quantifications also showed that clusterin levels were not different in patients and controls. On the contrary, serum amyloid P protein was upregulated in patients (Fig. 2). Leptin and ghrelin levels, which were elevated and decreased in presurgical patients, respectively (Fig. 2), did not correlate with LC scores or clusterin levels.

Table 2 Differential protein expression according to loss of control over eating in people with morbid obesity

	Clusterin	Serum amyloid P protein
Spot number	1109	6101
UniProt ID	P10909	P02743
Theoretical molecular weight	53,031	25,485
Theoretical isoelectric point	5.89	6.10
Score	236	72
Coverage (%)	19	26
Peptides matched	7	5
Tandem mass spectrometry sequence data	TLIEKTNEER	AYSLFSYNTQGR
	QQTHMLDVMQDHFSR	DNELLVYKER
	ASSIIDELFQDR	VGEYSLYIGR
	EPQDTYHYLPFSLPHR	QGYFVEAQPK
	RPHFFFPK	IVLGQEQDSYGGKFDR
	ELDESLQVAER	
	LFDSDPITVTVPVEVSR	
Correlation to LC	0.5263 (0.1214–0.7811)	0.3977 (− 0.04198 to 0.7084)
Proteomics	$p = 0.0119$	Not significant
Correlation to LC	0.6244 (0.1706–0.8596)	− 0.1233 (− 0.5684 to 0.3776)
(ELISA)	$p = 0.0097$	Not significant

The two proteins depicted in the table fitted the preliminary criteria to proceed with protein identification (significant differences between LC scores below or above the median value). They were identified by mass spectrometry and checked for correlations with LC scores in the whole sample of patients (Spearman's rho with 95% confidence limits and p value is shown)

Table 3 shows the results of the comparisons between serum proteomes of controls and patients before and after surgery. At the beginning of the study, controls and presurgical patients significantly differed in the optical densities of 16 spots that corresponded to apolipoprotein A4, complement C3, complement factor B, haptoglobin (spots 4–9), complement C4-A (spots 14–17), serotransferrin, apolipoprotein A1 and transthyretin. Five of these proteins were clearly upregulated in patients (apolipoprotein A4, complement C3, complement factor B, haptoglobin and serotransferrin), and two of them were downregulated (apolipoprotein A1 and transthyretin). Two of the spots identified as complement C4-A (spots 14 and 15) showed increased densities in patients, but the other two (spots 16 and 17) showed just the opposite. Baseline serum amyloid P protein upregulation in patients, as shown by ELISA (Fig. 2), was not paralleled by significant differences in the image analysis of proteomes.

One year after bariatric surgery, postoperative morbidity was limited to a patient who showed abdominal wound disruption with eventration, another who experienced dumping syndrome and a third patient with postoperative cholelithiasis. All the patients exhibited a reduction in body mass index (from 47.6 ± 1.2 to 31.1 ± 1.2 kg/m^2, with % excess weight loss = 74.8 ± 3.9 and % total body weight loss = 34.7 ± 1.8 ranging from 15.5 to 49.9%) as well as a decrease in serum leptin and low concentration of ghrelin (Fig. 2). An improvement in at least two comorbidities was recorded in 21.4% of the patients. Most of the proteins overexpressed before surgery declined to serum values closer to those of controls, even if these reductions did not correlate with weight loss (Table 3). ELISA confirmed surgery-induced corrections of serum amyloid P protein, complement C3 and haptoglobin, but not in the case of complement factor B (Fig. 2). In contrast, leucine-rich alpha-2-glycoprotein showed no significant elevations in patients from baseline that evolved to a marked increase 1 year after surgery. Apolipoprotein A1, and notably transthyretin, showed little evolution after surgery, and thus the latter remained markedly downregulated, as was the case with ghrelin. One form of serotransferrin (spot 10) and another one of complement C4-A factor (spot 18) also appeared to be downregulated postsurgery.

Discussion

The positive correlation between LC scores and serum clusterin levels in patients strongly suggests that this protein could be considered a potential biomarker of eating control in morbid obesity. Clusterin has been previously connected with obesity [13, 14]; however, we did not find significant differences between patients and controls and thus, the protein seems to be more directly related to food

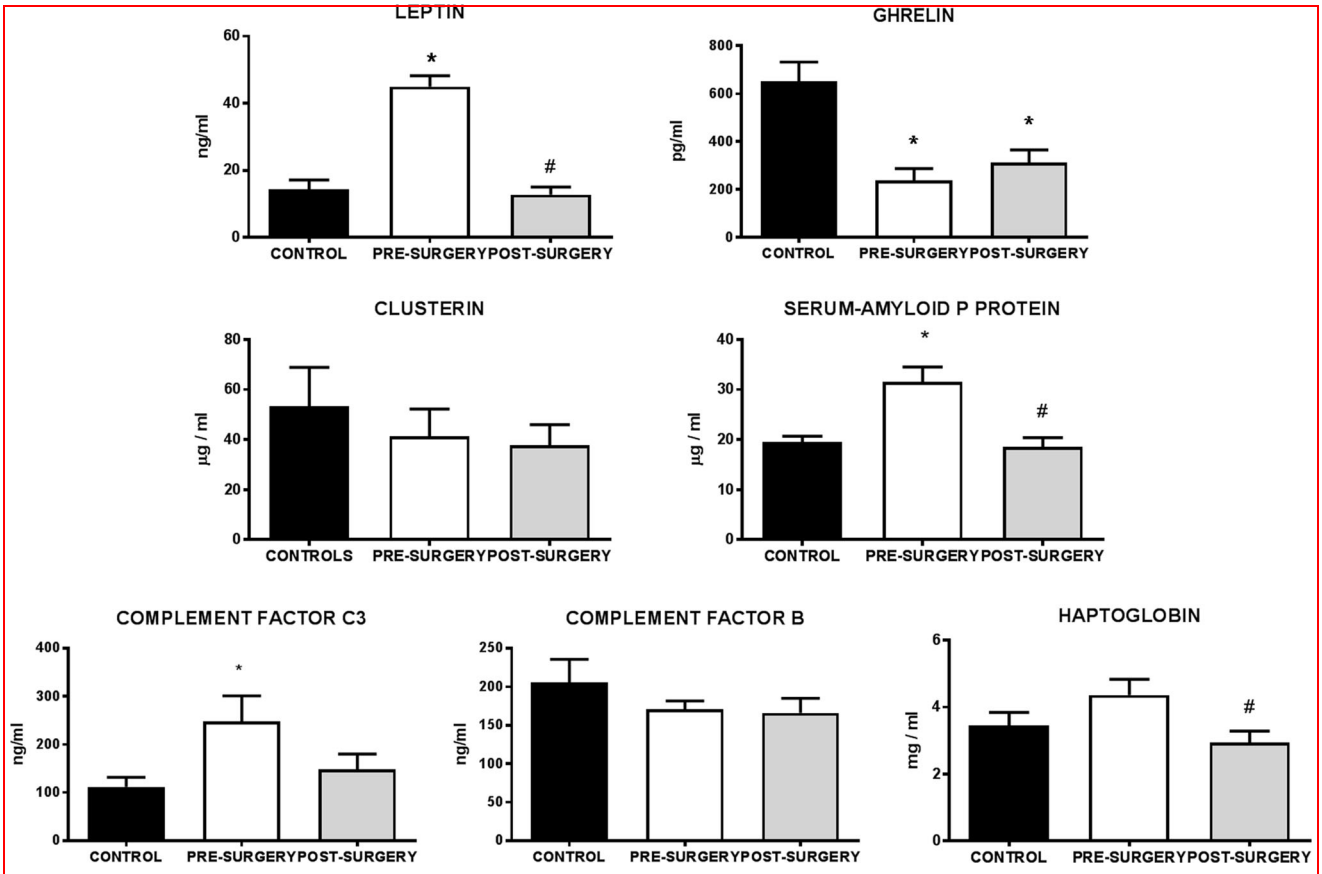

Fig. 2 ELISA quantifications of five proteins differentially expressed in proteomics. Bars represent mean ± SEM from at least 14 determinations. *$p < 0.05$ versus controls; #$p < 0.05$ versus presurgery

addiction than to body weight. In support of this idea, animal experiments have found that clusterin plays a significant role in feeding behavior given that it mediates the opposite effects of ghrelin and leptin on the activity of hypothalamic neurons and the homeostatic control of appetite [15]. The effects of clusterin on the activity of dopaminergic neurons in the brain reward system are presently unknown, but both leptin and ghrelin influence them in an opposite manner, as happens in the hypothalamus [16]. If similar regulatory mechanisms involving clusterin, leptin and ghrelin work in the hypothalamic and mesolimbic neurons, a close relationship between clusterin levels, neuronal activity and behavioral manifestations of food addiction (i.e., loss of control over eating) would not be surprising. Additional determinations in human subjects and experiments in animals are clearly needed to fully understand these interactions, but it is interesting to note that clusterin levels in our patients did not correlate with leptin or ghrelin, nor did these hormones correlate with LC scores. Hence, clusterin better fits the profile of a putative biomarker of behavioral control over eating and could behave as a common, final mediator of diverse hormonal influences.

In the case of serum amyloid P protein, we could not confirm an association with eating control. On the other hand, the increased levels of the protein observed in patients and the subsequent reduction after bariatric surgery are consistent with other reports and could reflect a favorable evolution of the inflammatory status of the patients [13]. Leptin and other proteins also differed between patients and controls and were significantly affected by surgery. None correlated with weight loss, showing that the latter variable does not necessarily indicate a parallel, general improvement in the biological alterations associated with obesity in a given subject. According to the literature, complement C3 and haptoglobin reductions after surgery may reflect attenuations of insulin resistance [17–19]. Complement factor B behaved similarly, which is in agreement with other authors [20], but was not confirmed by ELISA; probably, the two analytical procedures quantify different forms of the protein. Apolipoprotein A4 reduction postsurgery does

Table 3 Differential protein expression in normoweight controls and people with morbid obesity before and after bariatric surgery

Spot	Protein	Controls	Patients presurgery	Patients postsurgery
1	Apolipoprotein A4	7291.5 ± 2246.2	21057.9 ± 3323.8*	6301.9 ± 1736.7*#
2	Complement C3	572.5 ± 148.2	1336.3 ± 187.1*	891.6 ± 111.8#
3	Complement factor B	1637.6 ± 205.7	2659.5 ± 391.4*	1248.6 ± 240.0#
4	Haptoglobin	3786.2 ± 990.1	13781.7 ± 1933.0*	4726.1 ± 1122.3#
5	Haptoglobin	954.0 ± 367.0	4313.3 ± 1255.5*	997.1 ± 357.5#
6	Haptoglobin	7411.9 ± 1572.3	16573.3 ± 1853.3*	9708.0 ± 2387.18#
7	Haptoglobin	1696.8 ± 331.5	6669.0 ± 985.7*	2357.7 ± 626.4#
8	Haptoglobin	979.7 ± 238.5	3841.1 ± 1075.4*	1497.8 ± 568.5#
9	Haptoglobin	956.4 ± 260.7	2456.6 ± 586.2*	984.3 ± 326.0#
10	Serotransferrin	2025.9 ± 268.7	2558.5 ± 545.3	999.5 ± 159.4*#
11	Serotransferrin	2794.1 ± 520.0	5378.2 ± 1433.8*	1447.5 ± 276.1#
12	Apolipoprotein A1	9020.4 ± 1278.3	5410.7 ± 845.7*	6357.3 ± 778.9
13	Transthyretin	1185.2 ± 546.7	153.7 ± 73.7*	91.7 ± 60.2*
14	Complement C4-A	53.0 ± 30.1	607.5 ± 242.1*	178.0 ± 102.0#
15	Complement C4-A	197.9 ± 87.7	603.5 ± 213.5*	392.0 ± 120.5#
16	Complement C4-A	219.5 ± 60.8	100.3 ± 24.5*	69.3 ± 17.1*
17	Complement C4-A	1098.2 ± 242.9	439.3 ± 67.3*	331.0 ± 77.0*
18	Complement C4-A	5109.2 ± 1508.4	3091.1 ± 322.6	1648.9 ± 278.0*
19	Leucine-rich alpha-2-glycoprotein	298.6 ± 78.6	360.6 ± 127.4	802.9 ± 206.0*#
20	Serum amyloid P component	104.4 ± 30.5	106.8 ± 22.0	40.4 ± 13.5*#

Data represent optical densities (mean ± SEM). *$p < 0.05$ versus controls; #$p < 0.05$ versus presurgery

not support the lack of effect of gastric bypass previously reported; interestingly, this protein predicted the postsurgical evolution of insulin resistance in the latter study [21], and thus its role as a potential biomarker remains to be clarified. The same evolution pattern of initial elevation and postsurgical correction was noted for transferrin, but now the initial increase was unexpected [22–24] and could be attributed to iron deficiency secondary to dietary restriction, which is sometimes prescribed in our hospital prior to surgery [25].

Low levels of transthyretin may also reflect malnutrition in our patients [26]; however, in this case the deficit persisted 1 year after surgery, which is important for bariatric patient follow-up. The same persistent reduction in ghrelin could be explained by gastric fundus resection during surgery [27]. Similarly to transthyretin and ghrelin, apolipoprotein A1 was also initially downregulated in patients, and this reduction could be attributed to alterations in lipid metabolism and poorly controlled diabetes [28, 29]; the effects of surgery on this protein are unclear, if we consider the high dispersion of the data obtained. Nor was the behavior of complement C4-A clear: The five spots that corresponded to this protein experienced dissimilar evolutions, probably because they represent different forms and functions of the protein.

The notable increase in leucine-rich alpha-2-glycoprotein observed 1 year after surgery is of interest since it could reflect the emergence of new biological alterations. The protein has been shown to be differentially expressed in a variety of pathologies [30] and is also considered a biomarker of mortality [31]; therefore, further studies are strongly recommended to investigate possible associations with long-term adverse events of bariatric surgery.

It is important to note that the biomarkers of response to surgery identified in this study apply strictly to gastric bypass and not to other bariatric interventions that could lead to very different hormonal responses. Beyond this issue, the present study has two main limitations. First, the lack of data on eating control after surgery prevented us from providing additional evidence in support of clusterin as a biomarker. Second, the limited number of patients available did not permit us to establish subgroups according to key variables such as type 2 diabetes, thus limiting the interpretation of the results. Further validation studies focused on the identified biomarkers must include a higher number of patients, a more frequent sampling to better understand the biomarker progression along time and a parallel evaluation of eating control.

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Compliance with ethical standards

Conflict of interest None.

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