Title: Cocaine and amphetamine regulated transcript and Brain-derived neurotrophic factor in morbid obesity. One-year follow-up after gastric bypass.

Short title: Effect of gastric bypass on CART and BDNF.

ABSTRACT

The identification of biomarkers associated with obesity and response to treatment could represent an important advance in order to design more effective and personalized therapeutic strategies. The complexity of morbid obesity could be explained as the combination of genetic, biochemical, cultural and behavioral factors among others. The study of biomarkers should be considered a determinant factor taken into account into this equation.

The aim of this study was to define better biomarker profiles potentially associated to the short term outcome of bariatric surgery by paying attention to Cocaine and amphetamine regulated transcript (CART) and Brain-derived neurotrophic factor (BDNF), two neuropeptides related to eating behavior. Twenty-seven morbid obese subjects and thirty normoweight individuals matched by age and sex were selected for the study. Patients underwent bariatric surgery by Roux-en-Y gastric bypass (RYGB) and responded adequately in terms of weight loss and normalization of many biochemical parameters one-year post-surgery. A multivariate analysis showed that the hormonal/neuropeptidic profile explained 82% of the variability of the weight loss response.

The evolution of CART paralleled that of insulin and leptin: serum levels of this peptide were initially elevated in patients (4.24 ± 0.47 ng/mL) with respect to controls (2.94 ± 0.2 ng/mL), but this difference disappeared one year after RYGB (3.14 ± 0.26 ng/mL). BDNF levels were also decreased by RYGB (11.93 ± 0.96 ng/mL postsurgery vs. 15.3 ± 1.02 ng/mL presurgery), even when this peptide was not elevated in patients before surgery (14.23 ± 0.86 ng/ml in controls). The results suggest that CART and BDNF could be envisaged as new candidate biomarkers of short-term outcome after surgery.

KEYWORDS

bypass, roux en y gastric; morbid obesity; obesity biomarkers; BDNF; CART.

INTRODUCTION

The identification of biomarkers associated with obesity and response to treatment could represent an important advance in order to design more effective and personalized therapeutic strategies. In the case of morbid obesity, bariatric surgery provides the best success rates, which includes partial diabetes remission, improved glucose homeostasis, decreased cardiovascular risk and significant reduction in hepatic steatosis, inflammation, and fibrosis on nonalcoholic fatty liver disease, among other benefits[1–3]. However, the patient response may significantly vary depending on different factors. One of the most important source of variation is the eating behavior exhibited by the patients, which is known to be closely related to the peripheral levels of hormones such as ghrelin or insulin[4], but also to other neuropeptides less studied or more controversial like Cocaine and amphetamine regulated transcript (CART) and Brain-derived neurotrophic factor (BDNF).

To our knowledge, the serum levels of CART have not been studied in morbid obesity pre- and post-surgery, even though the facts that the peptide is widely known to mediate eating behavior in laboratory animals[5,6] and that CART polymorphisms have been associated with obesity susceptibility in humans[7]. It has been reported that CART levels tend to decrease in obese patients who are engaged in a physical activity program with calorie restriction diet[8], a finding that recommends further studies on the involvement of this neuropepetide in body weight regulation. Contrary to CART, BDNF levels have been reported to be reduced in the serum of human obese patients[9] and seem to recover after physical training[10]. This peptide has been related to the control of feeding[11] and thus

some mutations in the gene encoding BDNF lead to insatiable appetite and severe obesity[12]. Despite these antecedents, a direct relationship between obesity and BDNF has not been confirmed by all authors[13]. As in the case of CART, the evolution of BDNF levels after bariatric surgery has not been previously studied; thus, the aim of this work was to examine the effects of bariatric surgery on a cohort of morbid obese patients with a special attention to the evolution of CART and BDNF serum levels within 1 year after bariatric surgery.

SUBJECTS AND METHODS

Subjects and clinical assessments

This is a case-control study where morbid obese subjects were recruited along 18 months among candidates to bariatric surgery who met the principles of the International Federation for the Surgery of Obesity and Metabolic Disorders (IFSO). Inclusion criteria were 18-60 years of age, obesity lasting for at least 5 years, previous failure of non-surgical treatments, psychological stability and basic understanding of the technique. Exclusion criteria were obesity secondary to endocrine disorders or drug treatments, major psychiatric disease, mental retardation, severe eating disorders (bulimia nervosa) and alcohol or drug abuse. Patients fitting the criteria for laparoscopic sleeve gastrectomy (Body mass index, BMI > 60 and/or severe medical disease) were also discarded for this study, which was specifically focused on the effects of Roux-en-Y gastric bypass (RYGB). Normoweight controls were recruited at the same hospital and they were paired by sex and age with patients. Figure 1 reports the flowing diagram of study participants. Analytical determinations were performed at the beginning of the study and were repeated one year after surgery in the case of patients. The therapeutic response was evaluated by assessing weight loss and reduction of BMI, and was considered successful when overweight reduction exceeded 50% (%EBL [(preoperative BMI - current BMI)/(preoperative BMI - 25) x 100])[14].

Surgery

Laparoscopic gastric bypass was performed in 27 patients 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 constructed with 30 mm linear stapler and hand sewn.

Ethics statement

Patients and controls gave their informed consent prior to the participation in the study. All protocols used were approved by the Ethical Committee for Clinical Research (CEIC) of the hospital. The study meets The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and data was computerized according to the guidelines established by Spanish Law 15/1999 of Data Protection. A process quality monitoring of all records was implanted.

Biochemical determinations

Hemathology parameters in blood and serum samples such as blood count serum biochemical determinations were performed in the Laboratory of Clinical Analysis by conventional procedures routinely used in the hospital. Serum levels of hormones and neuropeptides were determined by enzyme immunoassays (EIA) or enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's protocols. The following kits were used: EZHADP-61K Human Adiponectin ELISA kit (Millipore, Billerica MA, USA); CYT306 ChemiKine Brain Derived Neurotrophic Factor ELISA kit (Millipore); Human CART EIA kit (RayBiotech, Norcross GA, USA); EZGRT-89K Human Ghrelin ELISA kit (Millipore); EZHI-14K Human Insulin ELISA kit (Millipore) and EZHL-80SK Human Leptin "Dual Range" ELISA kit (Millipore).

Statistical analysis

Statistical analyses were performed using the *SPSS* software (version 19.0 for Windows, IBM, USA) and graphics with R Studio 1.0.143. The applied statistical tests were bilateral and significance was established at $p<0.05$. Normality condition of quantitative variables

was checked by Shapiro-Wilk test. Student's t-test for paired samples was performed in comparisons of pre- and post-surgery patients groups. In patients (pre or post-surgery) versus controls comparisons, Student's t-test for independent samples was used with Levene's test to assess the equality of variances. When normality condition was not fulfilled, non-parametric tests were used: Wilcoxon signed-rank test for paired samples and Mann–Whitney U test for unpaired. Variables were classified into profiles according to their molecular nature. Multivariable analysis was also performed to determine factors associated with the response to surgery (with %EBL as main variable). We estimated regression coefficients (B) and 95% confidence intervals (CI) using multivariable adjusted linear regression models. The models were statistically analyzed by profiles and controlled for potential confounders based on published factors and those variables with p-values < 0.20 in the bivariate analysis.

RESULTS

The patient sample was formed by 27 subjects, 30% male, aged 42 ± 7 years, with BMI = 48.0 ± 1.0 kg/m^2. A control group of 30 normoweight controls paired by sex and age was recruited from the hospital personnel and university volunteers (male, 30%; age 37 ± 9 years, BMI, 22.6 ± 0.4 kg/m^2).

Figure 2 illustrates the marked reduction of body mass index provided by surgery in our patients; all of them but 3 achieved the objective of 50% loss of overweight, the average loss of BMI excess (%EBL) being 74.9 ± 3.3%.

Bivariate analysis of the analytical parameters is summarized in table 1. For **hematological, hepatic and renal profile,** leukocytes, GGT, ALT and C-reactive protein were significantly higher in pre-surgery patients than normoweight controls. After surgery, leukocytes, hemoglobin, creatinine, total protein, GGT, ALT and C-reactive protein significantly decreased, however AST still remained elevated and creatinine and total protein went below control levels.

In reference to **lipidic profile**, before surgery, HDL levels were lower in patients than in controls. Surgery decreased cholesterol, triglycerides and LDLs and elevated HDLs, however the latter remained low when compared to controls.

Attending to the **carbohydrate metabolism profile**, glucose and both homeostatic model assessment, HOMA β and HOMA IR, were elevated in patients at the beginning of the study and went down to control values after surgery. The opposite was observed regarding HOMA-2 %S.

For **phospocalcium metabolism** profile, results show low levels of vitamin D1, phosphorus, calcium, magnesium and iron in patients before surgery, while uric acid and

intact PTH were elevated. All these alterations were totally or at least partially corrected by surgery. Only the levels of vitamin B12 experienced a significant downregulation postsurgery.

Finally, the study of **hormonal and neuropeptidic profiles** revealed that patients showed higher levels of leptin, insulin and CART than normoweight controls before surgery, as well as lower levels of cortisol, adiponectin and ghrelin (table 1, figure 3). After surgery most of these differences were attenuated or even disappeared, as it was the case with CART; however, cortisol and ghrelin levels still remained significantly lowered in patients (table 1, figure 3). Interestingly, BDNF levels were similar in patients and controls at the beginning of the study, but a significant BDNF downregulation emerged in patients after surgery (table 1, figure 3).

Multivariable analysis was performed for each profile to evaluate possible factors associated with surgery outcome, using the %EBL as main variable. Only **hormonal and neuropeptide profile** showed significant differences (table 2). This model explains 82% of variability in %EBL after surgery and shows how the increase of each unit of adiponectin, ghrelin and BDNF levels, increase 0.155%, 0.024% and 0.828% of %EBL ($p=0.027$, $p=0.004$ and $p=0.030$) respectively. Contrariwise, the increase of each unit of leptin supposes a 1.366% reduction of %EBL ($p<0.001$).

Our patients showed a post-surgery increase of adiponectin and ghrelin with a leptin reduction (figure 3). According with the linear regression analyses (table 2) this represents an increase of %EBL (0.155%, 0.024% and 1.366% for each hormone, respectively). In contrast, BDNF was downregulated after surgery and this represented a 0.828% increase of %EBL per unit of BDNF decrease.

DISCUSSION

The importance of bariatric surgery for the treatment of morbid obesity has been extensively described. It seems clear that this therapeutic approach provides significant and lasting decrease of weight over time, accompanied by drastic reductions of comorbidities and improved quality of life[15–17]. The positive effects of surgery on Type-2 Diabetes Mellitus (T2D), metabolic syndrome and overall cardiovascular risk factors seems especially relevant for the reduction of mortality from 40 to 23%[11–13,15,16]. The results of our study are in general agreement with the literature and thus show that many biochemical alterations of the cohort of patients become significantly reduced or even completely disappeared one year after surgery. The beneficial effects applied to biomarkers of renal and hepatic function, inflammation, lipid, carbohydrate and phospho-calcium metabolism as well as the leukocyte count and serum levels of relevant ions, hormones and neuropeptides. Despite its benefits, bariatric surgery does not achieve the expected results in all patients and therefore some of them exhibit a poor therapeutic response or even experience emerging problems. Protein-calorie malnutrition and bone loss despite calcium and vitamin D supplementation are among other, the most important dysfunctions associated to the treatment[18–21]. In our case a slight, non-significant reduction of creatinine and total proteins were noted post-surgery; these deviations have been reported in the literature[22]. We also observed vitamin B12 deficiency, probably due to malabsorption poorly managed with the standard replacement therapy that needs to be revised. Therefore, the patients of our cohort showed the positive global response that was expected one year after surgery.

The results of the analysis carried out during the 6 months follow-up after RYGB[23] showed significant decrease in the levels of ghrelin. This decrease may be attributed to the resection of the gastric fundus during the operation, as this is responsible for ghrelin

secretion. It has been argued that ghrelin suppression is a key factor in the complex mechanism of weight loss after gastric bypass. According to Geloneze et al[24], the location of the gastric fundus resection line determines the decrease in the amount of postsurgery ghrelin and therefore the suppression of appetite. This rapid decrease in ghrelin after bariatric surgery has been reported to occur three months after surgery[25]. However, it is accompanied by a doubling of the increase at twelve months, in cases where no resection of the gastric fundus is performed, which is in line with our results.

The main novelty of our work is related to the study of CART and BDNF serum levels, a question insufficiently addressed up to the date in morbid obesity. CART seems to be a prominent mediator of eating behavior and body weight regulation in mammals: the peptide (and its mRNA) has been detected in several brain regions and peripheral tissues involved in regulation of feeding, where it has been suggested to play an inhibitory role on eating behavior[26]. Animal studies also showed that CART expression is regulated by both leptin and glucocorticoids, hormones associated with the regulation of body weight, and that CART gene mutations are associated with obesity in humans[26]. We have found that morbid obese patients showed an evolution of serum CART levels which ran parallel to that of insulin and leptin, therefore suggesting that this neuropeptide may be an additional biomarker of response to therapy that possibly quantifies the postsurgical normalization of the desire to eat that has been previously reported[27].

In the case of BDNF, a peptide that has been repeatedly associated to obesity[28,29], we did not observe significant differences in the serum concentration of our pre-surgery patients with respect to control subjects, a result that tends to confirm data from other studies that found no significant changes in serum BDNF associated with obesity in adults[13]. The reason for these apparent discrepancies may reside in a differential contribution of a variety of BDNF modulators from one study to the other. In fact, blood levels of BDNF

may be affected by several diseases (i.e. multiple sclerosis, rheumatoid arthritis, chronic kidney disease, Parkinson's disease, Alzheimer's disease or epilepsy)[30,31]; medications (i.e. clopidogrel, aspirin, anti-TNF alpha drugs, antidepressants or statins)[32–34]; dietary supplements (i.e. zinc, vitamin B, E, A, omega-3 fatty acids or folic acid)[32,35,36] and even physiological conditions such as the phase of the menstrual cycle[37]. Interestingly, we observed a significant reduction of BDNF levels below control values one year after surgery. To our knowledge, only one pilot study in morbid obese women whose BDNF levels were evaluated 3 months post-surgery already found significant decreases in the concentration of BDNF in all participants[38]. This post-surgical decrease of BDNF could be secondary to changes in any of the modulators previously cited, but may be also related to the emergence of feeding alterations in our patients: in fact, low serum BDNF may reflect a functional dysregulation of the peptide directly related to eating disorders[39]. This possibility highly recommends further monitoring serum BDNF levels in order to find out if they could anticipate post-surgical behavioral complications, i.e. "addiction transfer" from previous compulsive eating to drug and alcohol abuse[40].

By analyzing the hormonal and neuropeptide profile as a whole, the multivariate linear regression model brings forth the possibility of the establishment of a tool to facilitate the prediction of the evolution of surgery. Knowing the relationship between the variations in these parameters and its effect in the excessive BMI loss, some light may shed on how the treatments of this disease progress. Therefore, more groundbreaking studies will be necessary to consolidate these biomarkers as a way of measuring the patients' evolution.

LIMITATIONS OF THE STUDY

The follow-up time period was one year and some patient losses were suffered due to patients not attending their review appointment or choosing not to participate in the study. The low sample number may compromise extrapolation to a large population of individuals. However, the significance values, the level of statistical robustness and the percentage of variability explained by the presented linear regression analysis justify the results obtained. Further assessments of CART and BDNF as biomarkers of surgery outcome should include the collection of several samples to determine the intraday variability of the assays used. Finally, physical activity and a formal assessment of dietary intake would have been useful in explaining some of the outcomes observed.

CONCLUSIONS

The results suggest that CART and BDNF could be envisaged as new candidate biomarkers of surgery outcome.

STATEMENT OF INTEREST

None to declare.

FIGURE LEGENDS

Figure 1. Flow diagram of the study**.**

Figure 2. BMI boxplot of controls and patients pre- and one-year post-surgery. Data are expressed as mean ± SEM from at least 27 determinations. Outliers are represented by dots. *** $p<0.001$.

Figure 3. Hormone and neuropeptide boxplots of controls and patients pre- and one-year post-surgery. Data are expressed as mean ± SEM from at least 27 determinations. Outliers are represented by dots. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NS: Non-significant.

TABLE LEGENDS

Table 1. Bivariate analysis of analytical parameters. Non-paired t-Test for normal distribution and Mann-Whitney's test for non-normal distribution in Presugery vs Control and Postsurgery vs Control comparison. Paired t-Test for normal distribution and Wilcoxon's test for non-normal distribution in Postsurgery vs Presurgery comparison. Data are expressed as means ± SEM, 95% CI, from at least 27 determinations. *BMI: Body Mass Index; GGT: Gamma-glutamyltransferase; AST: Aspartate transaminase; ALT: Alanine transaminase; PCR: C-reactive protein; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; HOMA: Homeostatic model assessment; IR: Insulin Resistance; S: Sensibility; BDNF: Brain-derived neurotrophic factor; CART: Cocaine and amphetamine regulated transcript.*

Table 2. Multivariate linear regression analyses to identify hormonal and neuropeptidic profiles predictive of %EBL. Data are given as regression coefficients (B) and 95% confidence limits. Adjusted R-squared = 0.820 (Coefficient of determination, 82.0% of variability explained by the model).

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