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ORIGINAL PAPER

Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not

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Abstract Saliva is a non-invasive source of biomarkers useful in the study of physiological mechanisms. Moreover, this fluid has diverse functions, among which food perception and ingestion, making it particularly suitable for the study of obesity. The aims of this study were to assess changes in salivary proteome among morbidly obese women, with a view to provide information about mechanisms potentially related to the development of obesity, and to evaluate whether these changes persist after weight loss. Mixed saliva samples from morbidly obese women (N=18) who had been either subjected (group O-BS) or not (group O) to bariatric surgery and women with normal weight (N=14; group C) were compared for protein profiles, alpha-amylase abundance and enzymatic activity, and carbonic anhydrase (CA) VI abundance. Differences in salivary obese profiles were observed for 23 different spots. Zinc-alpha-2 glycoprotein-containing spots showed higher abundance in group O only, whereas cystatin S-containing spots presented higher abundance in the two groups of obese subjects. Most of the spots identified as salivary amylase were present at lower levels in group O-BS. With regard to the amylase enzymatic activity, increases were observed for group O and decreases for group O-BS. One interesting finding was the high correlation between levels of CA VI and body mass index in group O, which was not observed for groups O-BS or C. The differences between groups, mainly regarding salivary proteins involved in taste sensitivity and metabolism, point to the potential of using saliva in the study of obesity development.

Keywords Alpha-amylase · Bariatric surgery · Carbonic anhydrase VI · Obesity · Salivary proteome

E. Lamy (✉) · L. Rodrigues · A. R. Costa · C. Antunes
ICAAM (Instituto de Ciências Agrárias e Ambientais Mediterrânicas), Universidade de Évora, Apartado 94, 7002-554 Évora, Portugal
e-mail: ecsl@uevora.pt

C. Simões · A. R. Costa · C. Antunes
Departamento de Química, Universidade de Évora, Évora, Portugal

R. Vitorino · F. Amado
QOPNA (Química Orgânica Produtos Naturais e Agroalimentares), Universidade de Aveiro, Aveiro, Portugal

R. Vitorino
iBiMED, Institute for Biomedical Research, University of Aveiro, Aveiro, Portugal

C. Antunes
CNC (Centro de Neurociências e Biologia Celular), Universidade de Coimbra, Coimbra, Portugal

I. do Carmo
Faculdade de Medicina de Lisboa, Universidade de Lisboa, Lisbon, Portugal

Introduction

Obesity is regarded as having achieved epidemic proportions in both developed and developing countries, having major negative economic and personal impacts. Despite considerable investment in research, the prevalence of obesity has not decreased significantly,

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suggesting the lack of effectiveness of the methods currently used in its treatment and prevention [11]. The need for a better understanding of the pathophysiological mechanisms involved in the development of obesity is evident.

Saliva was first used as a non-invasive alternative to blood, aiding in the diagnosis of different pathophysiological conditions and providing an improved understanding of them. Besides comprising secretions from three pairs of major salivary glands and numerous minor glands, this fluid is also composed of substances derived from the blood which enter it intracellularly or through ultrafiltration at tight junctions between cells.

Concerning obesity, saliva composition has not been extensively studied. Nevertheless, recent studies reported the use of saliva for quantification of hormones as well as for the search of biomarkers of inflammation and insulin resistance [9] or of lipid metabolism [44].

Besides this potential of saliva as a source of biomarkers, an additional aspect that makes saliva particularly useful for the study of obesity is its role in oral food perception and ingestion. Poor dietary habits are one of the main factors suggested as contributing to the development of obesity, with high-calorie and palatable foods usually consumed rather than bitter-tasting and fibrous vegetables [13]. Since dietary choices depend to a large degree on oral perception, fluctuations in the factors that influence taste and other oral sensations can play an important role in weight gain. Changes in taste sensitivity have been referred to in studies of obesity, especially regarding sweetness and bitterness. Although there is some controversy as to whether these are perceived with less or equal intensity by individuals with high body mass index (BMI) [12], most studies report that obese individuals present higher liking ratings for sweetness [40] and more pronounced aversive reactions to bitterness [17] than normal-weight subjects.

Saliva composition has an influence on sensitivity to tastes and other oral sensations [38]. Salivary carbonic anhydrase VI (CA VI) has been suggested as being involved in bitter taste detection. Polymorphisms in the gene coding for this salivary protein appear to contribute towards explaining some of the variation in sensitivity to the bitter compound 6-*n*-propylthiouracil (PROP) [33]. Moreover, support for the role of saliva in taste perception has been provided by a number of studies reporting salivary proteins as being linked to sensitivity to PROP bitterness [6, 27], to the bitter compound caffeine [15], and even to the perception of fat [32]. For example, cystatins and zinc-alpha-2 glycoprotein have been observed to be present in higher or lower amounts, respectively, in saliva from children presenting higher acceptance for bitter taste. The exact effect of these proteins in taste perception is not known. Whereas zinc-alpha-2 glycoprotein is a protein with different functions, among which is initiation of lipid mobilisation [18], cystatins are cysteine protease inhibitors. These last are involved in the control of proteolysis within the oral cavity, and, for that reason, it was hypothesised that they could affect the accessibility of tastant molecules to taste receptors, influencing taste sensitivity [15]. Another salivary protein potentially involved in ingestion is alpha-amylase. Although a link between this protein and taste perception has not been reported, it is involved in carbohydrate digestion and absorption, having also a role in the perception of starchy foods [25]. The potential interest in examining alpha-amylase in the study of obesity is increased by the known relation between this protein's concentration levels and the activity of the sympathetic nervous system [31], which is involved in energy balance and implicated in the development and maintenance of obesity [42]. Despite what has been stated above, a limited number of studies on the biochemical characteristics of saliva in obese individuals have been carried out. Furthermore, most research has focused on parameters such as flow rate, electrolytes, and total protein amounts, mainly in the context of the development of dental caries (e.g. [28]). The aims of this study were to identify putative changes in salivary protein profiles in morbidly obese women and to evaluate the effect of weight loss on these profiles.

Methods and procedures

Study population and whole-saliva collection

The study population consisted of 18 women volunteer patients aged 22 to 56 years from the Department of Endocrinology, Diabetes and Metabolism of Santa Maria Hospital (HSM) in Lisbon, Portugal. Two groups of obese individuals were formed, one composed of morbidly obese individuals (group O, $N=11$; mean BMI 44.8 kg/m^2) and the other comprising obese individuals who had previously been subjected to bariatric surgery (gastric sleeve), having subsequently experienced body weight loss in the range 30–50 kg (group

O-BS, N=7; mean BMI 34.9 kg/m^2). Healthy women volunteers of normal weight in the same age range made up a control group (group C, N=14; mean BMI 21.1 kg/m^2). The study was approved by the local ethics committee and all participating individuals gave their written informed consent.

Unstimulated whole saliva was collected from 3.00 p.m. to 5.00 p.m. by direct draining into an ice-cold collection tube. Subjects refrained from eating and drinking for at least 1 h before collection. Samples were divided into different aliquots and stored at −20 °C.

Preceding analysis, saliva samples were thawed on ice and centrifuged for 30 min, 4 °C, 13,000*g*, for removal of cells and mucinous material. Supernatant total protein concentration was assayed using the Bradford method (Bio-Rad Protein Assay).

Salivary protein profile

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles were obtained for all individuals in 12 % polyacrylamide gels (80×100×0.75 mm) (Protean xi, Bio-Rad). The protein load per sample was 7 μg and each individual sample was run in triplicate. An electrophoretic run was performed at a constant voltage of 150 V. Gels were fixed for 1 h in 40 % methanol/10 % acetic acid, followed by staining for 2 h with Coomassie Brilliant Blue (CBB) G-250. Gel images were acquired using a scanning Molecular Dynamics densitometer with internal calibration and LabScan software (GE Healthcare), and images were analysed using GelAnalyzer software (GelAnalyzer 2010a by Istvan Lazar, www.gelanalyzer.com). Molecular masses were determined in accordance with molecular mass standards run with protein samples.

Given the small size of samples taken from individuals, and in order to reduce the influence of biological variation, the saliva of five individuals from each experimental group was pooled for 2D gel electrophoresis analysis. Pools were made up in such a way that each individual contributed the same amount of protein for the required total. Each pool was run in duplicate. In order to assess inter-individual variability within each group and confirm the results of the comparison of pools, individual gels from three different participants from each experimental group were run. The protein load was 160 μg both for pools and individual gels.

Saliva samples were desalted and concentrated using 3-kDa cut-off ultra-filtration microfuge tubes (Nanosep 3K omega, PALL Corporation) and then mixed with rehydration buffer {7 M urea, 2 M thiourea, 4 % (*w*/*v*) CHAPS [3-(3-cholamidopropyl dimethylammonio)-1 propanesulfonate], 2 % (*v*/*v*) IPG buffer, 60 mM dithiothreitol (DTT), and bromophenol blue 0.002 % (*w*/*v*)} to a final volume of 250 μL and loaded onto 13-cm pH 3–10 NL IPG strips (GE Healthcare) by passive in-gel rehydration overnight in a Multiphor Reswelling Tray (GE Healthcare). Strips were focused for 30 kVh at 18 °C, using a Multiphor II isoelectric focusing system (GE Healthcare). Following focusing, proteins in the IPG strips were reduced by soaking in 1 % (*w*/*v*) DTT; 50 mM Tris–HCl, pH 8.8; 6 M urea; 30 % (*v*/*v*) glycerol; and 2 % (*w*/*v*) SDS, and then alkylated with 65 mM iodoacetamide; 50 mM Tris–HCl, pH 8.8; 6 M urea; 30 % (*v*/*v*) glycerol; and 2 % (*w*/*v*) SDS. The equilibrated strips were then horizontally applied on the top of a 12 % SDS-PAGE gel (1×160×160 mm), and proteins were separated vertically at 18 °C, using a Protean II xi cell (Bio-R'ad) at a constant voltage of 150 V until the end of the run. Gels were stained with 0.1 % CBB-R250 (in 40 % methanol, 10 % acetic acid) and de-stained with 10 % acetic acid.

Digital images of the 2-DE gels were acquired using the same procedure as that described for SDS-PAGE image acquisition. Gel analysis was performed using Image Master Platinum v.7 software (GE Healthcare), with automatic spot detection, followed by manual editing for spot splitting and noise removal. A total of 15 gels were analysed (2 pool and 3 individual gels from each group), and the volume of each spot was normalised using relative spot volumes (% vol). In order to calculate fold change, the spot volume of obese subjects was divided by the spot volume of healthy subjects. Only protein spots that changed ≥1.5-fold and consistently changed in the same manner for individual gels were considered to be differently expressed. These spots were then removed and analysed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/TOF mass spectrometry (MS).

Protein identification

Tryptic digestion

Spots of interest were manually excised from gels, washed three times in 25 mM ammonium bicarbonate/50 % ACN, washed once in ACN, and dried in a

SpeedVac (Thermo Savant). Twenty-five microlitres of 10 μg/mL sequence-grade modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate was added to the dried gel pieces, and the samples were incubated overnight at 37 °C. Extraction of tryptic peptides was performed by means of the addition of 10 % formic acid (FA)/50 % ACN being dried in a SpeedVac (Thermo Savant).

Mass spectrometry

Tryptic peptides were re-suspended in 10 μL of a 50 % acetonitrile/0.1 % formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid prepared in 50 % acetonitrile/0.1 % formic acid. Aliquots of samples (0.5 μL) were spotted onto the MALDI sample target plate.

Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range from 800 to 4500 Da with ca. 1500 laser shots. For each sample spot, a data-dependent acquisition method was created in order to select the six most intense peaks, excluding those of the matrix, trypsin autolysis, and acrylamide peaks, for subsequent MS/MS data acquisition. Mass spectra were internally calibrated with autodigest peaks of trypsin (MH+: 842.5, 2211.42 Da) allowing for a mass accuracy of approximately 25 ppm.

Database search

Spectra were processed and analysed using a Global Protein Server Workstation (Applied Biosystems), using internal MASCOT software (v2.1.0, Matrix Science, London, UK) for searching the peptide mass fingerprints and MS/MS data. The Swiss-Prot non-redundant protein sequence database (March 2013) was used for all searches under *Homo sapiens*. Database search parameters were as follows: carbamidomethylation and propionamide of cysteine as a variable modification as well as oxidation of methionine, and the allowance for up to two missed tryptic cleavages. Peptide mass tolerance was 25 ppm and fragment ion mass tolerance was 0.3 Da. Positive identifications were accepted to a confidence level of 95 %. Criteria for positive identification were a significant MASCOT probability score (score >50; $p<0.05$).

Western blotting

Western blotting was used to verify the hypothesis evidenced by SDS-PAGE and 2D gels for changes in the abundance of CA VI and alpha-amylase. Protein load was adjusted to 3 μg per lane, and electrophoresis was carried out in 12 % polyacrylamide gels, using the protocol described above. Proteins were subsequently transferred to PVDF membranes at 350 mA constant current for 1 h. Membranes were stained with Ponceau S (0.2 % (*w*/*v*); 3 % (*v*/*v*) acetic acid) for the control of suitable protein load and transfer. After a blocking step in 5 % (*w*/*v*) powdered skimmed milk in TBS–Tween for 2 h, membranes were incubated with primary antibodies (Santa Cruz sc-46657 and sc-99173; 1:200 dilutions) overnight (4 °C). Salivary alpha-amylase and CA VI bands were detected using an alkaline phosphatase-linked secondary antibody against mouse IgG and rabbit IgG (GE Healthcare, 1:10,000 dilution), respectively, and a chemifluorescent substrate (ECF Plus Western Blotting Detection Reagents, GE Healthcare). Membranes were revealed in a transilluminator (Gel-doc system, Bio-Rad), and a semi-quantitative analysis of band expression was carried out using Quantity One software (Bio-Rad).

Salivary alpha-amylase enzymatic activity

Alpha-amylase enzymatic activity was determined using a colorimetric commercial kit (Liquid amylase, Sentinel Diagnostics), based on the catalysis of the hydrolysis of *p*-nitrophenyl-maltoheptaoside-4,6-ethylidene blocked by alpha-amylase with the formation of products which were subsequently hydrolysed into *p*-nitrophenol (PNP) and glucose. The increase in absorbance due to PNP formation was proportional to the activity of alpha-amylase in the sample examined. The protocol was performed in 96-well plates, in accordance with the manufacturer's instructions. For the assay, dilutions of 100, 200, 300, 400, and 500× from saliva samples were used. Absorbance at 405 nm was read each minute for 15 min. Linearity was observed in the interval between 5 and 10 min. Enzyme activity was expressed as the number of moles of PNP formed per minute, per milligram of protein (U/mg) or per liter of saliva (U/L).

Statistical analysis

All data were analysed using descriptive statistics, and normality and homoscedasticity were evaluated using

the Kolmogorov–Smirnov and Levene tests, respectively. To assess differences between treatments for parameters such as SDS-PAGE profiles, Western blot protein band expression, and alpha-amylase enzymatic activity, one-way ANOVA was performed. Means were compared using Duncan's test. Salivary parameters were related to BMIs by means of the Pearson correlation. Statistical significance was considered for $p<0.05$. All statistical analysis procedures were performed using the SPSS 21.0 software package (SPSS Inc., Chicago, USA).

Results

Characterisation of participants

The anthropometric characteristics of the women participating in the study are presented in Table 1. Differences in BMI among groups are clear. Although women from group O presented some weight loss since the start of their visits to the Department of Endocrinology, Diabetes and Metabolism of HSM in Lisbon, they were considerably lower than the ones derived from bariatric surgery experienced by women from group O-BS.

Total protein concentration

Total protein concentration in saliva was found to differ between groups. Obese individuals who had not been subjected to bariatric surgery (O) presented significantly higher values than those belonging to the two other groups, and the mean value for these individuals was almost twice as high as that of members of the control group (C) (850.3±99.0 vs. 483.1±44.7 μg/mL, respectively; $p=0.004$). The salivary protein concentration of obese individuals who had been subjected to bariatric surgery (O-BS) did not differ from that of the group of non-obese subjects (520.3±54.2 μg/mL in O-BS saliva; $p=0.372$).

Salivary protein profile

Salivary protein profiles observed after SDS-PAGE separation presented differences with regard to three protein bands. One protein band, with 92 kDa, was observed in higher abundance in group O (11.36±1.79 % vol) comparatively to group O-BS (8.42±3.2 % vol), although these groups did not significantly differ from normal-weight individuals. Protein bands with 57 and 42 kDa presented lower abundance in O-BS individuals as compared with the two other groups. [For the band with 57 kDa, 9.1±2.9 (O-BS) vs. 11.5±2.6 (O) and 10.0±3.7 % vol (C); p values, 0.048 for O-BS vs. O and 0.009 for O-BS vs. C. For the band with 42 kDa, 6.7±2.9 (O-BS) vs. 12.8±3.0 (O) and 9.5±2.7 % vol (C); p values, 0.024 for O-BS vs. O and 0.047 for O-BS vs. C.] One protein band with approximately 25 kDa presented higher abundance for group O only (11.6±2.2 for O vs. 9.2±1.8 and 10.0±2.2 % vol for groups O-BS and C, respectively; $p=0.005$ O-BS vs. O and $p=0.049$ O vs. C). In the region of 16 kDa, a tendency for higher protein expression in group O-BS (9.2±1.8 % vol) as compared with group C (7.6±2.0 % vol) was also observed, although these last differences were not statistically significant ($p=0.07$) and some intra-group variability was observed. This may be associated with the location of this band: in the vicinity of the lower end of the gel.

Table 1 Anthropometric characteristics of subjects

	C ($N=14$)		O ($N=11$)		O-BS ($N=7$)		p value
	Mean	SD	Mean	SD	Mean	SD	
Age	36.1	3.1	39.7	3.7	44.1	4.2	
Weight (kg)	57.5	2.5	114.9	5.6	94.2	7.5	$^{\S}0.0000$ $^{\#}0.0020$
Height (m)	1.66	0.02	1.60	0.02	1.64	0.03	
BMI (kg/m^2)	21.1	0.5	44.8	1.8	34.9	2.1	$^{\S}0.0000$ $^{\#}0.0003$
Weight loss (kg)	0.0	0.0	6.1	2.5	42.0	3.8	$^{\S}0.0000$ $^{\#}0.0000$

SD standard deviation

p value <0.05, statistically different group from the control group: § O and $^{\#}$ O-BS as compared with group C, respectively

A total of 91 and 101 protein spots were observed for 2-DE salivary profiles of pooled saliva samples from group C and the two groups of obese individuals, respectively. Of these, the % vol of 23 spots differed by more than twofold: for 19 spots, differences were observed between group C and the two groups of obese individuals (O and O-BS); for 2 spots, differences were observed only between the control group and group O; and for the other 2 spots, differences were observed only between the control group and group O-BS (Fig. 1).

The protein spots identified by means of mass spectrometry are listed in Table 2. Some differences in protein spot levels, in comparison with the control group, were observed to be specific for each obese group: (i) two different spots, identified as the protein zinc-α-2 glycoprotein (UniProt: P25311), presented higher levels for group O only and (ii) one spot, identified as transmembrane protease serine 13 (UniProt: Q9BYE2), presented increased levels and another one, identified as salivary amylase (UniProt: P04745), presented decreased levels in O-BS individuals only. With the exception of one spot (spot 137 in Fig. 1), all spots whose levels were decreased in obese subjects were identified as salivary amylase (UniProt: P04745). Meanwhile, five spots which were increased for both obese groups were also identified as salivary amylase (UniProt: P04745), but in this case, all with apparent molecular masses which were smaller than the theoretical molecular mass of this protein (three observed with apparent molecular masses of 27 kDa vs. theoretical molecular mass of 58 kDa). The non-identified protein spot 137 was located at the acidic end of the gel, presenting an apparent molecular mass of 25 kDa, and was stained violet with Coomassie R-250 after being de-stained with acetic acid only (not shown). This suggests the possibility of this being an acidic proline-rich protein [2]. Finally, one spot (108), observed to be increased in both groups of obese subjects, was identified as cystatin S (UniProt: P01036). The remaining unidentified spots may have either contained insufficient amounts of protein or belonged to proteins with fragmentation patterns which were unfavourable for reliable MS/MS sequencing.

Alpha-amylase abundance and enzymatic activity

Alpha-amylase was identified by means of Western blotting for two bands of 63 and 57 kDa (Fig. 2).

Differences were observed for the abundance of this protein, and the individuals from group O-BS presented lower levels.

The enzymatic activity of salivary alpha-amylase expressed per volume of saliva (U/L) was significantly increased in group O, presenting twice the level of activity of the control group ($245.7\times10^3\pm35.8\times10^3$ vs. $111.2\times10^3\pm43.8\times10^3$ U/L, respectively) (Fig. 3). It is interesting to note that the specific enzymatic activity (U/mg protein) did not present a statistically significant difference, comparatively to the control group (366.9± 82.0 and 198.4±68.6 U/mg, respectively). This is in accordance with protein expression results, obtained after Western blot and SDS-PAGE analysis, where no significant differences were observed between groups O and C. Group O-BS did not differ from normal-weight individuals ($130.0\times10^3\pm36.7\times10^3$ U/L and 242.0±83.0 U/mg).

Changes in CA VI abundance

One band, with an apparent molecular mass of 42 kDa, was identified as CA VI by means of Western blotting. Its expression in O-BS individuals was about half that observed for the control group (Fig. 4). This was in accordance with results obtained on the basis of SDS-PAGE profile analysis, whereas the band with an

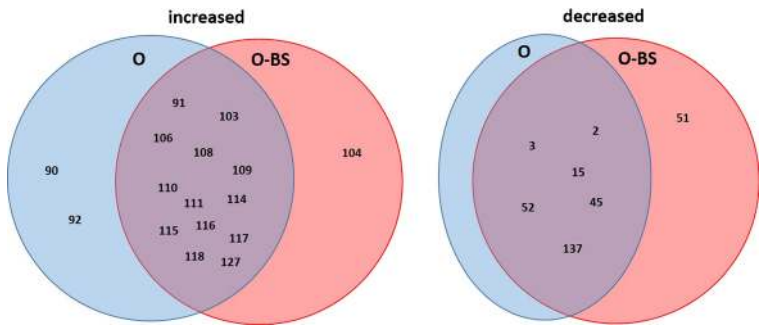

Fig. 1 Venn diagrams of the protein datasets differently expressed from the control group. Two pools for each group, each consisting in saliva from five different individuals, were used to produce the diagram

Table 2 MS identification of differentially expressed salivary proteins

Spot	Protein (UniProt entry reference)	Estimated/ theoretical MW (kDa)	Estimated/ theoretical pI	Id score/ PM	Fold of variation[a] (C vs. O)	Fold of variation[a] (C vs. O-BS)	Protein function/involvement in oral perception
2	Salivary amylase (P04745)	67/58	6.0/6.5	112/5	2.5	2.1	- Endohydrolysis of (1->4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1->4)-alpha-linked D-glucose units - Possible involvement in oral starch perception [25]
3		57/58	6.0/6.5	275/9	3.0	3.9	
15		67/58	6.7/6.5	113/4	5.7	18.8	
45		57/58	6.7/6.5	149/8	3.4	4.4	
51		67/58	5.5/6.5	108/6	–[b]	2.6	
52		57/58	5.5/6.5	80/3	2.0	4.0	
106		52/58	6.7/6.5	91/5	n.m./obs	n.m./obs	
116		27/58	6.5/6.5	103/4	n.m./obs	n.m./obs	
117		27/58	6.7/6.5	57/3	n.m./obs	n.m./obs	
118		27/58	7.7/6.5	80/3	n.m./obs	n.m./obs	
127		52/58	6.0/6.5	88/3	n.m./ct	n.m./ct	
90	Zinc-α-2-glycoprotein (P25311)	45/34	5.2/5.7	77/3	–[b]	n.m./obs	- Stimulates lipid degradation in adipocytes and causes extensive fat losses associated with some advanced cancers. May bind polyunsaturated fatty acids - Possible association to bitter taste sensitivity [29]
92		45/34	5.3/5.7	67/2	n.m./obs	n.m./obs	
104	Transmembrane protease serine 13 (Q9BYE2)	18/63	4.9/9.0	58/2	n.m./obs		- Serine protease activity, known to function in development, homeostasis, infection, and tumorigenesis
108	Cystatin S (P01036)	15/16	5.0/5.0	254/6	n.m./obs	n.m./obs	- Strongly inhibits cysteine proteases such as papain and ficin, among others - Potentially related to bitter taste sensitivity [14]

MW molecular weight, *C* control group, *O* obese group, *O-BS* obese subjected to bariatric surgery group, *PM* number of peptides matched, *n.m.* fold of variation not measureable because protein spot was only observed in the gels from one of the obese groups (*obs*) or only in gels from the control group (*ct*)

[a] Values represent the ratio of spot percentage volumes between the control and each group of obese individuals

[b] No differences relative to group C

apparent molecular mass of 42 kDa presented a decreased expression in this group, as stated before.

The abundance of CA VI was strongly correlated with BMI in obese individuals (r=0.701; p=0.001). This was particularly evident in the case of group O individuals (r= 0.67; p=0.018), with no correlation observed when considering only individuals from group O-BS. This suggests that, without intervention aimed at producing weight loss, there was a tendency for individuals with a higher BMI to present higher CA VI levels. On the other hand, for individuals with normal weight (the control group), the expression of this salivary protein did not correlate with BMI.

Discussion

During the last 20 years, the salivary proteome has been studied with regard to different pathologies, due to the potential of saliva as a non-invasive source of biomarkers of diseases and/or physiological conditions [7]. Although the knowledge of variations in salivary protein composition may have the potential for elucidating the physiological mechanisms involved in the development of obesity, or even for making inferences about individuals' susceptibility to this condition, few studies have been carried out on this topic. We recently

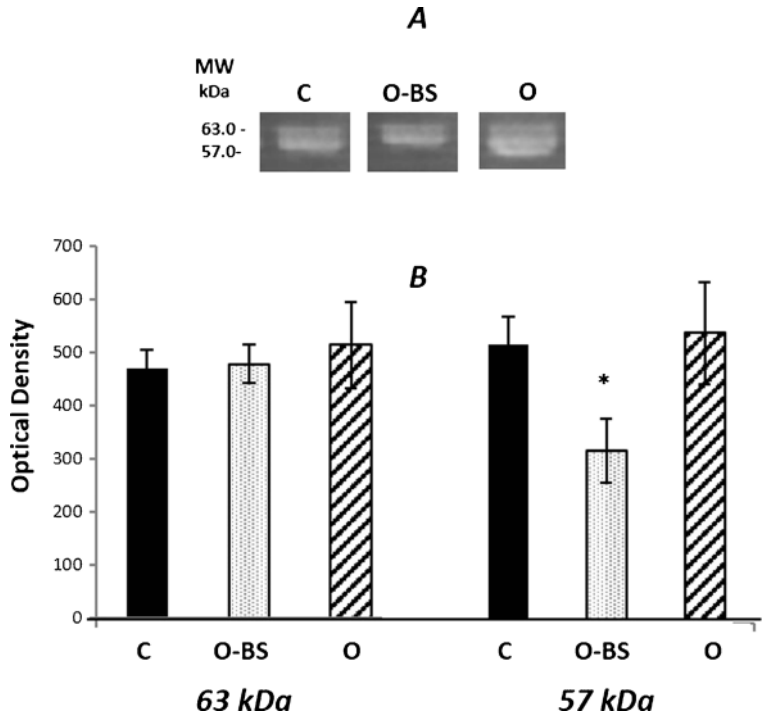

Fig. 2 Representative Western blot analysis of alpha-amylase in mixed saliva samples from the control group (*C*), the group of morbidly obese women (*O*), and the group of morbidly obese women who had been subjected to bariatric surgery (*O-BS*) (**a**). Optical density was obtained using densitometric analysis. These values comprehend background correction, which was applied for each band by normalising to an adjacent region of the membrane (**b**). Data are presented as mean±SEM (*$p<0.05$, statistically significant differences)

observed that young rats, prone to obesity, presented higher salivary alpha-amylase levels than animals resistant to the development of obesity, suggesting the possible use of saliva in signalling individuals susceptible to weight gain [37].

Because saliva composition is influenced by gender [21], only women were selected as subjects for the present study. A higher total protein concentration was observed in the saliva of obese individuals who had not been subjected to bariatric surgery, in line with reports on obese children and adolescents [34], and men [36]. Interestingly, women who had been subjected to bariatric surgery did not present increased levels of total salivary proteins, as compared to the control group, suggesting that weight loss may reverse such salivary change.

One major finding of the present study is the observation of differences in obese salivary protein profiles, as compared with those of individuals with normal weight, and some differences resulting from the existence or absence of weight loss induced by bariatric surgery.

Salivary alpha-amylase abundance was significantly reduced in the group of individuals who had been

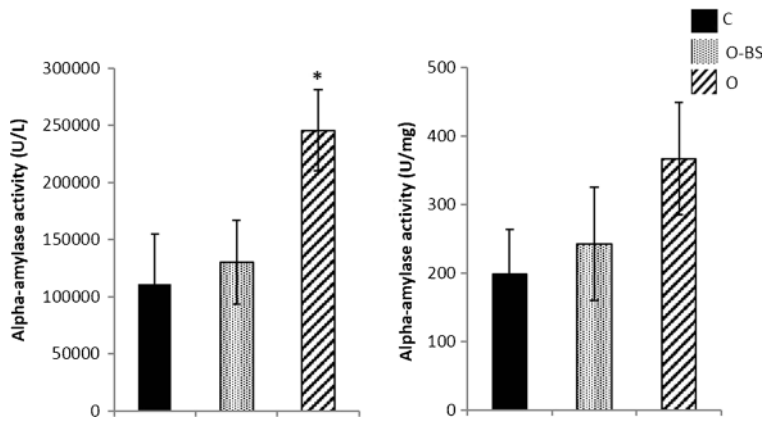

Fig. 3 Salivary alpha-amylase activities (U/L and U/mg) of mixed saliva samples from the control group (*C*), the group of morbidly obese women who had been subjected to bariatric surgery (*O-BS*), and the group of morbidly obese women (*O*). Data are presented as mean±SEM (*$p<0.05$, statistically significant differences)

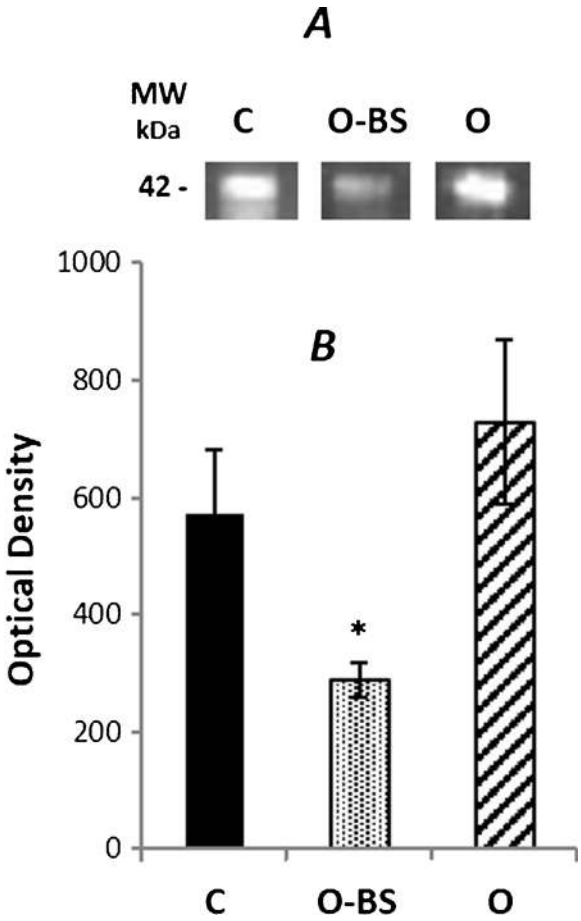

Fig. 4 Representative Western blot analysis of carbonic anhydrase VI in mixed saliva samples from the control group (*C*), the group of morbidly obese women who had been subjected to bariatric surgery (*O-BS*), and the group of morbidly obese women (*O*) (**a**). Signal volumes adjusted (optical density) were obtained using densitometric analysis. These values comprehend background correction, which was applied for each band by normalising to an adjacent region of the membrane (**b**). Data are presented as mean±SEM (*$p<0.05$, statistically significant differences)

subjected to bariatric surgery, observed in both SDS-PAGE profiles and Western blotting. Moreover, six of the spots from 2-DE profiles, identified as salivary amylase, also presented lower abundance for this group. Furthermore, the enzymatic activity was also significantly decreased as compared with group O. Studies carried out on diabetic individuals have suggested that alpha-amylase levels may be associated with insulin secretion [22], and there are reports of higher levels of amylase in individuals with type 2 diabetes (associated with hyperinsulinaemia) (e.g. [24]) and lower levels in type 1 diabetics (e.g. [23]). However, no information about insulin levels was obtained with regard to the subjects in the present study, almost all of those in group O reporting as being diabetics. Moreover, it is known that obesity is generally associated with hyperinsulinaemia, in the presence or absence of type 2 diabetes. In turn, sleeve gastrectomy has been reported to reverse metabolic changes, including lowering insulin levels in individuals who are fasting [8].

Salivary alpha-amylase abundance has also been suggested as a marker of stress and sympathetic nervous system activity [31]. Some studies suggest that obesity is associated with increases [20], whereas weight loss is related to decreases in sympathetic nervous system activity [42]. Thus, the high levels of this protein in group O and the low levels of the protein in group O-BS may partly reflect the activity of this branch of the autonomic nervous system. An interesting point to note is the different changes in the several spots identified as salivary alpha-amylase. Increases in salivary amylase spots with apparent molecular masses smaller than the native salivary amylase molecular mass (57 kDa) were observed in the two groups of obese individuals. Low molecular mass salivary amylase isoforms have previously been reported, although their biological role is yet to be investigated [19]. Consequently, a better knowledge of each isoform and its involvement in the development of obesity and/or weight loss may be useful.

Additionally, changes in salivary amylase levels in obese individuals may be of relevance due to the involvement of this protein in food ingestion and digestion. Despite a direct relationship between this salivary protein and taste not having been reported, a recent study described differences between individuals with variable levels of sensitivity to oleic acid, with one protein spot being identified as salivary amylase [30]. This protein produces changes in the viscosity perception of starchy foods [25] resulting in increases in the levels of the sweet molecules maltose and glucose in the mouth [39]. We therefore put forward the hypothesis that during the consumption of starch-containing foods, lower levels and lower enzymatic activities of this salivary protein in individuals who have been subjected to bariatric surgery may result in a lower concentration of sweet compounds in the mouth as compared with group O individuals. This in turn may result in different levels of sweetness perception, since changes in taste sensitivity due to the continuous stimulation of taste receptors by salivary constituents have already been suggested [26]. Although this is only a hypothesis, since no sensory evaluation was

performed with regard to the subjects in this study, the high enzymatic activity observed in group O may provide support for the studies reporting low levels of sweet taste sensitivity combined with strong preferences for sweet foods in obese individuals [1]. Furthermore, the small quantities of this enzyme present following bariatric surgery may be related to potential recovery of sweet taste sensitivity which had already been reported for these individuals [4].

Concerning the higher abundance of the spot identified as transmembrane protease serine in group O-BS as compared with the other two groups, no obvious conclusions can be drawn. This protease has been identified as a constituent of human saliva which has a role to play in histatin proteolysis [43]. Although it is associated with the human phenotype linked to starvation [3], its role in the composition of saliva is not well understood. Zinc-α-2 glycoprotein presented higher values only in obese individuals who had not been subjected to bariatric surgery. In another study, higher serum levels of this protein were found for obese individuals than for those with normal weight [45]. Consequently, the increased expression of this protein in the saliva of obese individuals, observed in our study, may reflect higher levels of the protein in the blood. Since changes in levels of this protein have been recently reported according to bitter taste acceptance in children [29], it is possible that differences between obese and normal-weight individuals are related to differences in oral food perception. Although identification by means of MS failed for one protein spot, observed as presenting decreased levels in the two groups of obese individuals, this spot was stained pink, suggesting that it may be a proline-rich protein (PRP). Despite the different functions attributed to these proteins, including oral protection, a significant role in the oral perception of astringency has been suggested for them (e.g. [10]) and one study has reported a positive relationship between levels of these proteins and bitter taste sensitivity [6]. Other salivary proteins potentially associated with taste sensitivity were observed to be differently expressed in obese individuals who had been subjected to bariatric surgery: one cystatin S spot was present at higher levels, whereas CA VI protein abundance was decreased. Meanwhile, cystatin S levels were observed to be increased in individuals with low bitter taste sensitivity, in both men [15] and women (Rodrigues et al., not published). Moreover, levels of salivary CA VI have been suggested as being positively associated with bitter taste perception [6], although there is some controversy regarding this issue [16, 35]. There are conflicting results regarding the effect of bariatric surgery on bitter taste perception (some authors report changes [41], while others do not [5]). However, if it is assumed that the reports of studies relating PRPs, cystatin S, and CA VI abundance with bitter taste sensitivity are correct, this surgical procedure may be suggested as being related to a decrease in bitter taste sensitivity. Similarly, the tendency for the higher protein abundance of CA VI in individuals with a higher BMI, observed only for group O-BS, may also be related to higher levels of bitter taste sensitivity. This matter requires further investigation since, if it is established that this is correct, this may be of significance in terms of the acceptance of healthy foods (e.g. vegetables), which are usually associated with increased bitter taste.

In conclusion, the results of this study clearly showed a salivary proteome for obese individuals which differed from that of normal-weight subjects. Moreover, some differences were not observed in the case of those who had been subjected to bariatric surgery. Some proteins presenting different levels may be related to the metabolic changes associated with obesity, whereas others are salivary proteins which have been referred to as being involved in taste perception. The findings of this study point to the possible use of saliva in the study of obesity and suggest a possible relationship between the salivary proteome and taste sensitivity in obese individuals, which can eventually result in different food intakes. Further research should be carried out in order to clarify the relation between the differences observed in this study and possible differences in oral food perception in obese individuals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval The study was approved by the local ethics committee and all participating individuals gave their written informed consent.

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