Relevance of obesity and overweight to salivary and plasma proteomes of human young adults from Brazil

Relevância da obesidade e sobrepeso para os proteomas salivares e plasmáticos de adultos jovens humanos do Brasil

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ABSTRACT

Obesity is a chronic condition related to multiple comorbidities such as hypertension, type 2 diabetes, periodontal and cardiovascular diseases. Obesity can lead to a metabolic change, creating a prolonged and low-intensity inflammatory process. This study aims to analyze the plasma and saliva proteomes of young adults with obesity and overweight comparing to normal weight individuals, to reveal if the rise on body mass influences the proteomic profiles. The reported population consisted of 18 students and/or employees of Rio de Janeiro State, Brazil, aged between 18 and 35 years. Individuals were categorized according to their anthropometric measures in the Normal Weight, Overweight and Obese

groups. Proteomic characterization was assessed by quantitative Mass Spectrometry (LC-ESI Q/TOF). In addition, cytokines were identified by Multiplex analysis. A total of 118 human proteins from saliva and plasma were identified, including 7 that were common between both fluids. The salivary and plasma proteomes seemed to be related to the body mass index, once the three groups showed distinct proteome profiles. Altogether 49 proteins presented different abundances between the obese, overweight, and normal weight individuals. The main functional category modified in both fluids was the immune response. Most of the modified proteins were previously reported as related to inflammatory diseases, such as cardiovascular diseases and Type 2 Diabetes Mellitus, in particular alpha-1 antitrypsin, C3 complement, alpha-1-antichymotrypsin, zinc-alpha2-glycoprotein, apolipoprotein AI and lysozyme, that could be tested to possible use as early biomarkers of obesity comorbidities.

Keywords: salivary proteome, plasma proteome, obesity, overweight, bioindicators.

RESUMO

A obesidade é uma condição crônica e inflamatória relacionada a múltiplas comorbidades como hipertensão, diabetes tipo 2, doenças periodontais e cardiovasculares. Este estudo tem como objetivo analisar os proteomas de plasma e saliva de adultos jovens com obesidade e sobrepeso em comparação com indivíduos com peso normal, para revelar se o aumento da massa corporal influencia os perfis proteômicos. A população relatada foi composta por 18 voluntários com idade entre 18 e 35 anos. A caracterização proteômica foi avaliada por Espectrometria de Massa quantitativa (LC-ESI Q/TOF). Além disso, as citocinas foram identificadas por análise Multiplex. Um total de 118 proteínas humanas da saliva e do plasma foram identificadas, incluindo 7 que eram comuns entre os dois fluidos. Os proteomas salivares e plasmáticos parecem estar relacionados com o índice de massa corporal, uma vez que os três grupos apresentaram perfis proteômicos distintos. Ao todo 49 proteínas apresentaram diferentes abundâncias entre os indivíduos obesos, com sobrepeso e magros. A principal categoria funcional modificada em ambos os fluidos foi a resposta imune. A maioria das proteínas modificadas foram relatadas anteriormente como relacionadas a doenças inflamatórias, como doenças cardiovasculares e Diabetes Mellitus tipo 2, em particular alfa-1 antitripsina, complemento C3, alfa-1-antiquimotripsina, zinco-alfa2-glicoproteína, apolipoproteína AI e lisozima, que poderiam ser testados para possível uso como biomarcadores precoces de comorbidades da obesidade.

Palavras-chave: proteoma salivar, proteoma plasmático, obesidade, sobrepeso, bioindicadores.

1 INTRODUCTION

In 2016, almost 39% of adults worldwide, over 1.9 billion people, were overweight, and 13%, about 650 million, were obese (WHO, 2021). In Brazil, according to Surveillance of Risk Factors and Protection for Chronic Diseases by Telephone Survey (VIGITEL), in 2019 the overweight rate in adults reached 55.4%, with a higher prevalence in men (57.1%) than in women (53.9%). The main consequence of this

situation is the negative effect on health services, due to the well-known comorbidities associated to individuals with overweight or obesity. Among those comorbidities, the most common are cardiovascular diseases (CVD), type 2 diabetes mellitus (DM2) and musculoskeletal disorders (Akil L & Ahmad, 2011).

Overweight and obesity are defined as an abnormal increase or excessive accumulation of adipose tissue (Schecaira *et al*., 2020; WHO, 2021). Adipocytes are responsible for energy storage and also play an endocrine role, through secretion of proteins and peptides. Thus, metabolic alterations are stimulated by the excessive amount of adiposity in overweight and obesity (Phillips & Perry, 2013). Obese individuals present a chronic inflammation characterized by an increase of the immune cell infiltration in the tissue and the production of inflammatory cytokines (Gregor & Hotamisligil., 2011).

The proteome of obese and lean individuals had already been demonstrated to be different in some kinds of fluids and tissues. The adipose tissue proteome of lean and obese mice showed that weight gain leads to a decrease in proteins from the Mitochondrial Oxidative Phosphorylation System machinery, increasing the risk of developing metabolic diseases. Differences in the serum proteome of pre-diabetic, non-diabetic and normal obese patients were also observed, modifying the expression of proteins such as Alpha-1-antitrypsin, Apolipoprotein A-I and haptoglobin (Kim., et al 2019; Schöttl., et al 2020; Kiseleva., et al 2021).

In this study, a quantitative mass spectrometry analysis was performed to picture the impact of weight gain on the salivary and plasma proteomes of Brazilian young adults from the state of Rio de Janeiro. A cross-sectional study was carried out, classifying volunteers into three states, according to BMI: normal weight, overweight and obesity. The comparative analysis allowed a better understanding of the influence of weight gain on salivary and plasma proteome, highlighting proteins that may play a role in the development of obesity comorbidities. Previous studies have reported data on normal weight and overweight proteomes. The aim of our study was to detach overweight individuals from the obesity group, in order to understand possible changes in proteomes that accumulate progressively with weight gain and according to different weight states.

2 MATERIALS & METHODS

2.1 EXPERIMENTAL DESIGN

The study protocol was approved by the Ethic in Human Research Committee of Unigranrio (#2.160.823). All participants gave written informed consent before entering

the study, which was carried out in accordance with the ethical criteria established in the 1964 Declaration of Helsinki and its subsequent amendments. They responded to the anamnesis questionnaire and underwent anthropometric examination, pressure measurement and biochemical laboratory tests.

Eighteen volunteers between 18 and 35 years of age, were categorized according to body mass index (BMI) in NW group (BMI ≥ 18.5 kg/m^2 and ≤ 24.9 kg/m^2), OW group (BMI ≥ 25 kg/m^2 and ≤ 29.9 kg/m^2) and OB group (BMI ≥ 30 kg/m^2). Participants were excluded if they had one of the following conditions: glucose intolerance or DM2; arterial hypertension; hepatopathy; nephropathy; endocrinopathies; neoplasms and CDV, autoimmune, hematological, psychiatric or inflammatory bowel diseases. Other exclusion criteria included gestation and lactation, smoking, use of drugs that interfere with body weight, metabolism of carbohydrates or lipids. These criteria were established to avoid the impact of these disorders on the salivary and plasmatic proteome in an independent nutritional status-associated manner.

2.2 CLINICAL EVALUATION

Clinical examinations were performed by calibrated examiners and include anthropometric examination: BMI, Height, Weight, Waist circumference, Hip circumference and Waist-hip ratio measurement. Volunteers were weighed and measured on the same scale with a stadiometer (Mod. 104A, Balmak). Weight was recorded in kg and height in cm. BMI was calculated by dividing weight (kg) by squared height (m²). Waist circumference, hip circumference and waist-hip ratio were measured with the aid of a clinical measuring tape. Blood pressure was measured by oscillometric method (OMRON). After 5 min of rest, three sequential measurements were performed, with intervals of 3 min, in order to calculate the mean blood pressure levels. Values with differences above 10 mmHg in systolic blood pressure and 5 mmHg in diastolic blood pressure were discarded. Laboratory examination of Total Cholesterol (mg/dl), HDL (mg/dl), Triglycerides (mg/dl), LDL (mg/dl), Glycose (mg/dl), HOMA-IR and Insulin (mcUI/ml) were performed at the clinical analysis laboratory at Unigranrio, Duque de Caxias, Brazil.

2.3 SAMPLE COLLECTION AND PREPARATION

Saliva was collected in the morning, after 12 hours fasting and 1 hour without consuming any liquid. Two milliliters of ultrapure water were used by participants to

rinse the mouth for 5 min and then it was collected using a sterile tube. Each sample was then mixed with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM ethylenediamine tetra acetic acid (EDTA; Sigma-Aldrich). Samples were kept on ice and rapidly centrifuged at 17,146 g for 10 min at 10 ºC, to separates microbial fraction. Supernatants were transferred to 1.5 mL sterile tubes and mixed with 1 mM Protease Inhibitor Cocktail (Sigma-Aldrich) (Gonçalves., et al 2010). Saliva supernatants were submitted to precipitation with 20% Trichloroacetic acid (TCA) for protein extraction, as previously described (Santos., et al 2011). Proteins were recovered by the suspension of precipitated pellets with 300 µL Ammonia Bicarbonate (50 mM). All biological material was stored at -80 ° C.

Blood samples (5 mL) were collected intravenously also after 12 hours fasting and stored in tubes containing EDTA as anticoagulant (Vacuplast EDTA k3). Plasma was separated by centrifugation at 3,1754 rpm for 10 min at 25°C, then transferred to 1.5 mL sterile tubes with 1 mM Protease Inhibitor Cocktail (Sigma-Aldrich) and preserved at -80° C until processing. For protein purification, 50 µL of plasma samples were depleted of albumin by using ProteoPrep Blue Albumin & IgG Depletion columns (Sigma-Aldrich).

2.4 PROTEIN PROCESSING FOR MASS SPECTROMETRY

The concentration of saliva and plasma proteins were measured using the Brarford reagent. Milli-Q water (158 µL) was added to 40 µL of Bradford reagent and 2 µL of sample. Reactions were incubated for 5 min on ELISA microtiter plate (96-wells), and then quantified using a spectrophotometer (Epoch Microplate Spectrophotometer; BioTek, Highland Park, USA) with absorbance of 595 nm. Bovine Serum Albumin (BSA) protein (Biorad Laboratories Inc., Hercules, CA, USA) was used as standard for the calibration curve (Bradford., 1976).

Saliva and plasma proteomes were submitted to trypsin digestion (shotgun method) individually following the methodology of Carnielli et al., 201. After that, the samples were filtered using Oasis HLB 1cc columns (Waters Corporation, Milford, MA, USA) to remove urea from the sample.

2.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS (HPLC-MS)

For this analysis, pools with saliva and plasma samples of individuals with the same group were carried out to reduce the use of the equipment and avoid analytical errors. Then, three pools were prepared with the collected samples from NW, OW and OB groups, to be analyzed together by MS. Six individual samples of tryptic peptides from the same group were combined with equimolar concentrations of the proteins from the samples. For MS analysis, all peptidome pools were analyzed by direct injection in the mass spectrometer for comparative evaluation of the mass profiles of peptides present in plasma and saliva. The pool samples were run once and in sequence for normalization, and then analyzed in three analytical replicates for quantitative evaluation, by using the relative ion count evaluation (Total Ion Account - TIC).

The tryptic peptidomes pools from saliva were analyzed by a nanoUPLC followed by an Electrospray Quadrupole Time-of-flight mass spectrometer (ESI-Q-TOF Synapt HDMS G1; Waters Corporation), in Inmetro, Rio de Janeiro, Brazil. Chromatographic mobile phase A consisted of formic acid 0,1% in water (v/v) and mobile phase B was formic acid 0,1% in acetonitrile (v/v). Sample desalting was performed with a nanoACQUITY UPLC Symmetry C18 Trap Colum (5 µm, 20 mm x 180 µm), using a 5 µL/min flow rate for 3 minutes. Chromatographic separation was achieved with a nanoACQUITY UPLC HSS T3 reversed-phase column (1.8 µm, 100 mm x 100 µm). A constant flow rate of 0.6 µL/min was used and the following linear gradient was applied: 7-40% B from 0-19.9 min, 40-85% B from 19.9-23.9 min, constant 85% B for another 4 min and then the column was re-equilibrated to initial conditions from 27.9-29.9 min and rinsed for another 7 minutes. Mass spectrometry assay was performed using a positive nano electrospray (nanoESI+) ionization source. Acquisition mode was MS^e, with data independent acquisition (DIA) and parallel fragmentation of all parent ions, with alternated acquisition at low and high-energy. At low-energy, continuum spectra from 50 to 2000 Da were acquired with a scan time of 0.8s, source temperature of 80 ºC, desolvation temperature of 100 ºC, 35 V sampling cone, capillary of 3.0 kV and constant collision energy of 6V. At high-energy, all parameters were the same, but collision energy was ramped from 15-55V during the scan time. Mass spectrometer was previously calibrated using a 300 fmol/µL solution of human (Glu1)-Fibrinopeptide B (Waters Corporation). This solution was used in the reference spray during analyses and scanned

every 30s at a 0.2 µL/min flow rate. Mass calibration of acquired data were corrected considering the doubly charged precursor ion of (Glu1)-Fibrinopeptide B (785.8426 Da).

For plasma proteomes and a second analysis of saliva pools, the MS system in LNBio, São Paulo Brazil, was employed exactly as previously described (Carnielli et al., 2018). The nano-chromatography was made in the Acquity Ultra Performance LC (Waters), coupled to an ESI-Q-Tof Premier Mass Spectrometer (Waters). The analyzed peptides were separated by a gradient of acetonitrile from 2 to 90% in 0.1% formic acid, using a C18 column, 100 µm x 100 mm (Waters), using a flow rate of 300 nL / min at over 212 min, where acetonitrile imparts a 35% gradient in 175 min. The nanoelectrospray voltage was set to 2.2 kV, and the source the temperature was set at 275°C (Carnielli et al., 2018).

2.6 MULTIPLEX ANALYSIS

Plasma cytokines were identified using Luminex xMAP magnetic technology. The technique sought to search for the following cytokines IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, bFGF, GCSF, GM-CSF, IFN-γ, IP-10, MCP-1 (MCAF), MIP-1α, MIP-1β, PDGF -BB, RANTES, TNFα, VEGF. TNFα. The assays were performed following the manufacturer's recommendations with 17 samples, individually. One of the samples presented analytical problems that make its results unfeasible. For the positive control, 20 ng/mL of TNFα was used. Before adding the magnetic beads to each well of the assay plate, calibration and validation of the BIO-Plex Magpix (Biorad Laboratories Inc., Hercules, CA, USA) were performed, in addition to reagent reconstitution and standard curve preparation. Before each step washing steps were performed, washing was performed using an automatic washing station (Biorad Laboratories Inc., Hercules, CA, USA). For the analysis, pearls were added before the supernatant, controls and standard samples, being incubated in the dark for 1h at 250rpm. The same incubation parameters were used for samples with detection antibodies. Later, Streptavidin-PE was added and incubated in the dark for 30 min at 350 rpm. For resuspension of the magnetic beads, assay buffer was used, shaken at 1200 rpm for 30 s and analyzed in the Bio-Plex Magpix device. The assay interference control was done with PLA-NP with IL-8 pattern determined by ELISA and IL-12 and VEGF by Magpix, suggesting that PLA-NP does not bind to secreted products. (Luz et al., 2017).

2.7 STATISTICAL RATIONALE

Differences among groups regarding demographic and clinical parameters were evaluated using Chi-square and Kruskal-Wallis tests in a statistical program (SPSS Statistics 20, IBM Brazil, São Paulo, Brazil). The kolmogorov-Smirnov normality test was performed on the research sample set to determine the normal distribution of the study samples. After verifying that they did not follow such distribution, the Kruskal-Wallis non-parametric statistical analysis was implemented. As this is a univariate analysis with more than two factors, a Post Hoc test of pairwise comparisons with Bonferroni correction was performed. Bonferroni's adjustment was made with the comparison of pairs to confirm the existence of significant results within the comparisons performed when this correction was applied.

To identify and detect variations in the proteomes, MS data were used in searches against a database of human proteins, excluding proteins from microorganisms, from UNIPROT (https://www.uniprot.org/) by using Progenesis tool (Waters), in the case of salivary proteome, and MaxQuant v1.3.0.3 software, with a minimal ratio count of 2 and a window of 2 min for matching between runs, for plasma samples (Cox & Mann., 2008 Carnielli et al., 2018). Any non-tryptic peptide passing from the filter criterion were discarded. Analysis was performed based on the ion matching requirements: minimal fragment ion matches per peptides, 3; minimal fragment ion matches per protein, 7; minimal peptide matches per protein, 1; trypsin missed cleavages, 1; carbamidomethylation of cysteines as a fixed modification and oxidation of methionine as a variable modification (Nagampalli et al., 2018). Polypeptides were identified if they matched with at least one unique and specific peptide from experimental data. Identification was sorted as real when detected in at least two analytical replicates (3 in saliva and 2 or more in plasma samples). The False Discovery Rate in all data was less than 0.3%. Protein abundance values were estimated by the average of the abundances of the first three matched peptides.

For quantitative analysis, variances were tested by ANOVA (Progenesis tool) and the T-test (Mascot Distiller program) with the level of significance of 5% or less was stablished for statistically acceptable differences between normalized abundances. Qualitative comparison was performed manually for detection of exclusive proteins. To assess the differences between the groups the MetaboAnalyst 5.0 program (https://www.metaboanalyst.ca/home.xhtml) was used, by performing Heatmap, Partial least squares-discriminant analysis (PLS-DA) and Principal Component Analysis (PCA).

Venn diagram was employed at Bioinformatics & Evolutionary Genomics website (bioinformatics.psb.ugent.be/webtools/Venn/).

3 RESULTS

3.1 DEMOGRAPHIC AND CLINICAL FINDINGS

All participants in NW and OW were female, while 50% of participants in OB group were female ($p = 0.005$, Chi-square test). As expect all anthropometric parameters evaluated were significantly ($p < 0.01$, Kruskal-Wallis test) higher in OB and OW groups compared to NW group, except for height (Supplementary table 1). The clinical data of the groups showed a few significant differences. Interestingly, a gradual increase in the mean of both arterial pressures, systolic and diastolic, was observed for OW and OB groups compared to NW group ($p = 0.004$).

3.2 SALIVA PROTEINS

Proteomic analysis allowed the identification of a total of 89 (Supplementary Figure 1A) proteins in saliva (Supplementary table 2). Most of them were related to the immune response, with 22 (32%) proteins in saliva. Five proteins displayed transport function, 5 were protease inhibitors and 3 were related to muscle contraction. Many other functional categories were identified too, as carbohydrate metabolic process and oxygen transport and muscle contraction (Supplementary Figure 1C).

3.3 BLOOD PLASMA PROTEINS

In plasma samples, 39 proteins were identified (Supplementary figure 1B), including 10 with equal and 29 with different abundances among the BMI groups (Supplementary table 3). More abundant proteins in plasma were serotransferrin, alpha-2-macroglobulin, fibrinogen alpha chain, serum albumin, immunoglobulins, alpha-1-antitrypsin and haptoglobin. The most plasma proteins were classified in the Immune response function (38%), followed by cholesterol binding and acute-phase response (Supplementary Figure 1D). Of those modulated proteins, 33 were found exclusively in plasma but not in saliva.

Our results indicate that the body mass rising probably modulate the proteome expression in saliva, and ether plasma. Generally, OW and OB groups showed more functional categories of proteins with increased abundance than the decreased ones (Supplementary figure 2A, 2B), in comparison to NW individuals. The immune response

was the most affected function in the expression profile, with proteins showing mainly abundance increase, followed by the cholesterol-binding and transport functions. Retina homeostasis and muscle contraction were categories exclusively enhanced with weight gain, while Tumor necrosis binding factor and DNA repair appeared only as reduced functions.

3.4 DIFFERENCES IN SALIVARY AND PLASMA PROTEOMES ACCORDING TO BMI

PCA of salivary and plasma proteomes showed that the samples of distinct nutritional status grouped separately (Figure 1A and 1B), endorsing the distinct characteristics between the proteomes in different BMI categories. The PLS-DA VIP score (variable importance in projection) was performed to detect the main proteins that influence these variations, which showed the 15 plasma (as Isoform of P01876, Immunoglobulin heavy constant alpha 1, Complement C4-B and Serum albumin) and salivary proteins (as Cystatin-S, Immunoglobulin heavy constant alpha 1 and Basic salivary proline-rich protein 2) that greatest impact on the separation of groups (Figure 2A and 2B). These VIP score proteins were used to construct the Heatmap (Supplementary figure 3A and 3B) to verify the variation of proteins amount in response to nutritional status.

Figure 1. Principal Component Analysis (PCA) score charts of identified proteins in the three study groups (normal weight, overweight and obese). (A) salivary proteins and (B) plasma proteins

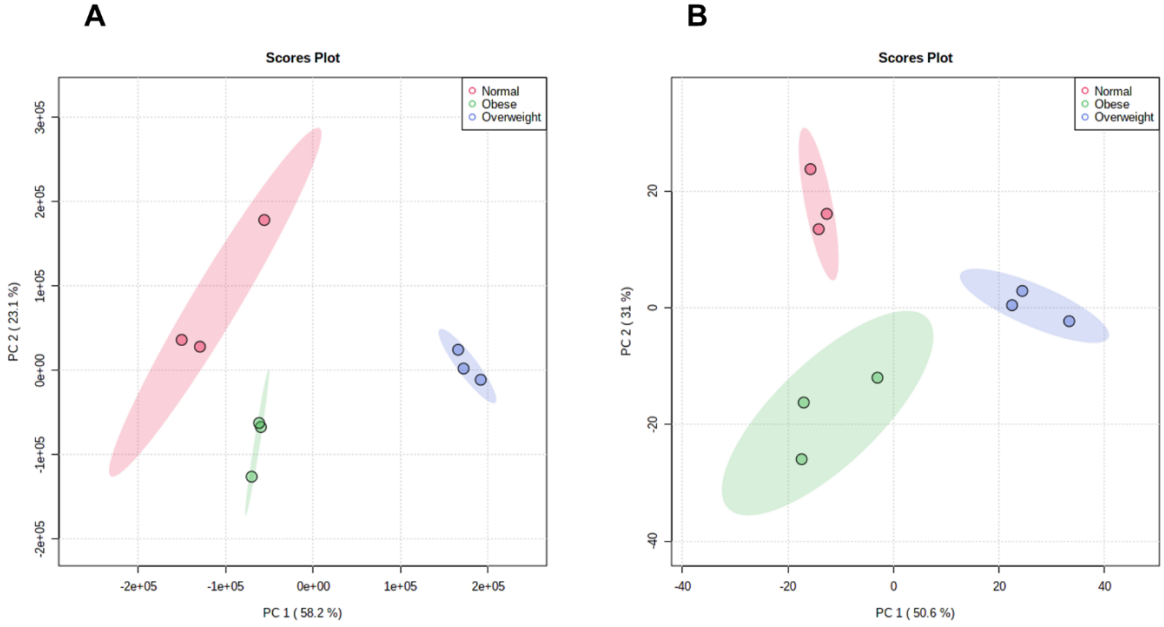
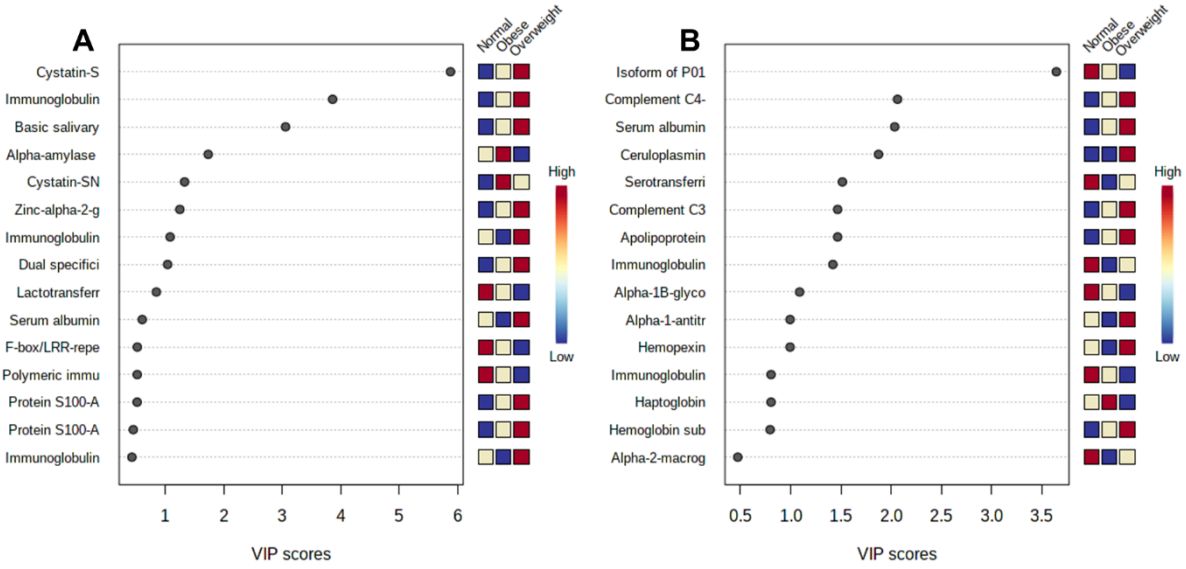


Figure 2. PLS-DA VIP score of the 15 most important salivary and plasma proteins that have the greatest impact on group differentiation. (A) salivary proteins and (B) plasma proteins



3.5 CYTOKINE'S SECRETION AND BMI

The secretion of cytokines on blood plasma was evaluated, by Bio-Plex Magpix, in response to the increase of body mass. Cytokines, as chemokine and growth factor activities, were analyzed in individual samples, but only 6 were identified and quantitatively different when groups were relatively compared (Supplemental table 4). Eotaxin, IL-4 and IL-7 showed an increase in the OW group compared to NW. IL-5 and bFGF presented higher expression in OB group compared to NW group. PDGF-BB was not identified in the overweight group and IL-7 were increased in OB group compared to OW group.

4 DISCUSSION

In this work, we performed a small but promising proteomic study with a local population of Rio de Janeiro, Brazil. Demographic data of this population was reported and the relative expression of salivary and blood plasma proteins of young adults with obesity and overweight was analyzed in comparison to normal weight individuals. The concentration of specific cytokines on plasma blood were also evaluated. Thus, our aim was to test the impact of the body mass rise on the salivary and plasma proteomes and its relevance to human young adult`s health.

Beyond the clinical parameters, only the blood pressure values were significantly influenced by the weight gain. In fact, our study group included young adults with no identified clinical diseases. Periodontal evaluation was performed because periodontal

diseases are inflammatory diseases related to obesity (Silva-Boghossian., 2013). None of the participants had destructive periodontal disease, however the prevalence of gingivitis was the same in all groups. So, it is plausible to assume that saliva and plasma proteins were not influenced by participants' periodontal conditions too.

Immune system was the most representative function in the two body fluids, with the highest amount of identified and regulated proteins. Overweight and obese individuals displayed the increment of salivary proteins from inflammation, transport and muscle contraction functions. These results reaffirm that the gain of mass can alter immunological aspects in the body, making them more prone to inflammatory processes and diseases associated with inflammation (Andersen, Murphy & Fernandez., 2016).

We present here the identification of 128 proteins, including 89 from saliva, 39 from plasma and 7 that were common to both fluids. Some protein changes identified here resemble conditions found in pathogenic situations. Various modulated proteins in the overweight and obese group are also observed in patients with CVD, type 1 and type 2 diabetes, acute kidney injury and cancer, which indicate the potential of OW and OB expressive proteins as good targets for diagnoses and therapeutic purposes, or for supporting decisions at clinical level. (Andersen, Murphy & Fernandez., 2016).

Twelve immunoglobulins (Ig) were identified in this study. Four immunoglobulins were uniquely identified in saliva and 3 in plasma. Overall, salivary Igs were increased in OW groups, including the lambda constant 2 and the lambda-like polypeptide 5, that were enhanced in obese proteomes too. Almost all plasmatic Igs were less abundant in OB and OW groups, except for the heavy gamma-chains 2 and 3, with a decrease only in obese proteomes.

Plasma levels of lambda-chain immunoglobulin 2 and alpha 1 heavy chain immunoglobulin are overexpressed in diseases, such as Rheumatoid Arthritis and inflammation (Yi et al., 2016). Curiously, in our work, both lambda-chain immunoglobulin 2 (IGLC2) and alpha 1 heavy chain immunoglobulin (IGHA1) presented a decrease in OB and OW plasma proteomes, compared to NW, but showed greater abundance in saliva.

Lysozyme is a conserved protein, which plays a fundamental role in host defense against oral microorganisms (Callewaert & Michiels., 2010). Furthermore, lysozyme participates in the activation of macrophages and neutrophils, contributing to pro-inflammatory pathways (Callewaert & Michiels., 2010). Here, we observed that lysozyme had a gradual increase along BMI rise. OB group had almost 3 times more the amount of

that protein than the NW. It could indicate that oral cavity of the obese population in this study was more inclined to inflammation conditions.

Fourteen proteins identified here were already related to CVD. Those are, ceruloplasmin, alpha-2-macroglobulin, alpha and beta subunits of hemoglobin, fibrinogen gamma chain, apolipoprotein A-I, alpha-1-acid glycoprotein 1, alpha-1-antichymotrypsin, and 6 cytokines (eotaxin, bFGF, PDGF-BB, IL-4, IL-5, IL-7). Notably, the hemoglobin subunits found in saliva samples were identified only in OB group. In plasma samples, hemoglobin alpha was not found in NW. Previous studies demonstrated that free hemoglobin is prone to oxidation and its products present pro-oxidant and inflammatory characteristics (Gram et al., 2013). In addition, hemoglobin levels increasing were observed in patients with hypertension (Lee, Rim & Kim 2015).

Interleukins are key mediators of the immune response in modulating its process (Bossa et al., 2014), and 3 of them were identified in our investigation (IL-4, IL-5 and IL-7) as increased in OB or OW plasma. Elevated levels of IL-4 were reported as associated to tropical endomyocardial fibrosis, a cardiomyopathy characterized by secondary valvular dysfunction, fibrous tissue removal from the endomyocardial and atrial entrapment (Bossa et al., 2014). Furthermore, increased levels of IL-5 have been linked to an increased risk for coronary heart disease. Regarding IL-7, it was demonstrated that have an important role in atherogenesis through endothelium, impacting on monocyte/ macrophages activation of PI3K/ AKT (Clarke et al., 2018). In general, our findings may suggest that the increase of interleukins levels in higher BMI might influence the immune processes related to CVD.

FGFs are a vast family of polypeptides, which have important biological effects. In humans, the FGF family has 22 members. Previous findings have indicated that several FGFs are involved in heart development, during health and disease (Ornitz & Itoh 2015). In our study, bFGF was found only in the plasma of obese and overweight individuals.

Eotaxin is a chemokine with the ability to promote the activation and migration of eosinophils by participating in a wide variety of allergic disorders, and it is overexpressed in tissues lesions (Rankin, Conroy & Williams 2000). Previous studies showed that it induces angiogenesis and influences vascular inflammation through endothelium activation (Salcedo et al., 2001). In our data, eotaxin had a significant increment in OW plasma compared to NW individuals. However, no significant difference was detected between the OB and NW groups.

Among proteins exclusively found in OB and OW plasma proteomes, the alpha-1-acid glycoprotein 1 (AGP) plays a role in cardiovascular diseases. It is an acute phase protein, synthesized by hepatocytes and immunological cells, but can also be expressed by adipocytes. The increase in its concentration was associated to adverse effects in acute heart failure (Agra et al., 2017). The fibrinogen gamma chain protein is pointed in the literature as related to coronary diseases and can even be used as a predictor for the disease. Fibrinogen gamma chain was detected in high amounts in individuals who suffered cardiovascular death (Melander et al., 2015). In our research we observed an increment of this protein in obeses. Obesity is notoriously known as a risk factor for cardiovascular disease. The alpha-1-antichymotrypsin protein (ACT), also known as SERPINA 3, showed an increase in the OW group, being identified only in plasma. The physiological function of the protein is not yet clear, but they can convert angiotensin-1 into active angiotensin-2 (Apha-1-antichymotrypsi., 2021). In addition, a study has pointed out that the protein is increased in calcified aortic stenosis (Martin-Rojas et al., 2017). Other correlated protein, ceruloplasmin (CP), was present only in OW individuals. Studies have observed that its increasing levels leads to a risk of heart failure and CVD; this intermediate elevation may suggest that the protein is a good early biomarker for CVD (Dadu et al., 2013). In our study, CP protein was identified exclusively in the overweight group. The increment of AGP, fibrinogen gamma chain, ACT and ceruloplasmin in higher adiposity conditions may demonstrates the danger of weight gain for problems related to CVD, probably triggering the future development of CVD-associated processes even in early adults.

Apolipoproteins have physiological importance associated with disorders such as CVD, dyslipidemia and neurodegenerative diseases (Apolipoproteins., 2021). The HDL-cholesterol/apolipoprotein A-I ratio was related to the risk of CVD developing, such as atherosclerosis, and mortality (Sung et al., 2015). Recently, apolipoproteins were associated to a higher risk for DM2. A strong association of apolipoprotein with BMI has already been demonstrated with a decrease in its concentration in overweight individuals (Wang, Blackett, Khan & Lee 2017). Some studies have observed a significantly BMI-related increase but with decrease in overweight individuals in the APOH protein levels in DM2 individuals compared to healthy individuals (Gómez-Cardona et al., 2017). Here, we identified the apolipoprotein A-I depletion in plasma of OW group compared to NW. Instead, obese group had increased amount of APOH, when compared to NW individuals.

Other 3 modulated proteins (APO A-II, alpha-1-antitrypsin and complement C3) were also directly associated with cases of DM (Brener et al., 2018). APO A-II showed an increase in overweight but decreased their expression in the obese group compared to the normal group. Alpha-1 antitrypsin (SERPINA1 or AAT) is a potent anti-inflammatory, with the ability to suppress inflammation, through the inhibition of IL-10 and IL-1 and the differentiation of regulatory T cells. Recent studies have observed that administration of SERPINA 1 can reduce the severity of patients with DM2, as well as preserve beta cell function in patients with type 1 DM (DM1). Studies have observed that the level of SERPINA1 in patients with DM2 is 50% lower than in healthy individuals (Brener et al., 201829). Initial Phase I and II studies using the administration of AAT, managed to improve the metabolic control, besides reducing the incidence of severe hypoglycemia (Kim, Cai & Oh., 2018). Our results showed a lower expression in SERPINA1 levels in the OB group compared to the NW. Here, we also observed the over expression of complement protein C3 according to BMI rise. Complement C3 protein is an inflammatory marker that can be used to predict both pre-diabetes and diabetes. The C3 protein was greatly enhanced the plasma of OW (more than five times) and OB groups. In general, studies have shown the raise of this protein in pre-diabetes plasma, which can be considered a risk indicator for the development of DM2 (Bao et al., 2016). Note that none of our participants had a diagnosis of DM1 or DM2, therefore, the modulation of SERPINA1 and C3 complement expression in obese and overweight individuals might constitute a potential biomarker for premature diagnoses of DM2.

A recent study identified seven proteins (Alpha-1-antichymotrypsin, Alpha-1-antitrypsin, Apolipoprotein A-I, haptoglobin, retinolbinding protein 4, transthyretin, and zinc-alpha2-glycoprotein) with significant differences between diabetic and pre-diabetic individuals. These proteins were also identified in our study, with the exception of haptoglobin and transthyretin, all seven proteins also showed the same significant differences against our control group (NW) (Kim et al., 2019). Thus, our results suggest that overweight and obese volunteers, without pre-diagnosed diabetes, possibly exhibited metabolic tendency to development of DM2.

In saliva proteomes, members 1 and 2 of BPI's B family (BPIFB1 and BPIFB2), and member 2 of the BPI A family were exclusive or overexpressed in the OB and OW groups. Proteins of the BPIF family have been associated with chronic lung diseases, such as cystic fibrosis (Vestbo et al., 2013). Previous studies showed that BPIFB1 levels are inversely related to respiratory capacity, in addition to a positive correlation with disease

severity (Vestbo et al., 2013). And BPIFA2 was reported with increased expression in mice without Nur, after 3 hours of induced kidney injury, followed by 7 and 8-fold increase after 6 and 24 hours of injury (Kota et al., 2017). Concerning the role of these proteins on immune response, we suppose that their abundance on OW and OB saliva might be triggered by obesity-related inflammatory processes.

Kallikrein and three proteins of POTE ankyrin domain family (members E, F and I) were exclusively identified in the OW group. In addition to BPIFA2, kallikrein was previously linked to kidney diseases. The kallikrein system is an important modulator of the cardiovascular, nervous and renal systems (Berger et al., 2019). This protein acts in the induction of genes and in the activation of pro-enzymes, molecules related to vasodilation, fibrinolysis and coagulation (Berger et al., 2019). Previous studies have shown that kallikrein is elevated in acute kidney injury, and this protein block may be a possible therapeutic alternative[62]. In other hands, POTEE, has been demonstrated to be elevated in patients with lung cancer, and with a low survival rate (Wang et al., 2015).

Some proteins associated with cancer were also identified here as incremented in response to weight gain. In proteomic and histochemical analysis studies, beta-actin-like protein 2 (ACTBL2) was found to be increased in colorectal tumors, indicating a possible use of the protein as an early biomarker (Ghazanfar et al., 2017). ACTBL2 protein was only observed in obese and the actin-gamma-enteric smooth muscle was also exclusively identified in OB and OW groups. The last one was related to aggressiveness of certain cancers (Ghazanfar et al., 2017).

The UV excision repair protein RAD23 homolog A appears to play a role in apoptosis, proteasomal degradation, as well as in some cancers (Marteijn et al., 2014). Here, we found an expressive increase in the levels of this protein in saliva of OW (about nine-fold) and OB (four-fold increase) groups, compared to NW. The investigation of the function of this protein on weight gain physiopathology's is primordial.

5 CONCLUSION

In conclusion, this study suggest a variety of proteins that seems to be modulated by the gain of weight in young adults, whose functions range diverse biological events, such as immune response, transport, neutrophil degranulation and other metabolic processes. From these some had already been reported as related to oral and cardiovascular inflammatory diseases, or possibly associated with the consequences of systemic inflammation processes. Despite the limited number of samples, it shows the

potential of saliva and plasma fluids as source of premature diagnoses biomarkers, mainly in case of obesity comorbidities. For future studies it is necessary not only to increase the number of volunteers, but also to balance the number of men and women. However, this work is improving the knowledge about the human metabolic response to obesity and overweight, in special for Brazilian populations, whose proteomic studies are so scarce.

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