



UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara



Camila Chierici Marcantonio

**Avaliação dos tecidos periodontais de ratos submetidos a força mecânica
ortodôntica em condições de saúde ou doença sistêmica**

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Orientador: Prof. Dr. Joni Augusto Cirelli

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**Avaliação dos tecidos periodontais de ratos submetidos a força mecânica
ortodôntica em condições de saúde ou doença sistêmica**

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“Quando ensinamos, deixamos um pouco de nós em cada aluno e levamos um pedaço deles dentro de nós”

Elcio Marcantonio*

* Frase dita pelo meu avô Elcio Marcantonio - Professor Doutor da Faculdade de Odontologia da Unesp, Câmpus de Araraquara, SP.

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RESUMO

Este estudo teve como objetivo avaliar in vivo os efeitos de doenças sistêmicas (obesidade e osteoporose) na remodelação dos tecidos periodontais de molares de ratos sob força mecânica ortodôntica. No primeiro estudo, foi avaliado o efeito da obesidade no perfil proteômico do ligamento periodontal de ratos, sob força mecânica ortodôntica. Foram utilizados 10 ratos divididos em dois grupos (n=5): grupo M (movimentação ortodôntica), e grupo OM (indução de obesidade e movimentação ortodôntica). A obesidade foi induzida por meio de ração hiperlipídica durante todo o período experimental. Após 15 dias de M, os animais de todos os grupos foram eutanasiados e suas hemimaxilas foram processadas para realização de microdissecção a laser (LCM) do ligamento periodontal (LP) dos primeiros molares e imuno-histoquímica (IHC). Os tecidos microdissecados foram analisados por cromatografia líquida e espectrometria de massas de alta resolução (LC- MS/MS). Os dados estão disponíveis via ProteomeXchange (código XD033647). Dentre as 109 proteínas abundantemente expressas, 49 estavam em OM ($p \leq 0,05$, Test-t). A análise de *Gene Ontology* (GO) apresentou proteínas enriquecidas associadas a categoria de componente celular ($p \leq 0,05$, teste exato de Fisher seguido por Benjamini) no grupo OM. Dentre estas, proteínas que possuem associação com obesidade: Vinculina, Cathepsina D e Osteopontina (Spp1) foram selecionadas e avaliadas por IHC, que confirmou a presença e localização delas. Os resultados demonstraram diferenças no perfil proteômico do LP submetido à M e OM, permitindo uma melhor compreensão dos mecanismos envolvidos nos processos de remodelação tecidual em situação de obesidade submetidos ao tratamento ortodôntico. No segundo estudo, foi avaliado o efeito do anticorpo monoclonal anti-esclerostina (Scl-Ab) nos tecidos periodontais de ratas ovariectomizadas sob movimentação ortodôntica. Foram utilizadas 48 ratas distribuídas em 6 grupos (n=8): C (controle – sem intervenção), OM (movimentação ortodôntica), OVX (ovariectomia), OVX+OM, OVX+Scl-Ab (ovariectomia e administração de Scl-Ab) e OVX+Scl-Ab+OM. Os animais foram submetidos a OVX ou cirurgia Sham. Após 30 dias (baseline), deu-se início à administração de Scl-Ab, mantida por todo o período experimental. 7 dias após o baseline, iniciou-se a OM e, após 15 dias, todos os animais foram eutanasiados (dia 21). A massa corporal dos animais foi registrada semanalmente e os úteros coletados na eutanásia. Foram avaliados volume (BV/TV) e densidade óssea (BMD) na tíbia e fêmur por microtomografia computadorizada (Micro-CT) previamente às cirurgias e no baseline. A movimentação dentária, BV/TV e BMD nas maxilas foi avaliada por Micro-CT. Foi avaliada a expressão gênica de Spp1, Il1b, TNFa e Rankl nos tecidos gengivais. A análise de colágeno birrefringente no LP foi realizada em três regiões da raiz distal em todos os grupos, nos lados de pressão e tensão. Os dados foram analisados estatisticamente por Shapiro-Wilk, seguido de ANOVA/Tukey ($p \leq 0.05$). Todos os animais submetidos à OVX apresentaram menor BMD e BV/TV na tíbia e fêmur 30 dias após a cirurgia, maior massa corporal e menor peso de útero. A expressão gênica de Tnfa foi significativamente maior no grupo OVX+OM em comparação com os grupos OVX e OVX+Scl-Ab+OM. No lado de pressão do LP, os animais dos grupos OM, OVX+OM e OVX+OM+Scl-Ab apresentaram uma redução no conteúdo de colágeno em comparação com os animais dos grupos não submetidos à movimentação dentária ortodôntica. Nas maxilas, os grupos OM e OVX+Scl-Ab+OM demonstraram maior tendência de movimento dentário comparados ao grupo OVX+OM. O grupo OVX+Scl-Ab+OM apresentou maior BMD e BV/TV que o grupo OVX+OM e menor que o grupo OVX+Scl-Ab. A administração de Scl-Ab reduziu os efeitos negativos da osteoporose nos tecidos periodontais submetidos à movimentação ortodôntica.

Palavras-chave: Técnicas de movimentação dentária. Obesidade. Osteoporose. Ovariectomia.

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ABSTRACT

This study aimed to evaluate *in vivo* the effects of systemic diseases (obesity and osteoporosis) on the remodeling of periodontal tissues of rat molars under orthodontic mechanical force. In the first study, the effect of obesity on the remodeling of periodontal tissues in rats under orthodontic mechanical force was evaluated. Ten rats were divided into two groups (n=5): group M (orthodontic movement), and group OM (induction of obesity and orthodontic movement). Obesity was induced by hyperlipidic feed throughout the experimental period. After 15 days of orthodontic movement, animals in all groups were euthanized and their hemimaxillae were processed for laser microdissection (LCM) of the periodontal ligament (LP) of the first molars, and immunohistochemistry (IHC). The microdissected tissues were analyzed by high-resolution liquid chromatography-mass spectrometry (LC-MS/MS). The data are available via ProteomeXchange with ID PXD033647. Among the 109 differentially abundant proteins, 49 were in OM ($p \leq 0.05$, Test-t). Gene Ontology (GO) analysis showed enriched proteins associated with cellular component category ($p \leq 0.05$, Fisher's exact test followed by Benjamini) in the OM group. Among these, proteins that have association with obesity: Vinculin, Cathepsin D and Osteopontin (Spp1) were selected and evaluated by IHC, which confirmed their presence and localization. The results showed differences in the proteomic profile of the LP submitted to M and OM, allowing a better understanding of the mechanisms involved in the processes of tissue remodeling in obese patients undergoing orthodontic treatment. The second study was to evaluate the effect of the monoclonal anti-sclerostin antibody (Scl-Ab) in the periodontal tissues of ovariectomized rats submitted to orthodontic movement. Forty-eight rats were distributed into 6 groups (n=8): C (control - no intervention), OM (orthodontic movement), OVX (ovariectomy), OVX+OM, OVX+Scl-Ab (ovariectomy and Scl-Ab administration) and OVX+Scl-Ab+OM. Animals were submitted to OVX or Sham surgery. After 30 days (baseline), Scl-Ab administration started and maintained throughout the experimental period. Seven days after baseline, OM initiated, and after 15 days, all animals were euthanized (day 21). The body mass of all animals was recorded weekly, and the uterus were collected and weighted at euthanasia. Bone volume (BV/TV) and bone density (BMD) in the tibia and femur were assessed by micro-computed tomography (Micro-CT) prior to surgery and at baseline. Tooth movement, BV/TV and BMD in the jaws were evaluated by Micro-CT. Gene expression of Spp1, Il1b, TNFa, and Rankl in the gingival tissues was evaluated. The measurement of collagen content in the LP was performed in three regions of the distal root in all groups. Data were statistically analyzed by Shapiro-Wilk, followed by ANOVA/Tukey ($p \leq 0.05$). All animals subjected to OVX showed lower BMD and BV/TV in the tibia and femur 30 days after surgery, higher body mass and lower uterus weight. The gene expression of Tnfa was significantly higher in the OVX+OM group compared to OVX and OVX+Scl-Ab+OM groups. In the pressure side of the LP, animals from OM, OVX+OM, and OVX+OM+Scl-Ab groups presented a reduction in the collagen content compared to animals from the groups not submitted to orthodontic tooth movement. In the maxillae, the OM and OVX+Scl-Ab+OM groups showed a tendency to greater tooth movement compared to the OVX+OM group. The OVX+Scl-Ab+OM group showed higher BMD and BV/TV than the OVX+OM group and lower than the OVX+Scl-Ab group. The administration of Scl-Ab reduced the harmful effects of osteoporosis on periodontal tissues submitted to orthodontic movement.

Keywords: Tooth movement techniques. Obesity. Osteoporosis. Ovariectomy.

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1 INTRODUÇÃO

Na Odontologia, o tratamento ortodôntico visa corrigir as más oclusões dentárias por meio da aplicação de forças mecânicas, que induzem um remodelamento dos tecidos periodontais, e consequente movimento dentário^{1,2}. Nesse processo, células do ligamento periodontal, como fibroblastos e osteoblastos, produzem sinais biológicos por meio de mediadores para que se inicie um processo inflamatório local, a fim de promover reabsorção óssea do lado de pressão (sentido em que vai ocorrer o movimento dentário) e aposição óssea no lado de tensão (sentido oposto em que vai ocorrer o movimento dentário)¹⁻⁴. Portanto, durante a movimentação ortodôntica, diversas citocinas pró- inflamatórias são liberadas, como a interleucina 1-beta (IL-1b), interleucina 6 (IL- 6) e fator de necrose tumoral alfa (TNFa)⁵⁻⁷. É importante ressaltar que apesar do processo inflamatório causado na movimentação dentária, essa inflamação não é decorrente de uma doença como no caso da doença periodontal, e sim de fatores externos, como a aplicação de uma força, para que ocorra o deslocamento dentário, sendo assim chamada por muitas vezes de processo de “inflamação aguda asséptica”². Dito isto, a presença de inflamação exacerbada, como a doença periodontal ou doenças sistêmicas inflamatórias, pode afetar o processo de remodelação tecidual ocorrido durante a movimentação ortodôntica, levando a um aumento na osteoclastogênese e maior destruição dos tecidos periodontais⁸⁻¹¹.

A obesidade é uma doença metabólica crônica caracterizada pelo acúmulo excessivo de gordura no corpo, gerando impactos negativos na saúde¹². É definida quando o cálculo do índice de massa corporal (IMC) resulta em valor maior ou igual a 30 kg/m²¹³. Segundo os últimos dados da Organização Mundial de Saúde (OMS), no ano de 2016, 13% da população mundial adulta apresentava obesidade. Já em 2019, 38,2 milhões de crianças abaixo de 5 anos apresentavam obesidade ou sobrepeso. Além disso, a OMS faz um alerta de que o sobrepeso e a obesidade estão em ascensão também nos países de baixa renda, principalmente em centros urbanos, e não mais somente nos países de alta renda¹⁴. A obesidade está associada a diversas patologias como a doença cardiovascular, a artrite reumatoide, a diabetes mellitus tipo 2 e alguns tipos de cânceres¹⁵⁻¹⁷. O estímulo responsável por desencadear os processos inflamatórios na obesidade ainda não foi totalmente elucidado. Entretanto, uma das hipóteses seria que o excesso de nutrientes presentes nas células

metabólicas, como adipócitos, macrófagos e células T, promovem ativação de células imunes provocando um aumento na quantidade de citocinas e adipocinas^{18,19}. Além do aumento no número de adipócitos há também aumento no volume destes. Este aumento de volume determina a secreção de citocinas inflamatórias, TNF α , IL-6, IL-1b e causa hipóxia. Portanto, há um aumento na inflamação local e sistêmica^{20,21}.

Durante o movimento ortodôntico em pacientes com obesidade há um aumento nos níveis de concentração de leptina, uma adipocina pro-inflamatória, na saliva destes pacientes quando comparados a pacientes sem obesidade²². Além disso, foi demonstrado que a leptina aumenta a expressão de fatores pró-inflamatórios como IL-6 e ciclo-oxigenase-2 (COX-2) em células de ligamento periodontal submetidas à força mecânica²³. Além desta, outras adipocinas pró-inflamatórias, como a resistina, estão presentes no fluido crevicular gengival e em níveis séricos de pacientes obesos^{24,25}. Animais com obesidade submetidos ao movimento dentário ortodôntico apresentam maior perda óssea devido ao aumento da osteoclastogênese do que animais saudáveis sob tratamento ortodôntico²⁶. Em um estudo anterior recente do nosso grupo demonstramos que animais submetidos à indução de obesidade e ao movimento ortodôntico apresentaram redução nos parâmetros de volume e densidade óssea em comparação com animais não obesos submetidos à força ortodôntica²⁷. Além disso, animais e pacientes com obesidade possuem uma tendência a um movimento dentário mais rápido do que aqueles que não apresentam esta doença^{24, 27}.

A osteoporose pós-menopausa é uma doença ósteometabólica, de etiologia multifatorial de alta prevalência em mulheres^{28,29}. Esta é caracterizada pela redução de massa óssea em ossos longos e vértebras que ocorre devido à diminuição dos níveis de estrogênio³⁰. Essa condição faz com que haja um desequilíbrio entre osteoblastos e osteoclastos, causando um aumento na reabsorção óssea desproporcional à formação óssea^{31,32}. A osteoporose é diagnosticada através do exame de densitometria óssea, onde é comparado o resultado com a média de adultos jovens do mesmo gênero e raça (população que apresenta pico de massa óssea). Esta comparação é chamada de T-score ou desvio padrão, e quando este apresenta valores 2,5 desvios padrão abaixo da média, a osteoporose é diagnosticada³³. Anualmente, 8,9 milhões de fraturas ósseas são causadas devido à osteoporose,

resultando em uma fratura a cada 3 segundos³⁴. Além disso, a osteoporose leva à perda da massa óssea não somente em ossos longos e na coluna vertebral, mas também no osso alveolar³⁵.

Mulheres em pós-menopausa apresentam aumento na severidade da doença periodontal, com maior perda clínica de inserção³⁶⁻³⁸. A intensidade de aplicação da força ortodôntica pode influenciar e causar danos ósseos, como colapso da microestrutura do osso trabecular, podendo levar a uma maior chance de perda dentária em situações de osteoporose pós-menopausa³⁹. Em ratas ovariectomizadas submetidas à movimentação ortodôntica há um aumento no número de osteoclastos no osso alveolar quando comparadas a ratas sem indução de osteoporose sob movimentação ortodôntica⁴⁰.

As drogas mais utilizadas para o tratamento da osteoporose pós- menopausa são os medicamentos anti-reabsortivos⁴¹⁻⁴⁴, com destaque para os bisfosfonatos. Estes atuam inibindo osteoclastos para que a perda óssea ocasionada por doenças metabólicas seja estabilizada⁴⁵. Além disso, também é demonstrado a sua ação direta em osteoblastos, impedindo a neoformação óssea^{46,47}. Os bisfosfonatos estão relacionados a efeitos adversos graves como a osteonecrose de maxilares, fraturas femorais atípicas e fibrilação atrial^{41,44}. Devido a estes efeitos, não é recomendado a realização de alguns procedimentos odontológicos em pacientes em uso de bisfosfonatos, pois podem levar a lesões ósseas necrosantes⁴⁸⁻⁵². Recentemente, um novo medicamento para o tratamento de osteoporose pós-menopausa foi aprovado (Romosozumabe), que consiste em um anticorpo monoclonal anti-esclerostina (Scl-Ab)⁵³. Este medicamento age promovendo aumento na atividade e no número de osteoblastos, resultando assim em aumento na formação óssea⁵⁴⁻⁵⁷. Segundo as recomendações da Sociedade de Endocrinologia, para que haja uma redução nos riscos de fraturas vertebrais e de quadris em mulheres com osteoporose pós-menopausa severa ou com histórico de múltiplas fraturas vertebrais, é recomendado receitar o uso de Romosozumabe por um ano^{58,59}.

A esclerostina é codificada pelo gene SOST e é produzida principalmente por osteócitos maduros⁶⁰. Esta proteína é antagonista da via de sinalização de Wnt – um dos elementos chave para formação óssea. Quando ativada, esta via inibe a reabsorção óssea por osteoclastos, induzindo a expressão de osteoprotegerina (OPG)

por osteoblastos e osteócitos^{61,62}. Além disso, a esclerostina estimula a expressão do ligante do recetor ativador do fator nuclear kappa B (RANKL) por osteócitos, regula negativamente a expressão de proteína morfogenética óssea (BMP) e impede a via de sinalização canônica de Wnt^{63,64}. Na movimentação ortodôntica, esta proteína promove reabsorção óssea e inibe a nova formação óssea^{65,66}. Além disso, a intensidade da expressão de esclerostina está relacionada a magnitude da força⁶⁷. No lado de tensão, há uma redução na expressão de esclerostina, anulando assim seus efeitos negativo sob a nova formação óssea⁶⁶.

A inibição de esclerostina favorece a osseointegração e a regeneração óssea ao redor de implantes demonstrando ser uma potencial terapia para redução do tempo de reparo após a instalação de implantes^{68,69}. O uso sistêmico de Scl-Ab promove regeneração óssea e de cimentos dentários em defeitos ósseos ao redor de dentes⁷⁰. Além disso, um estudo demonstrou aumento da formação óssea em um modelo de defeito femoral de tamanho crítico em ratos após administração de Scl-Ab⁷¹. Em modelos de periodontite experimental, a administração de Scl-Ab promoveu aumentou do volume ósseo, da densidade mineral óssea e da altura do osso alveolar⁷². Durante a movimentação ortodôntica em animais SOST KO, a atividade osteoclástica no lado de pressão foi reduzida com deficiência nos níveis de esclerostina. Além disso, observaram nesses animais que as mudanças encontradas na atividade osteoclástica e na expressão de esclerostina eram similares, sugerindo um efeito direto da esclerostina na reabsorção óssea durante o movimento dentário⁷³.

Neste contexto e levando em consideração a procura de pacientes adultos para tratamento ortodôntico. Estudos que avaliem os efeitos de doenças sistêmicas na remodelação dos tecidos periodontais sob força mecânica ortodôntica são necessários para maior previsibilidade e escolha adequada de tratamento.

2 PROPOSIÇÃO

Avaliar in vivo os efeitos de doenças sistêmicas (obesidade e osteoporose) na remodelação dos tecidos periodontais de molares de ratos sob força mecânica ortodôntica. Para responder a este objetivo, a tese foi dividida em dois estudos:

Estudo 1: Avaliar o efeito da obesidade no perfil proteômico do ligamento periodontal de ratos, sob força mecânica ortodôntica.

Estudo 2: Avaliar o efeito do anticorpo monoclonal anti-esclerostina nos tecidos periodontais de ratas ovariectomizadas sob movimentação ortodôntica.

3 PUBLICAÇÕES

A presente tese gerou duas publicações: um artigo que foi aceito na *Journal of Proteomics* (Qualis A1; Fator de impacto 3,855) (ANEXO A) e outro artigo que está em fase final de redação.

3.1 Publicação 1*

Obesity affects the proteome profile of periodontal ligament submitted to mechanical forces induced by orthodontic tooth movement in rats.

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HIGHLIGHTS

- LCM is a suitable technique for PDL tissues isolation for proteomic analysis.
- Obesity modulates the proteome profile of rats PDL during orthodontic movement.
- Proteins in the Phagosome and Lysosome pathways are abundant in obesity.
- Periodontal tissues remodeling proteins are up-regulated in obesity.

ABSTRACT

The prevalence of obesity has increased significantly worldwide. Therefore, this study aimed to evaluate the influence of obesity on the proteomic profile of periodontal ligament (PDL) tissues of rat first maxillary molars (1M) submitted to orthodontic tooth movement (OTM). Ten Holtzman rats were distributed into two groups (n = 5): the M group (OTM), and the OM group (obesity induction plus OTM). Obesity was induced by a high-fat diet for the entire experimental periods. After that period, the animals were euthanized and the hemimaxillae removed and processed for laser capture microdissection of the PDL tissues of the 1M. Peptide extracts were obtained and analyzed by LC-MS/MS. Data are available via ProteomeXchange with identifier PXD033647. Out of the 109 proteins with differential abundance, 49 were identified in the OM group, including Vinculin, Cathepsin D, and Osteopontin, which were selected for in situ localization by immunohistochemistry analysis (IHC). Overall, Gene Ontology (GO) analysis indicated that enriched proteins were related to the GO component cellular category. IHC validated the trends for selected proteins. Our study highlights the differences in the PDL proteome profiling of healthy and obese subjects undergoing OTM. These findings may provide valuable information needed to better understand the mechanisms involved in tissue remodeling in obese patients submitted to orthodontic treatment.

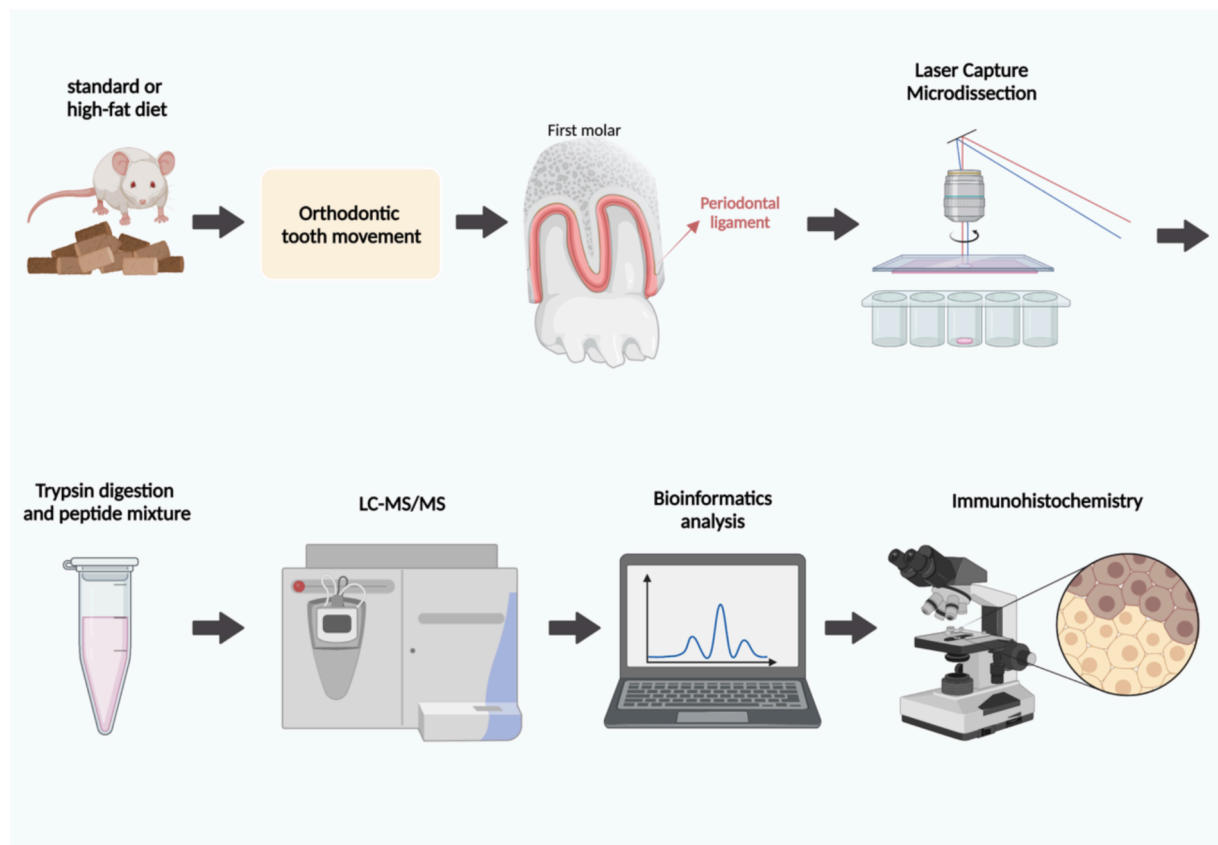
KEYWORDS: Proteomics; Obesity; Orthodontic Tooth Movement; Vinculin; Osteopontin; Cathepsin D

SIGNIFICANCE

The prevalence of obesity is increasing worldwide. Emerging findings in the field of dentistry suggest that obesity influences the tissues around the teeth, especially those in the periodontal ligament. Therefore, evaluation of the effect of obesity on periodontal tissues remodeling during orthodontic tooth movement is a relevant research topic.

To our knowledge, this is the first study to evaluate proteomic changes in periodontal ligament tissue in response to the association between orthodontic tooth movement and obesity. Our study identified a novel protein profile associated with obesity by using laser microdissection and proteomic analysis, providing new information to increase understanding of the mechanisms involved in obese patients undergoing orthodontic treatment which can lead to a more personalized orthodontic treatment approach.

GRAPHICAL ABSTRACT



INTRODUCTION

Obesity is a chronic metabolic disease that is caused by excessive fat accumulation in the body and results in a myriad of health problems [1, 2]. It is associated with comorbidities such as type 2 diabetes, cancer, and stroke and, more recently, was demonstrated to be a risk factor for severe forms of coronavirus disease 2019 (COVID-19) [3, 4]. The adipose tissue produces and releases adipokines, bioactive molecules that participate in the inflammatory and metabolic processes responsible for insulin resistance and energy output [5]. These adipokines can be pro- or anti-inflammatory; the former are increased in obese subjects, leading to systemic inflammation [6]. Therefore, the systemic inflammation present in obesity could be the pathogenic link between this disease and other inflammatory disorders [5].

Orthodontic tooth movement (OTM) occurs in response to mechanical forces on the supporting periodontal tissues, consisting of the alveolar bone, periodontal ligament (PDL), gingiva, and cementum. The PDL and alveolar bone cells are sensitive to mechanical loading, promoting genome-environment communication; for this reason, these cells play an essential role in OTM [7]. The PDL is a connective tissue that consists predominately of fibroblasts [8]. It is an important and complex structure that transmits and absorbs mechanical stress and provides a vascular supply and nutrients to the surrounding tissues [9]. During OTM, the mechanical forces induce the production of biological signals by cells of the PDL, such as fibroblasts and osteoclasts, generating an aseptic local inflammatory response through the release of several proinflammatory cytokines [10-12], which enables tooth movement via bone remodeling [13, 14].

There is evidence that obese animals submitted to OTM present greater inflammation of periodontal tissues and more alveolar bone loss by increased osteoclastogenesis than normal-weight animals [15, 16]. In addition, recent in vitro studies demonstrated that leptin, a proinflammatory adipokine overexpressed in obese individuals, increases the expression of proinflammatory factors, such as interleukin-6 (IL-6) and prostaglandin-endoperoxidase synthase- 2 (PTGS2), in human periodontal ligament fibroblasts (hPDLF) submitted to mechanical strain [17] and influences the effect of compressive forces on cementoblasts, increasing the cell apoptosis rate [18].

Considering the increased prevalence of obesity worldwide [1] and the previous evidence of its influence on periodontal tissue remodeling during OTM, studies evaluating the molecules and mechanisms involved in this biomechanical process are

an important point for in-depth investigation. In this context, this study evaluated the influence of obesity on the proteomic profile of PDL tissues of rats submitted to orthodontic force.

MATERIAL AND METHODS

Experimental design

This study was conducted on the same animals as a previously published study from our group, which included lipidic, serum, histological, microtomographic, and gene expression results [16]. The study was approved by the Ethical Committee on Animal Experimentation of Sao Paulo State University, School of Dentistry at Araraquara, Brazil (protocol number 16/2015) and followed the Animal Research: Reporting of In Vivo Experiments guidelines. Therefore, for this study, we used 10 male adult Holtzman rats (average weight 300 g, 8 weeks old). Animals were randomly assigned to one of the following experimental groups: 1 – M group: animals received a standard diet, and 75 days later the first maxillary molar was randomly selected to be submitted to OTM for 15 days ($n = 5$), and 2 – OM group: animals received a high-fat diet to induce obesity, and 75 days later the first maxillary molar was randomly selected to be subjected to OTM for 15 days ($n = 5$) (Fig. 1A). All animals housed at a controlled temperature (22-25 °C) and received water ad libitum with a 12-hour light/dark cycle.

Obesity induction

To induce obesity, animals of the OM group received a high-fat diet. In contrast to the standard diet (Labina/Purina, Ribeirão Preto, Brazil) administered to the M group, which presents around 2.25 kcal/g and 22 g of protein, 48 g of carbohydrate, 4 g of total fat, 8 g of fiber, and 200 mg of sodium per 100 g of diet [19], the high-fat diet contained around 3.82 kcal/g; was composed of 27.5% of standard rat chow plus 25% peanuts, 25% milk chocolate, and 12.5% sweet biscuits; and contained 20 g of protein, 48 g of carbohydrate, 20 g of total fat, and 4 g of fiber per 100 g of diet [20]. This diet was started 75 days before baseline (M initiation) and feed replaced daily (Fig. 1A) [16].

Orthodontic tooth movement

The procedure to induce OTM was performed as previously described [16, 21, 22]. General anesthesia was performed by intramuscular injection of ketamine

hydrochloride 10% (0.08 mL/100 g body weight) and xylazine hydrochloride 2% (0.04 mL/100 g body weight). A closed-coil nickel-titanium spring (Sentalloy, GAC Central Slip, New York, USA) connected the maxillary first molar and central incisors with a steel wire (CrNi, 0.20 mm, 55.01.208; Morelli, Sorocaba, Brazil). To position the spring, grooves were prepared around the incisor, and a thin layer of composite resin was used to stabilize the system, whereas the spring was positioned on the occlusal surface of the first molar and stabilized using composite resin. To avoid occlusal interference, the lower first molars were extracted [16, 22, 23]. The nickel-titanium spring provided a constant force of approximately 25 g during all the experiments.

Sample processing, laser capture microdissection, and protein extraction

Fifteen days after OTM, animals were euthanized by anesthetic overdose (Fig. 1A). This period was selected on the basis of previous studies by our group, as it represents a period in which significant tooth movement occurs [16, 21, 22]. Following euthanasia, the maxilla of each animal was collected and histologically processed as previously described [24]. Tissues were fixed in 10% buffered formalin (Fisher Diagnostics, Middletown, VA, USA) at 4 °C for 24 hours, rinsed three times in phosphate-buffered saline (PBS, pH 7.4, Applied Biosystems, Foster City, CA, USA) for 30 min at 4 °C, and decalcified in 20% ethylenediaminetetraacetic acid (EDTA, Merck/Millipore, Burlington, MA, USA) for 30 days at 4 °C under agitation. The maxillae were trimmed, washed in PBS for 30 minutes at 4 °C, dehydrated in 100% ethanol (Sigma-Aldrich, San Luis, MO, USA) at 4 °C for 30 minutes, diaphanized in xylene (Arcturus Bioscience, San Francisco, CA, USA) for 20 minutes at room temperature, and embedded in paraffin (Merck/Millipore, Burlington, MA, USA). Serial parasagittal 5- μ m-thick sections of the first molar were obtained and mounted on polyethylene naphtholate membrane glass slides (PEN, Applied Biosystems, Foster City, CA, USA) (Fig. 1B). Sections were deparaffinized in two changes of xylene (Arcturus Bioscience, San Francisco, CA, USA) for 2 minutes and one change for 5 minutes and air-dried for 5 minutes. Sections were used immediately for microdissection. Tissues of the PDL were microdissected as previously described [25] (Fig. 1B). Eight to ten sections per sample were microdissected and the total captured area was calculated and used to normalize the amount of tissue/protein for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Caps containing the captured tissues were incubated with 30 μ L of 8 M urea (Sigma- Aldrich, San Luis, MO USA) for 30 minutes

at room temperature for protein extraction. Samples were sonicated and centrifuged briefly. Whole protein extracts were reduced by incubation in 5 mM dithiothreitol (DTT, Sigma-Aldrich, San Luis, MO USA), alkylated with 14 mM iodoacetamide (Sigma-Aldrich, San Luis, MO USA), and digested by 2 µg of sequencing-grade modified trypsin (Promega, Madison, WI, USA), as described previously [24]. The resulting tryptic peptide samples were equilibrated to pH 2.0 with formic acid (Sigma-Aldrich, San Luis, MO USA). Peptide mixtures were desalted and purified using microcolumns (ZipTip[®] C18, P10 Pipette Tips, Merck Millipore, Billerica, Massachusetts, USA), dried in a vacuum concentrator, and reconstituted in 0.1% formic acid for analysis.

LC-MS/MS and bioinformatics analysis

Mass spectrometry analysis was performed in the BIOMASS Core Facility for Scientific Research of the University of São Paulo, Brazil. Peptide mixtures were analyzed on an LTQ-Orbitrap Velos ETD (Thermo Scientific, Waltham, Massachusetts, USA) mass spectrometer coupled to an Easy NanoLC II (Thermo Scientific, Waltham, Massachusetts, USA) ion source. Peptides were separated through a 2%-95% acetonitrile gradient in 0.1% formic acid using a C18 PicoFrit analytical column (C18 PepMap, 75 µm id × 10 cm, 3.5 µm particle size, 100 Å pore size; New Objective, Ringoes, NJ, USA), at a flow rate of 300 nL/min for 105 minutes. The LTQ-Orbitrap Velos was operated in positive ion mode with data-dependent acquisition. The full scan was obtained in the Orbitrap with an automatic gain control (AGC) target value of 10^{-6} ions and a maximum fill time of 500 ms. Each precursor ion scan was acquired at a resolution of 60,000 FWHM in the 400–1500 m/z mass range. Peptide ions were fragmented by CID MS/MS using a normalized collision energy of 35. The 20 most abundant peptides were selected for MS/MS and dynamically excluded for a duration of 30 seconds. The samples were analyzed in two technical replicates that were combined to perform the database search. All raw data were accessed in the Xcalibur software program (Thermo Fisher Scientific) and processed using Proteome Discoverer (version 1.4.0.288, Thermo Finnigan, San Jose, CA, USA). The MS/MS spectra (msf) generated from raw files were searched against the UniProt Rattus_norvegicus_Protein Database (released 2020_01 (26-Feb-2020); 178,316,438 entries), assuming trypsin digestion with parameters set to a maximum of two missing cleavages sites, with a parent ion tolerance of 20.0 ppm for the MS search and a

fragment ion mass tolerance of 0.6 Da. Oxidation of methionine (+ 16 Da) was set as a dynamic modification, and carbamidomethylation in cysteine residues (+ 57 Da) was set as a static modification. Proteins with at least two valid values in at least two samples were considered for further analysis. An independent samples t-test was applied to test for differences in protein intensities between the M and OM sample groups. Protein ratios were calculated from the average of normalized spectral protein intensities. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [26] partner repository with the dataset identifier PXD033647. Principal Component Analysis (PCA) was performed to determine PDL sample variability between the proteome profiles of both groups. A volcano plot was generated to represent the differentially abundant proteins in the PDLs of each group. PCA and volcano plot generation were carried out using the R software. For PCA, the raw values were converted to the log₂ scale followed by use of the R statistical software function “prpcomp” to determine the values of the principal components, and the “ggfortify” package (from “ggplot2”) for graphical representation. The volcano Plot was constructed using the “plot” function in R statistical software. The plot consists of p values converted to the “-log₁₀” scale on the Y-axis and log₂(fold change) values on the X-axis. The fold change values were calculated using the following criteria: (1) When the mean of raw values of M is zero, fold change is equal to the mean of OM; (2) When the mean of raw values of OM is zero, fold change is equal to - (mean of M); (3) When the result of the equation (mean of OM / mean of M) is < 1, and the fold change is equal to -(mean of M/ mean of OM); otherwise, the value of fold change is (mean of OM /mean of M). When log₂(fold change) is greater than zero, the protein is more abundant in OM, otherwise, it is more abundant in M. Sample clustering and heat map graphic organization were performed by converting NSC into z-scores and submitting them to the EXPANDER software. Gene ontology (GO) enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway assignment were performed using the Database for Annotation, Visualization, and Integrated Discovery platform (DAVID v 6.8, released 2020-11-26; <https://david.ncifcrf.gov>). Differential abundance of proteins was determined by t-test followed by the Benjamini-Hochberg multiple test correction. The fold change between differentially regulated proteins was also determined. All statistical tests were calculated using a $p \leq 0.05$, and only statistically significantly enriched GO terms were reported in the text. The protein- protein interactions of proteins with differential OM

abundance was evaluated using the Protein-Protein Interaction Networks Functional Enrichment Analysis platform (STRING v 11.0, released 2020-11-26; <https://string-db.org>) with high confidence (0.700) [27].

Immunohistochemistry

Selected proteins identified in the PDL tissues by proteomic analysis were confirmed by immunohistochemistry (IHC) using additional histological sections from the animals assessed by LC-MS/MS (n = 5 animals/group). IHC was performed on 5- μ m-thick mesiodistal paraffin sections (two sections per animal) mounted on silanized slides (Perfecta, Sao Paulo, SP, Brazil), using an avidin- biotinylated peroxidase enzyme complex (ABC)-based kit (Vector Labs, Burlingame, CA, USA). The sections were incubated with primary antibodies, including: VCL/Vinculin (1:50, LS-B5770, LifeSpan BioSciences), SPP1/Osteopontin (1:100, LS-B425, LifeSpan BioSciences) and CTSD/Cathepsin D (1:50, LS-B9108, LifeSpan BioSciences) at 4 °C overnight. Negative controls lacking primary antibodies were performed. Slides were stained with 3,3'- diaminobenzidine (Dako, Glostrup, Denmark) as a chromogen substrate and counterstained with Carazzi's hematoxylin. These analyses were performed by a blinded and calibrated examiner using Leica DMLS microscopy (Leica DMLS; LeicaReichert Diastar Products & Jung, Wetzlar, Germany) with a magnification of 400x. The areas of interest were selected and photographed by a digital camera (Leica DFC 300 FX; Leica– Reichert Diastar Products & Jung, Wetzlar, Germany), and the captured images were archived in TIFF (Tagged Image File Format) format. The number of positive cells was measured using an image-analysis system (Image J 1.37b National Institutes of Health, USA) (Fig. 5A).

Statistical analysis

The statistical t-test with a Bonferroni test correction was used to determine the differential protein abundances on normalized spectral count values ($p \leq 0.05$). Fold-change was calculated as the ratio of OM/M values. For the IHC analysis, intergroup comparisons were performed using the Shapiro-Wilk Student test for normality, and to determine any significant differences between groups, a Student's t-test was used (Graph- Pad Prism 9 Software: Inc, La Jolla, San Diego, CA, USA) at a significance level of $p \leq 0.05$.

RESULTS

Proteomic analysis of PDL

Considering the pivotal function of PDL cells in periodontium remodeling induced by OTM, proteomic analysis was specifically focused on the PDL tissues, aiming to identify molecules involved in the regulation of that biomechanical process. A total of 796 proteins were identified, and 630 (79%) of them were found in both M- and OM-derived PDL samples (Supplemental Table S1), 125 (16%) exclusive to OM (Supplemental Table S2), and 41 (5%) exclusive to M (Supplemental Table S3), as shown in the Venn diagram (Fig. 2A). Interestingly, we found 109 (14%) differentially abundant proteins, and among these, 49 proteins presented differential abundance in the OM group ($p \leq 0.05$) (Supplemental Table S4) and 60 in the M group ($p \leq 0.05$) (Supplemental Table S5). In the present investigation, PCA analysis was performed to evaluate intrinsic variation among samples and showed that OM-derived PDL samples were highly homogeneous, whereas M-derived PDL samples featured a level of heterogeneity (Fig. 2B). Proteins with a statistically significant ($p \leq 0.05$) > 1.5-fold difference in the M versus OM PDL samples are included in the volcano plot (Fig. 2C, Supplemental Tables S4, and S5). A heatmap analysis illustrates the top 50 differentially regulated proteins (Fig. 2D-F), showing the abundance profile among the M and OM samples.

Characterization of differentially abundant proteins in OM group

Protein-protein interactions among all differentially abundant proteins in the OM group were analyzed using STRING software. Strong protein-protein interactions were displayed at a high confidence level (0.700). The proteins Cttd, Lysozyme C-1 (Lyz2), Vcl, and Importin subunit beta-1 (Kpnb1) showed strong interactions amongst them, while the protein Spp1 presented a strong interaction with Integrin beta-1 (Itgb1). Both proteins Spp1 and Vcl showed a strong interaction with the Fibrinogen alpha chain (Fga) (Fig. 3A, Supplemental Table S6). The identification of protein-protein interactions plays an important role in revealing the principles of cellular organization and function. Proteins without any interactions did not appear in the network (Fig. 3A).

Biological characterization of OM differentially abundant proteins

The KEGG pathway assignment of differentially abundant proteins present in the OM group were analyzed using DAVID Software. As a result, the following pathways were

found: “tuberculosis (rno05152),” “phagosome (rno04145),” “lysosome (rno04142),” “platelet activation (rno04611),” “renin secretion (rno04924),” “alcoholism (rno05034),” “pertussis (rno05133),” “gastric acid secretion (rno04971),” “salivary secretion (rno04970),” “estrogen signaling pathway (rno04915),” “rap1 signaling pathway (rno04015),” “circadian entrainment (rno04713),” “melanogenesis (rno04916),” “glucagon signaling pathway (rno04922),” “amoebiasis (rno05146),” “leukocyte transendothelial migration (rno04670)” (Fig. 3B, Supplemental Table S7).

In the GO enrichment analysis, performed by DAVID software, identified proteins were analyzed according to cellular component (CC), biological process (BP), and molecular function (MF) terms (Supplemental Table S8 and S9). The most enriched GO CC terms, determined by Fisher’s Exact test and corrected by Benjamini-Hochberg multiple tests, with adjusted P-values ≤ 0.05 , were “alveolar lamellar body (GO:0097208)” featuring 3 proteins, “lysosomal membrane (GO:0005765)” and “late endosome (GO:0005770)” with 5 proteins for each term, “vesicle (GO:0031982)” with 6 proteins, “lysosome (GO:0005764)” containing 7 proteins, “membrane raft (GO:0045121)” presenting 8 proteins, “perinuclear region of cytoplasm (GO:0048471)” with 9 proteins, “extracellular space (GO:0005615)” containing 12 proteins, “cytosol (GO:0005829)” with 15 proteins, “membrane (GO:0016020)” featuring 19 proteins, “cytoplasm (GO:0005737)” presenting 28 proteins and “extracellular exosome (GO:0070062)” with a total of 37 proteins (Fig. 4A, Supplemental Table S9). Among the 37 proteins associated with “extracellular exosome (GO:0070062)” terms, Vcl, Cttd and Spp1 were chosen for IHC (Fig. 4B).

Subcellular localization and expression pattern of selected proteins determined by IHC analysis

The presence of the proteins Vcl, Cttd, and Spp1 in the PDL, identified by the proteomic analysis, was confirmed by IHC staining. This analysis showed a significant difference in the number of positive cells between the OM and M groups for all proteins (Fig. 5B- C), confirming that the representation pattern of these proteins in the PDL is higher in the presence of obesity during orthodontic tooth movement.

DISCUSSIONS

The influence of systemic conditions such as obesity on the OTM has been increasingly studied and elucidated [16, 17, 28-30]. Thus, proteome analysis of PDL tissues under OTM may provide the opportunity to expand our knowledge of such relevant biological events. In this context, to our knowledge, the present study is the first to demonstrate the effects of obesity on the proteomic profile of PDL tissues from rats submitted to OTM. Proteomic approaches have been used to determine the impact of various challenges on a given tissue and/or cells on their protein abundance profile and have already been demonstrated effective at providing valuable information to allow for a deeper understanding of biological processes [24, 31-34]. Some studies evaluated the proteome profiling of hPDLF [35, 36] and demonstrated how differences in the proteome profiling of human gingival fibroblasts (hGF) and hPDLF might reflect on the function of each tissue [35]. In orthodontics, proteomic methods were used to evaluate the saliva proteome profiles of patients during OTM [37, 38], and during different clinical conditions, e.g., orthodontically inflammatory root resorption [39], and under accelerated osteogenic orthodontic treatments [40]. On the other hand, Zhang and collaborators [41] performed plasma proteome profiling on patients submitted or not to controlled stress during OTM. Recently, a study by our group evaluated the effect of obesity on the proteomic profile of rats with periodontitis. The regulation of proteins with roles in inflammation and tissue remodeling, such as Spondin 1, Tartrate-resistant acid phosphatase (TRAP), and Vcl, were investigated [42].

An important aspect of our study was the use of laser capture microdissection (LCM) technology to separate the targeted tissue (e.g., PDL) from other periodontal tissues [43]. In a recent study by Lira Dos Santos and collaborators [44], they used LCM technology to separate the cellular cementum in teeth submitted to orthodontic movement in rats and identified a total of 168 proteins. This method was demonstrated to guarantee 95% cell purity and is used to study pathology and physiology and to discover unknown processes, contrasting diseases or health conditions [45, 46]. In our present study, the 796 proteins detected in PDL tissues confirmed the abundance of proteins obtained from microdissected samples, as proposed by Salmon and collaborators [24].

Our data demonstrate similarities between the samples of each group, which in turn were distinct from each other, as shown in the PCA graphic, confirming the homogeneity of our samples. Among all identified proteins, 49 were differentially

abundant in the OM group, indicating a potential influence of obesity on OTM. Those proteins were, therefore, the main target of our analysis.

Among the differentially abundant proteins found in the OM group, proteins involved in the phagosome (rno04145) and lysosome (rno04142) pathways were identified by KEGG analysis (Fig. 3B). These proteins are related to autophagy, a catabolic pathway associated with the cellular component self-degradation process that responds to extra- or intracellular stress and signals [47]. Previous studies have demonstrated that autophagy is related to orthodontic forces, regulation of the inflammatory response and bone turnover, and the PDL rearrangement during tooth movement [48, 49]. Furthermore, defects in autophagy homeostasis are related to metabolic disorders such as obesity and promote local or systemic metabolic dysregulation [50]. Therefore, the increased detection of proteins from these pathways in the present study may indicate that obesity is related to greater tissue remodeling, leading to an accelerated tooth movement. Furthermore, in our previous study, we observed, albeit without a statistical difference, a tendency toward greater tooth movement in the animals from the OM group than in the M group animals, corroborating our present proteomic findings [16]. When evaluating the same phenomenon in humans, consistent with our findings, Saloom and collaborators [51] demonstrated that obese patients presented a trend toward greater tooth movement. An increase in bone resorption, associated with a higher number of osteoclasts on the compression side during OTM in animals that received a high-fat diet, could be the reason for this accelerated tooth movement in obese subjects [15].

Our GO enrichment analysis identified that the CC term “extracellular exosome (GO:0070062)” was associated with the most proteins. Extracellular exosomes, also called exosomes, are vesicles produced and released by various types of cells. They are involved in several processes, such as autophagy, cellular homeostasis, immunomodulation, and inflammation [52]. Furthermore, exosomes are present in most body fluids; for this reason, they are demonstrated to serve as potential biomarkers [53, 54]. In orthodontics, exosomes contained up- and down-regulated proteins related to root resorption during tooth movement [55]. Corroborating this, in obesity, there is a higher release of exosomes and microvesicles from the adipose tissue into the systemic circulation, resulting in the induction of a proinflammatory microenvironment in adipose tissue and promoting insulin resistance [56].

In our STRING analysis, Cttd and Vcl showed a strong interaction between Lyz2 and Kpnb1. Lyz2, an inducible marker of tissue macrophage activation [57, 58] is associated with higher plasma levels in obese subjects [59]. Kpnb1 is a protein that mediates nuclear import and is most often associated with cancers, such as melanoma, lung cancer, gastric cancer, and lymphomas [60, 61]. Among these related proteins, we focused on certain proteins involved this interaction, such as Vcl and Cttd due to their relation to obesity and/or tooth movement. Conversely, Spp1 presented a strong interaction with Itgb1. Itgb1 belongs to the integrin family. This protein plays a role in cell adhesion via interaction with collagen and is an essential regulator of stem cell differentiation [62]. Itgb1 is related to osteoblast differentiation in response to tensile strain [63] and was shown to be expressed in adipocytes; in visceral adipose, its expression was negatively correlated with insulin sensitivity [64]. As Spp1 presented a strong interaction with Itgb1, we selected Spp1 for protein validation for its function in obesity and orthodontics. Osteopontin (Spp1), also known as secreted phosphoprotein-1, is a proinflammatory cytokine that is key to the cellular immune response [65] and is secreted by macrophages under inflammatory conditions, participating in the adhesion, migration, differentiation, and phagocytosis of monocytes [66-69]. This protein belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family, consisting of non-collagenous proteins involved mainly in bone metabolism [70]. Furthermore, Spp1 facilitates osteoclast adhesion to the matrix of bone tissue [69] and was demonstrated to be an important factor in bone remodeling during OTM, participating in osteoblast differentiation [71-73]. In obesity, Spp1 participates in the developing of insulin resistance, contributing to inflammation and accumulation of macrophages in adipose tissues [74, 75]. Furthermore, it was demonstrated that obese subjects presented a higher circulating Spp1 concentration than non-obese subjects [76]. These findings justify the higher presence of Spp1 in the PDL tissue from the OM group.

Cttd is a lysosomal aspartyl endopeptidase that breaks down proteins into several polypeptide fragments that digest other lysosomal endopeptidases and exopeptidases [77]. It is considered a biomarker for mammary cancer [78] due to its stimulatory effects on cancer cells, angiogenesis, and metastasis [79]. Furthermore, this protein is related to several inflammatory diseases, such as rheumatoid arthritis, Alzheimer's disease, and arteriosclerosis [80-82]. In obesity, corroborating our findings, Cttd plays a role in the control of adipogenesis due to its higher abundance during adipocytes

differentiation [83] and in the development of dyslipidemia, where, during hepatic inflammation, the inhibition of Ctsd activity improves lipidic levels, such as those of cholesterol and triglycerides [84].

Vcl is an actin-binding protein involved in extracellular matrix adhesion and the intracellular link between actin filaments [85, 86]. It participates in osteoclastogenesis, regulating osteoclast function [87]. Although Vcl regulation is reduced in diabetes [88], the opposite was observed in obesity [89]. Interestingly, Vcl regulation in obesity was reduced following administration of a bodyweight reducing agent [89]. Considering these previous data from literature and our present results, we suggest that the higher abundance of these proteins in the OM group is associated with obesity and favored higher bone remodeling activity. In addition, these data also corroborate with a previously published study from our group that used the same animals as this study. In this study, the OM group presented a tendency for faster tooth movement and decreased bone mineral density compared to the M group [16]. Based on these data, we can speculate that the chronic systemic inflammation in obesity may affect the local inflammatory condition induced by the orthodontic forces in the periodontal tissues through the increase of proteins such as Vcl, Spp1 and Ctsd, leading to an exacerbation of the inflammatory condition, increase in the osteoclast's activities and adipocytes differentiation. When a mechanical force is applied to the supporting periodontal tissues to promote tooth movement, the tooth shifts its position within the PDL space, resulting in PDL compression in some areas and PDL tension in others [90]. The mechanisms involved in these areas differ. A methodologic limitation of the present study is that we did not separate the PDL compression and tension areas to analyze the proteome profile. In addition, this was a preclinical study in animals, a degree of caution is necessary when extrapolating the present results to humans.

CONCLUSIONS

This is the first study to evaluate the proteome profile of PDL tissues during OTM and to provide evidence that obesity affects the proteomic profile of the PDL tissues during OTM. In addition, differentially abundant proteins in the obese group were identified in enriched GOs and pathways related to tissue remodeling, suggesting that obesity influences this biomechanical process during orthodontic treatment. The identification of these factors and proteins associated with obesity provides new information to increase understanding of the mechanisms involved in obese patients undergoing

orthodontic treatment and could thus lead to a more personalized orthodontic treatment approach.

AUTHOR'S CONTRIBUTION:

Camila Chierici Marcantonio: Conceptualization, Validation, Formal analysis, Investigation, Writing - Original Draft.

Maria Eduarda Scordamaia Lopes: Investigation, Writing - Review & Editing.

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Cristiane Ribeiro Salmon: Software, Writing - Review & Editing.

Francisco Humberto Nociti Junior: Conceptualization, Writing - Review & Editing.

James Deschner: Conceptualization, Writing - Review & Editing.

Joni Augusto Cirelli: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration.

Andressa Vilas Boas Nogueira: Conceptualization, Methodology, Writing - Review & Editing, Supervision.

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Figure 1. A) Study design and timeline of the experiment (OTM = orthodontic tooth movement). B) Image demonstrating the structures before and after laser capture microdissection (LCM), as well as the captured periodontal ligament (PDL) tissues. The red arrows show the empty space left by the microdissection.

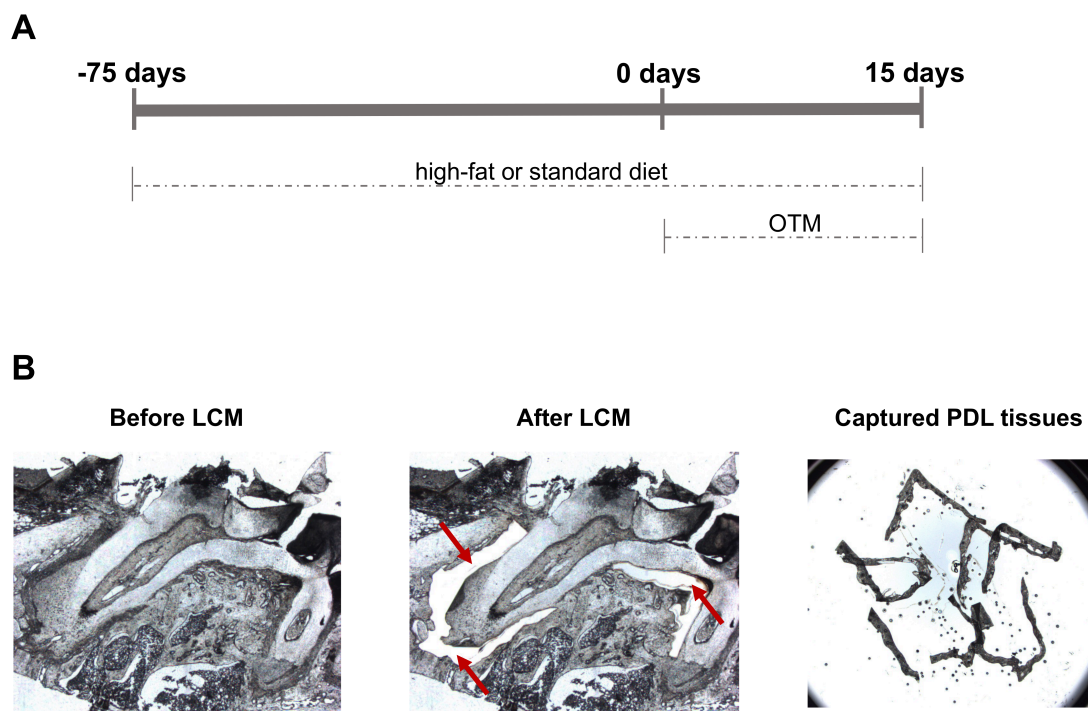


Figure 2. Protein profile summary of the M and OM groups. A) Venn diagram showing the distribution of the total proteins identified in both groups. B) Principal component analysis graph (PCA) showing the difference between the samples of the M (blue) and OM (yellow) groups and the similarity between the samples within each group. C) Volcano plot showing the distribution of significantly differentially abundant proteins determined by statistical t-test followed by Bonferroni correction ($p \leq 0.05$) in the PDL of animals from the M (left side - blue) and OM (right side - yellow) groups. D-F) Heatmap showing hierarchical clustering of top 50 up- and down-regulated proteins from both groups (M and OM), ranked by P-value ($p \leq 0.05$; t- test) and generated with Z-score values (from the normalized spectral counts values), applying the Euclidian distance method and average linkage.

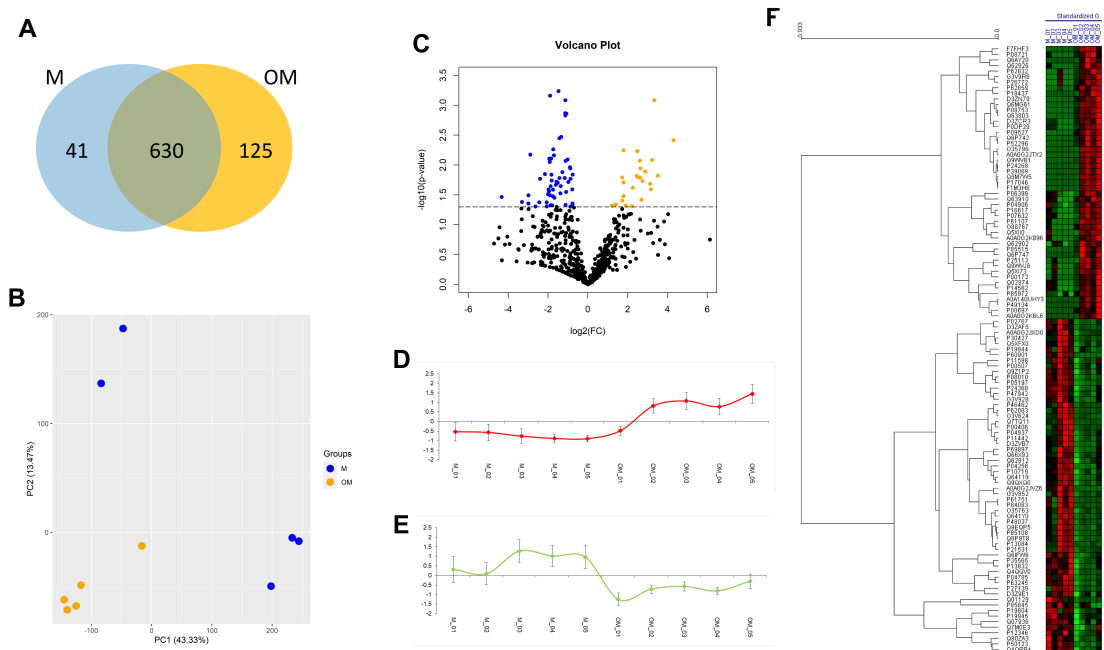


Figure 3. Analysis of differentially abundant proteins in the OM group. A) High-confidence (0.700) protein-protein interaction network analysis of the differential abundant proteins of the OM group (disconnected nodes in the network are hidden). B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for differentially abundant proteins in the OM group and the number of proteins associated with each KEGG pathway term.

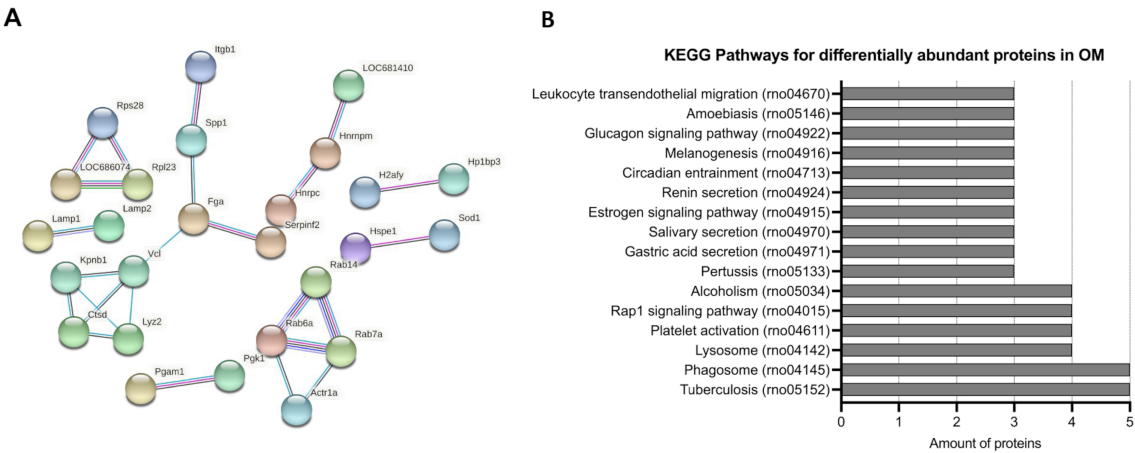


Figure 4. Gene Ontology (GO) enrichment term analysis of differentially abundant proteins of the OM group, obtained by DAVID software. A) Top enrichment GO of Cellular Component (CC) terms for differentially abundant proteins and the number of proteins associated with each GO enrichment term (Fisher's Exact test, followed by Benjamini-Hochberg multiple tests, adjusted P- value ≤ 0.05). B) Proteins associated with the GO CC enrichment term "extracellular exosome (GO:0070062)". The proteins in red were selected for immunohistochemistry.

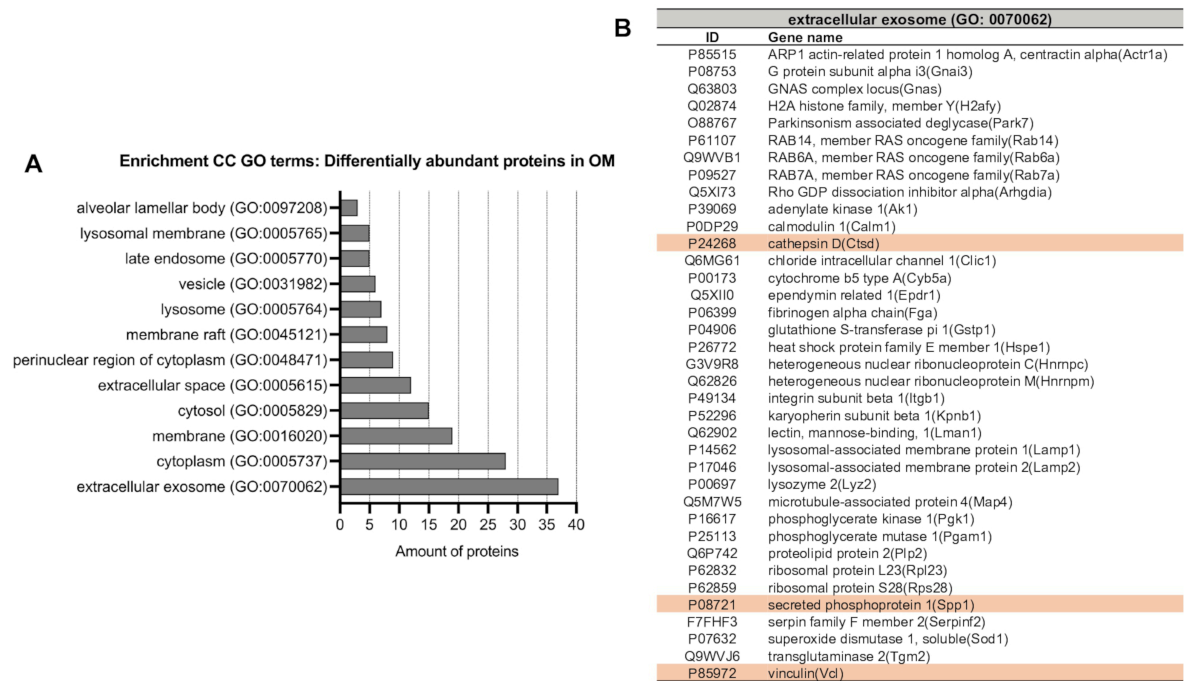
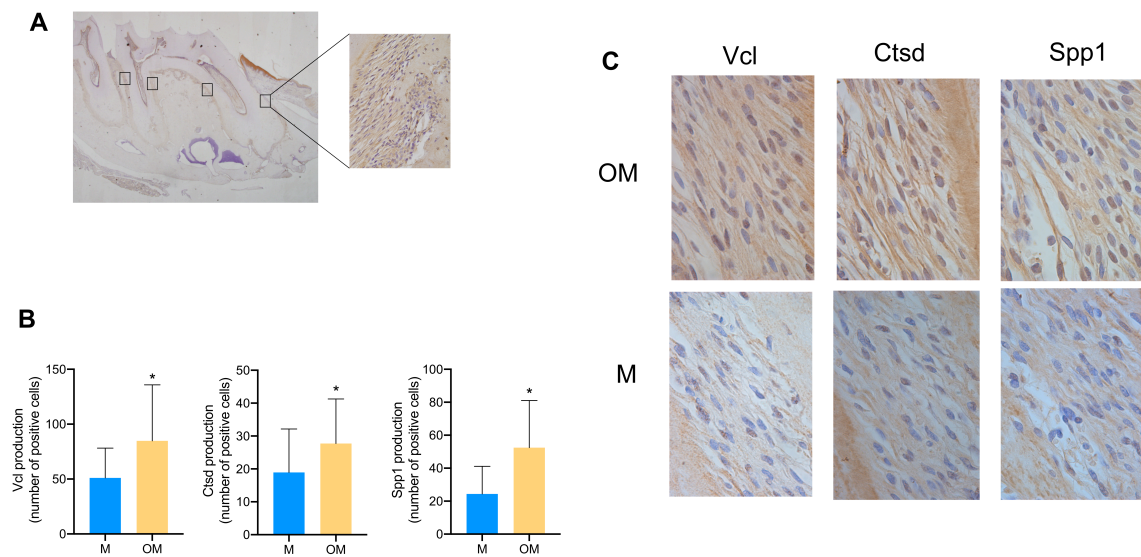


Figure 5. Validation of proteomic data by immunohistochemistry (IHC). A) Image demonstrating the IHC analysis. B) Graphs of mean and standard deviation (SD) showing the number of positive cells for Vinculin (Vcl), Cathpesin D (Ctsd), and Osteopontimn (Spp1) IHC staining. *Difference between groups OM and M ($p \leq 0.05$; t-test). C) Representative images (1,000x magnification) of IHC staining for Vcl, Ctsd, and Spp1, respectively, in tissue sections from the OM and M groups.



3.2 Publicação 2*

Influence of the monoclonal anti-sclerostin antibody on osteoporotic female rats' periodontal tissues during orthodontic tooth movement.

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* O presente estudo está em fase final de redação e será apresentado a seguir em formato de artigo.

ABSTRACT

Women's postmenopausal osteoporosis can modify the maxillary and mandibular bone tissue remodeling during orthodontic movement. The sclerostin expression and bone repair after treatment with Scl-Ab demonstrate that sclerostin inhibition is a viable approach for developing anabolic agents in diseases characterized by bone loss. Therefore, this study aims to evaluate the effect of a new drug based on an anti-sclerostin antibody (Scl-Ab) on ovariectomized rats' periodontal tissues during orthodontic tooth movement. A total of 48 rats were randomly distributed into 6 experimental groups (n=8/group): C (control), OM (orthodontic tooth movement), OVX (osteoporosis induction by ovariectomy surgery), OVX+OM, OVX+Scl-Ab (osteoporosis induction and Scl-Ab administration), and OVX+Scl-Ab+OM. All animals were submitted to OVX or Sham surgery (C and OM groups) 30 days before day 0 (baseline). Scl-Ab administration started on baseline and OM on day 7. The body weight was recorded weekly, and after 21 days, all animals were euthanized by anesthetic overdose. All the animals were evaluated for alveolar bone volume (BV/TV), bone density (BMD), trabecular bone thickness (Tb.Th), separation (Tb.Sp), and number (Tb.N) by *in vivo* computed microtomography analysis (micro-CT) after surgery and at baseline. After euthanasia, the maxillary was collected and separated into two hemimaxillae for BV/TV, BMD, Tb.Th, Tb.N, Tb.Sp, and tooth displacement analyses by Micro-CT. The gene expression of osteopontin, TNF α , IL-1 β , and RANKL in gingival tissues was evaluated. The measurement of collagen content in the periodontal ligament was performed in three regions of the distal root in all groups. All data were tested for normality by the Shapiro-Wilk test and analyzed by the One-Way ANOVA and Tukey's post-hoc tests, using a 5% significant level. A significant increase in body weight and a decrease in uterus weight were observed in all groups submitted to OVX. Furthermore, all ovariectomized animals significantly reduced BMD and BV/TV parameters 30 days after the surgery. The OM and OVX+Scl-Ab+OM groups showed a tendency for higher tooth movement when compared to the OVX+OM group. In addition, the OVX+Scl-Ab+OM group showed an increase in BMD and BV/TV compared to OVX+OM and a decrease in these parameters compared to the OVX+Scl-Ab group. The gene expression of Tnfa was significantly higher in the OVX+OM group compared to OVX and OVX+Scl-Ab+OM groups. In the pressure side of the periodontal ligament, animals from OM, OVX+OM, and OVX+OM+Scl-Ab groups presented a reduction in the collagen content compared to animals from the groups not submitted to orthodontic tooth movement. Our study showed that the Scl-Ab administration in ovariectomized rats reduces the harmful effects of osteoporosis on periodontal tissues submitted to orthodontic tooth movement.

KEYWORDS: Osteoporosis, Orthodontic Tooth Movement, Antibodies, Periodontium

INTRODUCTION

The orthodontic treatment aims to correct malocclusions by promoting tooth movement, which occurs in response to external mechanical forces, generating an unbalance of the physiological forces that maintain the tooth in its position^{1, 2}. This mechanism results in bone resorption on the pressure side and bone apposition on the tension side of the periodontal ligament. This bone remodeling occurs in the presence of fibroblasts, osteoclasts, osteoblasts, T lymphocytes, and other immune system cells³, leading to the release of several pro-inflammatory cytokines such as IL1b, IL6, and TNFa during orthodontic movement⁴⁻⁶. Therefore, mechanical forces are directly related to the bone condition⁷. Furthermore, exacerbated local or systemic inflammation may affect the periodontal remodeling during orthodontic movement^{8, 9}.

Postmenopausal osteoporosis is characterized by loss of bone mass in long bones and vertebrae and occurs due to estrogen deficiency. This condition causes an unbalance in bone remodeling, causing an increase in bone resorption disproportionate to bone formation¹⁰. Ovariectomized rats are a widely used model to mimic postmenopausal osteoporosis^{10, 11}. Rats submitted to ovariectomy (OVX) exhibit loss of bone mass and an increase in alveolar bone remodeling^{12, 13}. In addition, the estrogen deficiency during tooth movement in ovariectomized rats leads to an increase in OPG and RANKL expression¹⁴ and a consequent increase in the number of osteoclasts¹⁵.

Antiresorptive drugs, such as bisphosphonates, are the most used drugs for the treatment of postmenopausal osteoporosis¹⁶⁻¹⁹ and are related to serious adverse effects such as osteonecrosis of jaws, atypical femoral fractures, and atrial fibrillation^{16, 19}. During orthodontic treatment, these drugs act on the osteogenesis and structures of the periodontium, leading to a decrease in the amount of tooth movement^{20, 21}.

Recently, a new drug consisting of an anti-sclerostin monoclonal antibody (Scl- Ab) was approved to treat postmenopausal osteoporosis²². This drug promotes an increase in the activity and number of osteoblasts, augmenting bone formation²³⁻²⁶. The sclerostin expression and bone repair after treatment with Scl- Ab in postmenopausal women and animal models demonstrate that sclerostin inhibition is a viable approach for the development of anabolic agents in diseases characterized by bone loss²⁷⁻³¹.

Sclerostin is a glycoprotein encoded by the SOST gene primarily expressed in osteocytes³². This protein is antagonistic to the Wnt signaling pathway - one of the key

elements for bone formation. When activated, this pathway inhibits bone resorption by osteoclasts through induction of osteoprotegerin (OPG) expression by osteoblasts and osteocytes^{33, 34}. Therefore, sclerostin reduces bone formation and increases bone resorption³⁵⁻³⁷. Several factors can regulate sclerostin expression by osteocytes, such as mechanical stress, parathyroid hormone, or the presence of cytokines³⁸⁻⁴⁰. Sclerostin inhibition favors osseointegration and bone regeneration around dental implants showing to be a potential therapy to reduce the repair period after implant installation^{41, 42}. In addition, the systemic use of the Scl-Ab promotes bone and cementum regeneration in alveolar bone defects⁴³ and favors bone formation in periodontal disease⁴⁴. On tooth movement, sclerostin was shown to directly affect bone resorption when its deficiency was associated with a reduction of osteoblastic activity on the pressure side in SOST KO mice⁴⁵. Furthermore, when local injections of sclerostin were administered on the pressure side, it accelerated tooth movement in rats⁴⁶.

Studies have shown that osteoporosis can alter the bone tissue of the maxilla and mandible during orthodontic treatment^{47, 48}. Given that, studies to evaluate the effect of this new drug for osteoporosis on periodontal tissues during orthodontic movement are necessary. Therefore, this study aimed to evaluate the effect of a new drug for osteoporosis based on an anti-sclerostin antibody (Scl- Ab) on female rats' periodontal tissues during orthodontic movement.

MATERIAL AND METHODS

This study was approved by the Ethics Committee on Animal Experimentation (protocol number 38/2020). The sample size was calculated by ANOVA, based on the results of micro-CT analysis performed in a previous study by our group⁹. Forty-eight females adult Holtzman rats, with an average weight of 300 g, were used. These animals were maintained in the university's animal facilities in plastic cages, under controlled temperature (22–25 °C) with a 12-hour light/dark cycle and received a standard laboratory diet (Labina/Purina, Ribeirão Preto, Brazil) and water ad libitum. The animals were randomly assigned into six experimental groups: 1) control/without any intervention (C), 2) experimental orthodontic tooth movement (OM), 3) osteoporosis induction by ovariectomy surgery (OVX), 4) experimental orthodontic tooth movement and osteoporosis induction by ovariectomy surgery (OVX+OM), 5) osteoporosis induction by ovariectomy surgery and Scl-Ab administration (OVX+Scl-

Ab), 6) experimental orthodontic tooth movement, osteoporosis induction by ovariectomy surgery and Scl-Ab administration (OVX+Scl-Ab+OM). The animals' body mass was recorded weekly.

Osteoporosis induction by ovariectomy surgery (OVX)

Thirty days before baseline (Figure 1A), bilateral ovariectomies were performed under general anesthesia by intramuscular injection of ketamine hydrochloride 10% (0.08 mL/100 g body weight, Francotar, Virbac do Brasil Ind. Com. Ltda., Sao Paulo, SP, Brazil) and xylazine hydrochloride 2% (0.04 mL/100 g body weight, Virbaxyl 2%, Virbac do Brasil Ind. E Com. Ltda, Sao Paulo, SP, Brazil). Through small bilateral incisions in the skin and muscle layers, the ovaries of the rats were removed. Sham operations were performed in the C and OM groups, in which all procedures were the same, except for the removal of the ovaries. The animals received 0,1 ml/100g of penicillin by intramuscular administration in a single dose and 0,03 ml/100g of ketoprofen by subcutaneous administration twice a day for three days immediately after the surgery.

Monoclonal anti-sclerostin antibody (Scl-Ab) administration

At baseline, all animals from OVX+Scl-Ab and OVX+Scl-Ab+OM groups received a subcutaneous injection of monoclonal anti-sclerostin antibody (25mg/kg, Evenity[®], Scl-Ab VI, Amgen Inc., Thousand Oaks, CA, USA), twice weekly (Figure 1A). The animals from C, OM, OVX, and OVX+OM received a subcutaneous injection of PBS 1x, twice weekly. The dose of Scl-Ab was determined based on previous studies^{43, 44}. As the body weight was measured weekly, the drug doses were adjusted accordingly.

Orthodontic tooth movement

After 7 days from baseline, the procedure to induce OTM was performed as previously described^{9, 49} in the groups: OM, OVX+OM, and OVX+Scl-Ab+OM (Figure 1A). General anesthesia was performed as previously described. A closed-coil nickel-titanium spring (Sentalloy[®], GAC Central Slip, New York, USA) connected the maxillary first molar and central incisors with a steel wire (CrNi, 0.20 mm, 55.01.208; Morelli, Sorocaba, Brazil). To position the spring, grooves were prepared around the incisor, and a thin layer of composite resin was used to stabilize the system, whereas the spring was positioned on the occlusal surface of the first molar and stabilized using

composite resin. To avoid occlusal interference, the lower first molars were extracted^{9, 49}. The nickel-titanium spring provided a constant force of approximately 25 g during all the experiments. The animals received Paracetamol[®] 200 mg/Kg by oral administration immediately after the position of the springs.

Animal sacrifice and analyses

All animals were sacrificed after 21 days from baseline (Figure 1A). The maxillary jaws were collected for micro-computed tomography (micro-CT) analysis, aiming to evaluate mineralized periodontal tissues. Gingival tissue around the maxillary first molars was excised to analyze cytokine gene expression by reverse- transcription quantitative polymerase chain reaction (RT-qPCR). Uterus tissues were removed and weighed on a precision scale.

Micro-computed tomography analysis (micro-CT) on long bones

To confirm the establishment of osteoporosis by OVX, the animals of all groups were evaluated regarding bone volume fraction (BV/TV, %), bone mineral density (BMD), trabecular bone thickness (Tb.Th), trabecular bone separation (Tb.Sp) and trabecular bone number (Tb.N) by in vivo micro-CT, before OVX and 30 days after OVX (baseline). The animals were submitted to general intramuscular anesthesia as previously described in this paper, placed in lateral decubitus position, and had their right femur and tibia scanned using a microcomputed tomography device (μ CT Skyscan 1176; Bruker, Aartselaar, Kontich, Belgium). The x-ray generator was operated at 50 kVp, with beam current at 497 μ A, integration time of 360 ms, 0.5 mm aluminum filter, voxel size of 17.48 μm^3 , and an image resolution of 12.45. The images were reconstructed with the aid of specific software (NRecon version 1.6.1.5 - Bruker, Kontich, Belgium) in all three spatial dimensions, then oriented using Data Viewer software (Data Viewer version 1.4.3.1 - Bruker, Kontich, Belgium) and then analyzed using CTan software (CT Analyser version 1.10.1.0 - Bruker, Kontich, Belgium).

Micro-CT analysis on hemimaxillae

The hemimaxillae of all animals were fixed in 4% paraformaldehyde for 48 hours and stored in 70% ethanol before micro-CT scanning (μ CT Skyscan 1176; Bruker, Aartselaar, Kontich, Belgium). Scanning was performed using an x-ray generator

operating at 50 kVp, beam current at 497 μ A, with a 0.5 mm aluminum filter and image resolution of 18 μ m. The images were reconstructed, reorientated, and analyzed with the same software used on the micro-CT analysis of long bones. A region of interest (ROI) was delimited from the root apices of the first and second maxillary molars to the alveolar crest in the apical–coronal direction. In the mesial-distal direction, the limits were the mesial face of the second molars, and therefore all the alveolar bone around the first molars was included in the ROI. Tooth roots and periodontal ligaments were excluded from the ROI (Figure 1C). The microarchitecture parameters evaluated were bone volume (BV), tissue volume (TV), bone volume fraction (BV/TV, %), and bone mineral density (BMD). The BMD measurement was performed with phantom rods at densities previously established by the manufacturer (Bruker, Kontich, Belgium). To calculate tooth movement, the horizontal distance between the distal surface of the first molars and the mesial surface of the second molars was measured using the Data Viewer software (Data Viewer, v.1.4.3.1, Skyscan, Bruker, Kontich, Belgium) (Figure 1B).

RT-qPCR

Total RNA was extracted from the gingival tissues collected using a RNeasy Mini kit (RNAeasy Plus Mini kit catalog number 74134; Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration was determined using the Synergy H1 Hybrid Reader (Biotek, Winooski, VT, USA) and 500 ng of total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (High-Capacity cDNA Reverse Transcription Kit catalog number 4368814; Applied Biosystems, Waltham, USA) according to the manufacturer's instructions. The gene expression of osteopontin (Spp1) (Rn01449972-M1), Rankl (Rn00589289-M1), Tnfa (Rn01525859_g1), Il1b (Rn00580432_m1), and Gapdh (Rn01775763-G) was analyzed by RT-qPCR in a qPCR thermocycler (StepOnePlus Real-Time PCR System, Thermo Fisher Scientific, Waltham, MA, USA), using TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, Foster City, CA, USA) systems and specific primers (TaqMan® Gene Expression Assays, Thermo Fisher Scientific, Foster City, CA, USA). Amplification was carried out under the following conditions: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and combined annealing/extension at 60 °C for 1 min. Data were analyzed using the comparative cycle threshold (Δ CT) method.

Measurement of collagen content in the periodontal ligament

For the collagen content estimation in the periodontal ligament, the sections were stained with 0.1% picrosirius red solution and analyzed under light microscope (BX51, Olympus) equipped with filters to provide polarized illumination. In each specimen, the content of collagen was measured in the pressure and tension sides of the periodontal ligament from distal root of first molars. Three standardized areas (cervical, middle, and apical portions) of each side (pressure and tension) of the periodontal ligament were captured at 400x.

Birefringent collagen content was obtained using an image analysis software (ImageJ®, <http://rsbweb.nih.gov/ij>) according to the methodology described by Manni et al⁵⁰. Data of each hue (red, orange, yellow and green) was determined as percentage of the total number of collagen pixels which in turn was expressed as a percentage of the total number of pixels in the image⁵¹.

Statistics

Analyses were performed using GraphPad Prism Software (Graph-Pad Prism 9 Software: Inc, La Jolla, San Diego, CA, USA). The Shapiro-Wilk test was used to confirm the normal distribution of all data ($p > 0.05$). One-way analysis of variance (ANOVA) was followed by a post hoc Tukey's test for multiple comparisons among groups ($p \leq 0.05$).

RESULTS

OVX was effective in promoting osteoporosis induction

Osteoporosis was confirmed by in vivo micro-CT, body weight, and uterus weight analysis. Animals submitted to OVX presented a significant reduction in BMD, BV/TV, Tb.Th, Tb.Sp, and Tb.N parameters 30 days after the surgery (Figure 2A). These data demonstrate the bone changes in the tibia and femur bones after osteoporosis induction. Furthermore, a significant increase in body weight and a decrease in uterus weight were observed in all groups submitted to OVX (Figures 2B and 2C).

Scl-Ab administration improves bone volume and density parameters in OVX animals associated with orthodontic tooth movement

The animals in the OVX+Scl-Ab+OM and OM groups presented significantly high values of BV/TV and BMD in comparison to the OVX+OM group ($p \leq 0.05$), indicating

an increase in bone volume and density parameters around the first maxillary molars in OVX animals submitted to orthodontic tooth movement that received Scl-Ab administration (Figure 3A). In addition, the OVX+Scl-Ab+OM group showed a significant decrease in those parameters compared to the OVX+Scl-Ab group ($p \leq 0.05$). In the OVX+OM group, there were significantly lower bone volume and density parameters and significantly higher values in the Tb.Sp parameter compared to C, OVX and OVX+Scl-Ab groups ($p \leq 0.05$). In addition, the Tb.Th parameter was significantly lower in all groups submitted to orthodontic tooth movement (OM, OVX+OM, and OVX+Scl-Ab+OM) compared to all other groups (C, OVX, and OVX+Scl-Ab) ($p \leq 0.05$).

No significant difference was found in the amount of tooth displacement between groups submitted to orthodontic tooth movement. However, the OM and OVX+Scl-Ab+OM groups showed a tendency for higher tooth movement when compared to the OVX+OM group (Figure 3B).

Scl-Ab administration did not affect gene expression

There was no statistically significant difference in the gene expression of Spp1, Il1b, and Rankl between the groups (Figure 4). The gene expression of Tnfa was significantly higher in the OVX+OM group compared to OVX and OVX+Scl-Ab+OM groups ($p \leq 0.05$) (Figure 4).

The administration of Scl-Ab did not change the collagen content in the periodontal ligament

The groups submitted to orthodontic tooth movement (OM, OVX+OM, and OVX+OM+Scl-Ab) presented a significant reduction ($p \leq 0.05$) in the collagen content in the pressure side of the periodontal ligament compared to animals from the groups not submitted to orthodontic tooth movement (C, OVX and OVX+Scl-Ab) (Figure 5). In the tension side, the quantitative analysis showed a significant reduction in the collagen content estimation on animals from the C group compared to animals from OVX+OM and OVX+Scl-Ab groups ($p \leq 0.05$) (Figure 5).

DISCUSSION

The new drug based on Scl-Ab is being recommended to treat postmenopausal osteoporosis^{22, 52, 53}. However, there are little published data about the effects of the

systemic administration of Scl-Ab in dentistry. In this context, to our knowledge, the present study is the first to evaluate the effects of systemic administration of Scl-Ab in the periodontal tissues of ovariectomized rats during orthodontic tooth movement.

It is well established in the literature that ovariectomy surgery is effective in mimicking postmenopausal osteoporosis in rats^{10, 11}. To confirm the development of osteoporosis, the animals in the current study were weighed weekly, and at the time of sacrifice, the uterus was collected and weighed on a precision scale. Furthermore, 30 days after ovariectomy, the tibia and femur from all animals were evaluated by *in vivo* micro-CT analysis regarding BV/TV, BMD, Tb.Th, Tb.Sp, and Tb.N. From the first week onwards, animals submitted to OVX presented higher body weight than animals without OVX (Figure 2B). Meanwhile, animals from these groups presented higher uterus weight than animals from the other groups (Figure 2C). These data demonstrate the effectiveness of our methodology in inducing osteoporosis⁵⁴. Furthermore, 30 days after surgery, all OVX rats presented lower micro-CT parameters values than no-OVX animals (Figure 2A), confirming a significant worsening in bone quality.

The literature diverges on the ideal period after OVX to wait for the osteoporosis establishment⁵⁴⁻⁵⁸. After 30 days from OVX, the distobuccal root of rats' maxillary first molars presented a decrease in BV/TV and BMD parameters compared to animals without OVX⁵⁴. At this same timepoint, Sirisoontorn et al⁵⁹ found that OVX animals presented a higher volume of root resorption craters in all roots from the maxillary first molar; otherwise, Seifi et al⁶⁰ demonstrated a lower surface of root resorption craters after orthodontic force in the OVX group.

During orthodontic movement, the periodontal tissues undergo complex biological changes when submitted to mechanical force, characterized mainly as an aseptic inflammatory condition, which results in bone resorption on the pressure side and bone apposition on the tension side². Osteoporosis increases bone loss in long bones, spinal column, and alveolar bone⁶¹. Therefore, as expected, the OVX+OM group presented a decrease in BMD and BVTV, and an increase in Tb.Sp when compared to C, OVX, and OVX+Scl-Ab groups, as demonstrated by micro-CT analysis. Diverging from the literature⁶², though no significant statistical difference, OVX+OM presented lower tooth movement compared to OM and OVX+Scl-Ab+OM groups. However, in the picosirius staining, animals without tooth movement (C, OVX, and OVX+Scl-Ab groups) had significantly higher amounts of collagen on the pressure side than animals with experimental tooth movement (OM, OVX+OM, and OVX+Scl-Ab+OM groups). These

data corroborate with previous studies from our group that used the same methodology to induce tooth movement^{9,49}. Although both osteoporosis and Scl-Ab administration have a role in bone metabolism⁶³, the collagen content was not affected by OVX and Scl-Ab.

Our main findings were that animals from OM and OVX+Scl-Ab+OM groups presented similar BV/TV and BMD parameters, according to the micro-CT analysis. Additionally, animals from these groups showed higher BV/TV and BMD values than the OVX+OM group. Together, these data suggest that the systemic administration of Scl-Ab reduced the harmful effects of osteoporosis on periodontal tissues submitted to orthodontic tooth movement. Although gingival tissue is widely used in the literature to analyze gene expression^{8, 9, 64}, we did not find significant statistical difference in the RT-qPCR analysis when we investigated genes that have roles specially in bone metabolism, as Rankl and Spp1. Probably the overexpression of these molecules may have occurred during the first few days of mechanical stimulus. When we investigated pro-inflammatory genes in the RT-qPCR analysis, as Il1b and Tnfa, we found a significant increase on Tnfa expression in the OVX+OM group compared to OVX and OVX+Scl-Ab. Although no significant difference, the Il1b expression was either higher in the OVX+OM group. Both Il1b and Tnfa are involved in osteoclastogenesis⁶⁵. During initial stages of orthodontic movement, Il1b was found to be one of the most abundant cytokines on the compression side and induce the expression of COX-2^{66,67}. Additionally, Tnfa can directly bind to TNF receptor-1 to induce Rankl expression of osteocytes by increased sclerostin expression⁶⁸. Therefore, these results are in line with the lower BV/TV observed in the OVX+OM group compared to the other groups with orthodontic movement.

A methodologic limitation of the present study is that we used a humanized Scl- Ab, having immunogenic properties in rodents. Therefore, anti-drug antibodies (ADA) analysis of each animal serum would be necessary to guarantee that the drug was effective in our animals^{69,70}. However, considering our results and from other studies that used the same drug in rats⁷⁰⁻⁷³, a significant effect of the drug on the animals can be noticed. In addition, this was a preclinical study carried out in animals with induced postmenopausal osteoporosis and orthodontic tooth movement; therefore, a degree of caution should be considered when extrapolating the present results to humans.

CONCLUSION

The results of the present study showed that the Scl-Ab administration in ovariectomized rats seems to improve the alveolar bone quality, guaranteeing normal orthodontic tooth movement and reducing the harmful effects of osteoporosis on periodontal tissues submitted to orthodontic tooth movement.

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FIGURES

Figure 1. A) Study design and timeline of the experiment (OVX = ovariectomy; OM = orthodontic tooth movement; Scl-Ab = anti-sclerotin antibody administration). **B)** Representative image showing the selected region in the interproximal area between the first and second molars used for the micro-CT measurement of tooth movement analysis by Data Viewer software. **C)** Image demonstrating the region of interest (ROI) used for the analysis of micro-CT measurement of alveolar bone volume (BV/TV), bone density (BMD), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N) and trabecular bone separation (Tb.Sp) using a specific tool (custom processing) from CTAn software, excluding the roots and the periodontal ligament.

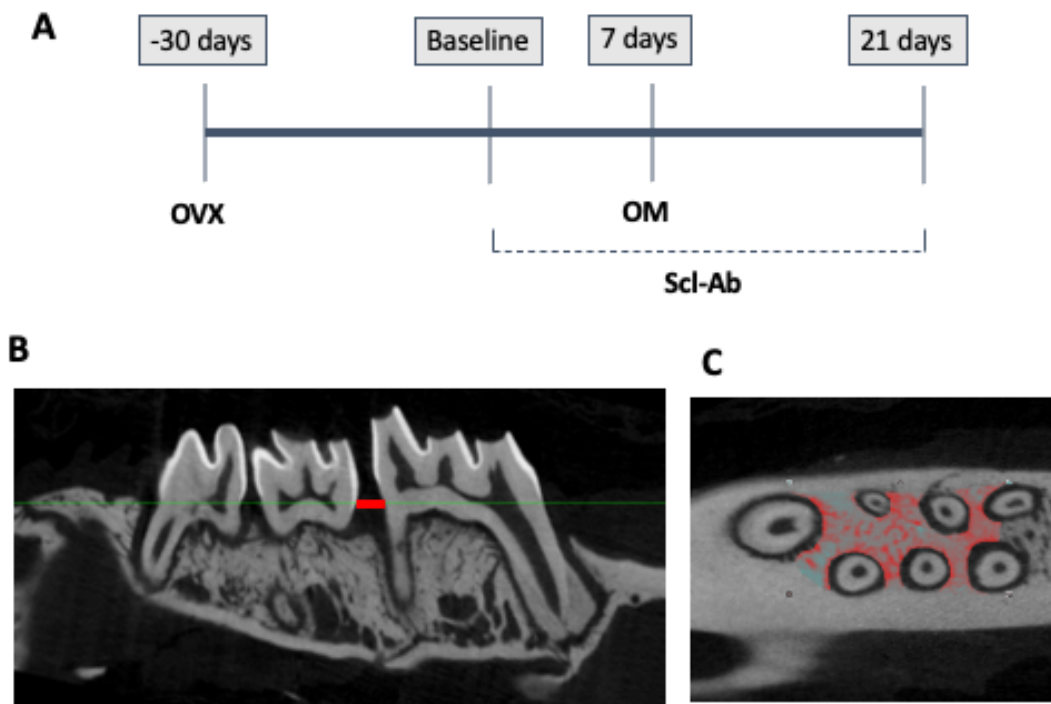


Figure 2. A) Mean and standard deviation (SD) micro-CT analysis of alveolar bone volume (BV/TV), bone density (BMD), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N) and trabecular bone separation (Tb.Sp) in tibia and femur of all animals submitted or not to ovariectomy (OVX), before (-30d) and 30 days after surgery (baseline). **B)** Mean and SD body weight of animals submitted or not to OVX during the experiment. **C)** Mean and SD uterus weight of animals from all groups after euthanasia. *Significant difference between ovariectomized (OVX) and non-ovariectomized (no OVX) animals ($p \leq 0.05$). **Significant difference with the groups: OM, OVX, OVX+OM, OVX+Scl-Ab, and OVX+Scl-Ab+OM ($p \leq 0.05$). #Significant difference with the groups: C, OVX, OVX+OM, OVX+Scl-Ab, and OVX+OM+Scl-Ab ($p \leq 0.05$).

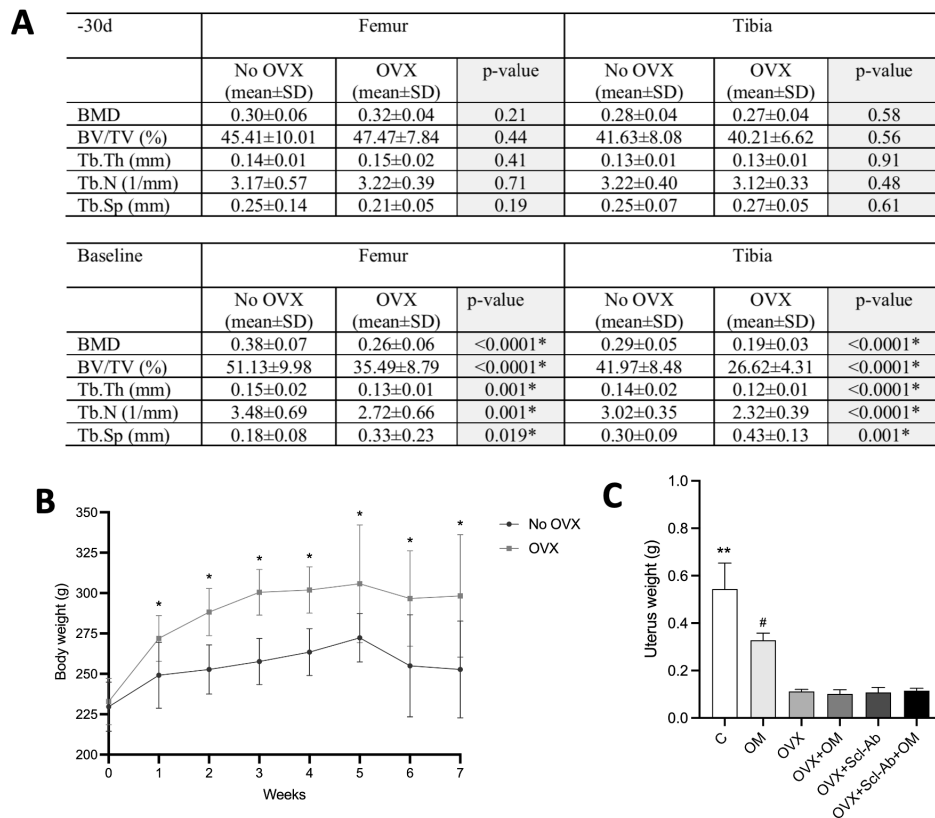


Figure 3. A) Three-dimensional occlusal, lingual, and facial micro-CT views of maxillary molars of representative animals from each group C, OM, OVX, OVX+OM, OVX+Scl-Ab, and OVX+Scl-Ab+OM. **B)** Mean and SD comparison of trabecular bone number (Tb.N) (μm), trabecular bone thickness (Tb.Th) (μm), trabecular bone separation (Tb.Sp) (μm), bone volume (BV/TV) percentage, bone density (BMD) (mg/cm^3), and first molar displacement (μm) in micro-CT analysis. *Significant difference with the C, OVX, and OVX+Scl-Ab groups ($p \leq 0.05$). **Significant difference with the OVX+Scl-Ab+OM group ($p \leq 0.05$). #Significant difference with the OVX+OM group ($p \leq 0.05$).

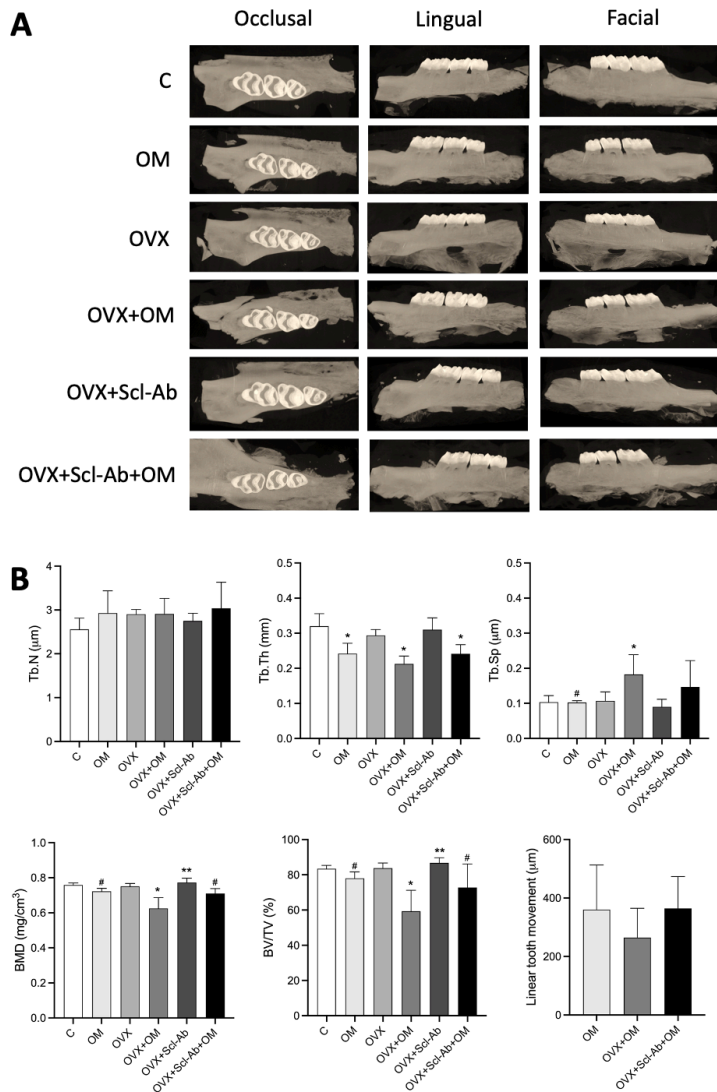
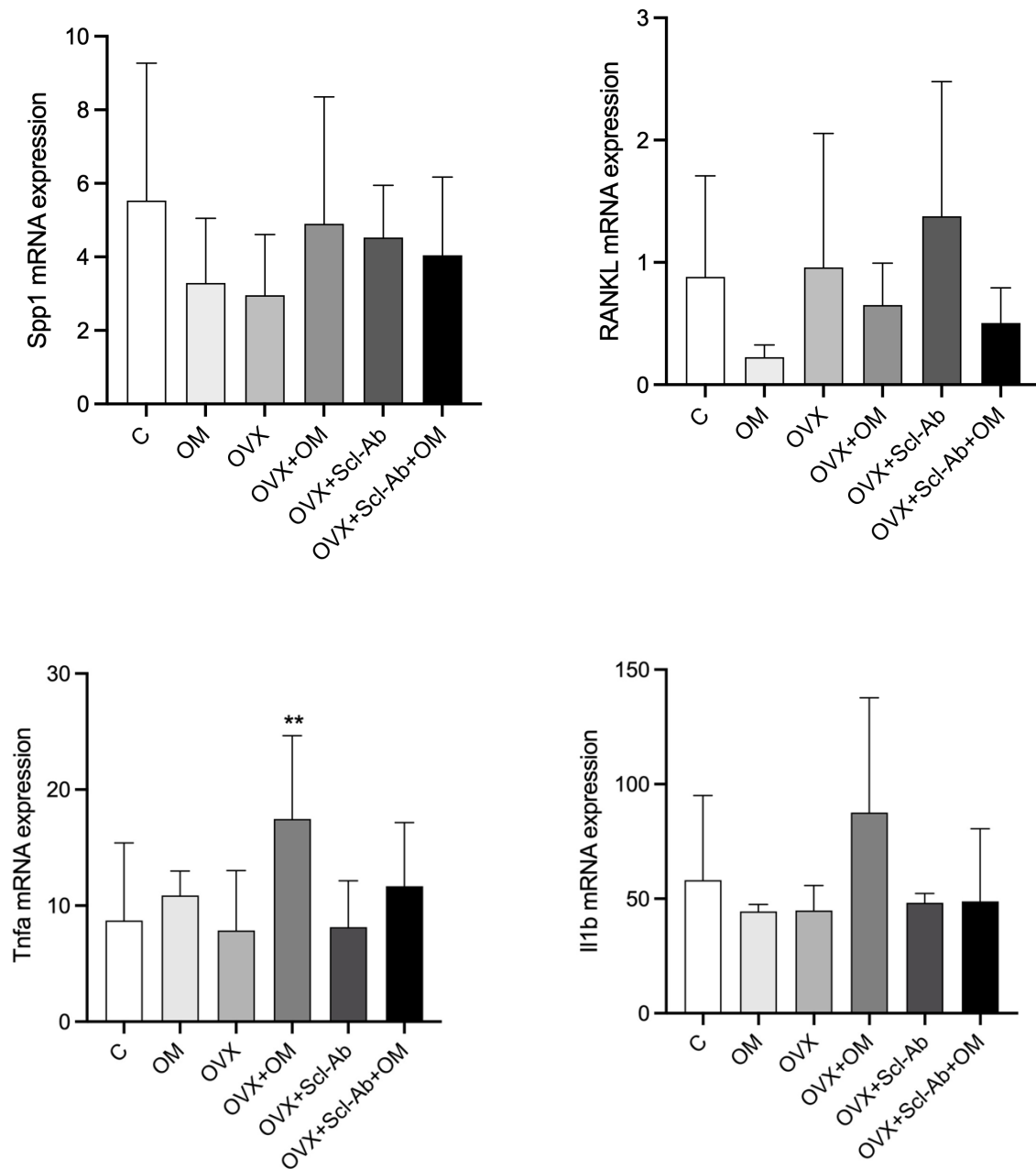
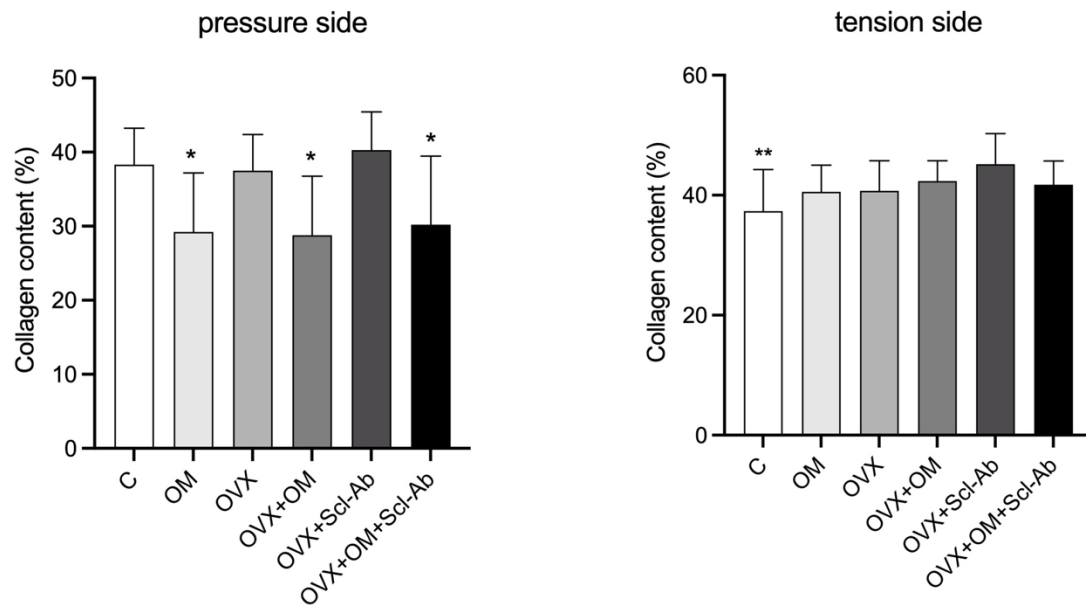


Figure 4. Mean (and SD) mRNA expression of Spp1, Rankl, TNFa, and Il1b by RT-qPCR. Cytokine expression was normalized to the expression of the housekeeping gene GAPDH. **Significant difference with OVX and OVX+Scl-Ab groups ($p \leq 0.05$).



Figures 5. Mean and SD comparison of birefringent collagen content in the pressure and tension sides in all groups. *Significant difference with the OM, OVX+OM, and OVX+Scl-Ab+OM groups ($p \leq 0.05$). **Significant difference with OVX+OM, and OVX+Scl-Ab groups ($p \leq 0.05$).



4 CONSIDERAÇÕES FINAIS

O objetivo deste estudo foi avaliar in vivo os efeitos de doenças sistêmicas (obesidade e osteoporose) na remodelação dos tecidos periodontais de molares de ratos sob força mecânica ortodôntica. De uma forma geral, as doenças sistêmicas e seu tratamento (no caso da publicação 2 – administração de Scl-Ab), afetaram a remodelação tecidual durante a movimentação ortodôntica.

Em relação à publicação 1, onde foi avaliado o efeito da obesidade no perfil proteômico do ligamento periodontal de ratos, sob força mecânica ortodôntica, identificou-se proteínas diferencialmente abundantes nos animais obesos submetidos à movimentação ortodôntica, em relação aos animais sistemicamente saudáveis submetidos à mesma movimentação ortodôntica. Essas proteínas estão relacionadas à obesidade e ao remodelamento tecidual. Além de promover novos dados e informações a respeito do mecanismo envolvido durante a movimentação dentária em obesos, nossos achados demonstraram que a obesidade promove alterações no mecanismo de remodelação tecidual durante o movimento ortodôntico.

Na publicação 2, teve-se como objetivo avaliar o efeito do anticorpo monoclonal anti-esclerostina (Scl-Ab) nos tecidos periodontais de ratas ovariectomizadas sob movimentação ortodôntica. Os resultados obtidos demonstraram que o tratamento para osteoporose à base de Scl-Ab, não influencia negativamente o remodelamento tecidual durante a movimentação ortodôntica. Pelo contrário, animais ovariectomizados que receberam Scl-Ab apresentaram qualidade óssea semelhante àqueles que não foram ovariectomizados. Portanto, a administração de Scl-Ab possui potencial de redução dos danos causados pela osteoporose em animais sob movimentação ortodôntica.

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APÊNDICE A – SUPPLEMENTARY MATERIAL

Estudo 1 – Obesity affects the proteome profile of periodontal ligament submitted to mechanical forces induced by orthodontic tooth movement in rats.

Table S1. All proteins identified on both M and OM groups list.

Protein_ID
A0A096MK61
A0A0G2JSL0
A0A0G2JSV6
A0A0G2JTX7
A0A0G2JUA5
A0A0G2JV24
A0A0G2JW12
A0A0G2JW41
A0A0G2JXD0
A0A0G2JXF0
A0A0G2JXT8
A0A0G2JYA4
A0A0G2JZI2
A0A0G2JZS9
A0A0G2JZV7
A0A0G2K014
A0A0G2K051
A0A0G2K1A2
A0A0G2K290
A0A0G2K2B5
A0A0G2K3Z9
A0A0G2K695
A0A0G2K6I4
A0A0G2K776
A0A0G2K7W6
A0A0G2K808
A0A0G2K896
A0A0G2K8K0
A0A0H2UHM7
A0A0U1RVI9
A0JN30
A1L1J9
A2RUV9
A7VJC2
A9UMV8
B0BNA5
B0BNI5
B0BNK1
B0BNN3

B1WC34
B2GUZ5
B2GV73
B2RYM3
B2RZ72
B2RZ77
B3GNI6
B4F7C2
B5DEN5
B5DF65
B5DF91
B5DFC3
B5DFD8
C0JPT7
D3Z952
D3Z9E1
D3ZAF5
D3ZAN3
D3ZBN0
D3ZCR3
D3ZE00
D3ZFA8
D3ZFH5
D3ZG05

Table S2. All OM exclusive proteins list

Protein_ID	Description
A0A0G2JTX2	PRA1 family protein OS=Rattus norvegicus (Rat) OX=10116 GN=Prpf2 PE=1 SV=1 - [A0A0G2JTX2_RAT]
A0A0G2JVP9	RCG63136 OS=Rattus norvegicus (Rat) OX=10116 GN=Psme1-ps1 PE=1 SV=1 - [A0A0G2JVP9_RAT]
A0A0G2JW60	Utrophin OS=Rattus norvegicus (Rat) OX=10116 GN=Utrn PE=1 SV=1 - [A0A0G2JW60_RAT]
A0A0G2JWC7	PH domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Fermt2 PE=1 SV=1 - [A0A0G2JWC7_RAT]
A0A0G2K1D2	RAP1, GTP-GDP dissociation stimulator 1 (Predicted), isoform CRA_a OS=Rattus norvegicus (Rat) OX=10116 GN=Rap1gds1 PE=1 SV=1 - [A0A0G2K1D2_RAT]
A0A0G2K4I8	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000057681 PE=1 SV=1 - [A0A0G2K4I8_RAT]
A0A0G2K6L6	Ribosomal_S10 domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000059138 PE=3 SV=1 - [A0A0G2K6L6_RAT]
A0A0G2K7P6	IgV domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000057376 PE=4 SV=1 - [A0A0G2K7P6_RAT]
A0A0G2K980	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000055375 PE=1 SV=1 - [A0A0G2K980_RAT]
A0A140UHY3	EWS RNA-binding protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Ewsr1 PE=1 SV=1 - [A0A140UHY3_RAT]
B0BNJ1	LOC683667 protein OS=Rattus norvegicus (Rat) OX=10116 GN=Sri PE=1 SV=1 - [B0BNJ1_RAT]
B1WC26	AFP-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Nans PE=1 SV=1 - [B1WC26_RAT]
B2GV06	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Oxct1 PE=1 SV=1 - [SCOT1_RAT]
B2RZ08	RGD1563325 protein OS=Rattus norvegicus (Rat) OX=10116 GN=Tmem263 PE=1 SV=1 - [B2RZ08_RAT]
B2RZ37	Receptor expression-enhancing protein 5 OS=Rattus norvegicus (Rat) OX=10116 GN=Reep5 PE=1 SV=1 - [REEP5_RAT]
B2RZB7	Small nuclear ribonucleoprotein Sm D1 OS=Rattus norvegicus (Rat) OX=10116 GN=Snrpd1 PE=1 SV=1 - [B2RZB7_RAT]
B2RZD1	Protein transport protein Sec61 subunit beta OS=Rattus norvegicus (Rat) OX=10116 GN=Sec61b PE=1 SV=1 - [B2RZD1_RAT]
B4F7A5	CD99 molecule (Xg blood group) OS=Rattus norvegicus (Rat) OX=10116 GN=Cd99 PE=1 SV=1 - [B4F7A5_RAT]
B5DFD2	Tll13 protein OS=Rattus norvegicus (Rat) OX=10116 GN=Tll13 PE=2 SV=1 - [B5DFD2_RAT]
D3ZC54	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000030235 PE=1 SV=2 - [D3ZC54_RAT]
D3ZEP5	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000054128 PE=1 SV=3 - [D3ZEP5_RAT]
D3ZK86	Uncharacterized protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000040060 PE=4 SV=3 - [D3ZK86_RAT]
D3ZM69	FERM domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Epb41l2 PE=1 SV=2 - [D3ZM69_RAT]
D3ZN79	Uncharacterized protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000030364 PE=3 SV=1 - [D3ZN79_RAT]
D3ZQR5	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000048182 PE=1 SV=1 - [D3ZQR5_RAT]
D3ZY51	Plakophilin 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Pkp1 PE=4 SV=2 - [D3ZY51_RAT]
D4A5F0	Family with sequence similarity 114, member A1-like 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Fam114a1l1 PE=4 SV=2 - [D4A5F0_RAT]
D4A885	ABC transporter domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Abca13 PE=4 SV=3 - [D4A885_RAT]

D4A8A0	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase OS=Rattus norvegicus (Rat) OX=10116 GN=Cad PE=1 SV=2 - [D4A8A0_RAT]
D4AAR7	Coiled-coil domain-containing 136 OS=Rattus norvegicus (Rat) OX=10116 GN=Ccdc136 PE=1 SV=3 - [D4AAR7_RAT]
D4AAT4	Sm domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Snrpf PE=1 SV=1 - [D4AAT4_RAT]
D4ABT8	Heterogeneous nuclear ribonucleoprotein U-like 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Hnrnpul2 PE=1 SV=1 - [D4ABT8_RAT]
D4AEL1	PNMA family member 5 OS=Rattus norvegicus (Rat) OX=10116 GN=Pnma5 PE=4 SV=1 - [D4AEL1_RAT]
F1LMV6	SH3 domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Dsp PE=1 SV=1 - [F1LMV6_RAT]
F1M3H8	Hypothetical protein LOC681410 OS=Rattus norvegicus (Rat) OX=10116 GN=LOC681410 PE=1 SV=3 - [F1M3H8_RAT]
F1M5Q4	Fibrillin 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Fbn2 PE=4 SV=3 - [F1M5Q4_RAT]
F7FF45	Nuclear mitotic apparatus protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Numa1 PE=1 SV=2 - [F7FF45_RAT]
F7FHF3	SERPIN domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Serpinf2 PE=1 SV=1 - [F7FHF3_RAT]
G3V6S7	IL6Ra-bind domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Il5ra PE=4 SV=2 - [G3V6S7_RAT]
G3V816	Nucleoside diphosphate kinase OS=Rattus norvegicus (Rat) OX=10116 GN=Nme3 PE=1 SV=1 - [G3V816_RAT]
G3V8S9	CAP18_C domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Camp PE=1 SV=1 - [G3V8S9_RAT]
M0R692	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000050652 PE=1 SV=1 - [M0R692_RAT]
M0RBK4	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000061576 PE=1 SV=2 - [M0RBK4_RAT]
O35186	Cathepsin K OS=Rattus norvegicus (Rat) OX=10116 GN=Ctsk PE=2 SV=1 - [CATK_RAT]
O35567	Bifunctional purine biosynthesis protein PURH OS=Rattus norvegicus (Rat) OX=10116 GN=Atic PE=1 SV=2 - [PUR9_RAT]
O35796	Complement component 1 Q subcomponent-binding protein, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=C1qbp PE=1 SV=2 - [C1QBP_RAT]
O88600	Heat shock 70 kDa protein 4 OS=Rattus norvegicus (Rat) OX=10116 GN=Hspa4 PE=1 SV=1 - [HSP74_RAT]
P00564	Creatine kinase M-type OS=Rattus norvegicus (Rat) OX=10116 GN=Ckm PE=1 SV=2 - [KCRM_RAT]
P00697	Lysozyme C-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Lyz1 PE=1 SV=2 - [LYSC1_RAT]
P01830	Thy-1 membrane glycoprotein OS=Rattus norvegicus (Rat) OX=10116 GN=Thy1 PE=1 SV=1 - [THY1_RAT]
P02600	Myosin light chain 1/3, skeletal muscle isoform OS=Rattus norvegicus (Rat) OX=10116 GN=Myl1 PE=1 SV=2 - [MYL1_RAT]
P02764	Alpha-1-acid glycoprotein OS=Rattus norvegicus (Rat) OX=10116 GN=Orm1 PE=2 SV=1 - [A1AG_RAT]
P04905	Glutathione S-transferase Mu 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Gstm1 PE=1 SV=2 - [GSTM1_RAT]
P05370	Glucose-6-phosphate 1-dehydrogenase OS=Rattus norvegicus (Rat) OX=10116 GN=G6pdx PE=1 SV=3 - [G6PD_RAT]
P05371	Clusterin OS=Rattus norvegicus (Rat) OX=10116 GN=Clu PE=1 SV=2 - [CLUS_RAT]
P05943	Protein S100-A10 OS=Rattus norvegicus (Rat) OX=10116 GN=S100a10 PE=1 SV=2 - [S10AA_RAT]
P08081	Clathrin light chain A OS=Rattus norvegicus (Rat) OX=10116 GN=Clta PE=1 SV=1 - [CLCA_RAT]
P08699	Galectin-3 OS=Rattus norvegicus (Rat) OX=10116 GN=Lgals3 PE=1 SV=4 - [LEG3_RAT]

P08753	Guanine nucleotide-binding protein G(i) subunit alpha OS=Rattus norvegicus (Rat) OX=10116 GN=Gnai3 PE=1 SV=3 - [GNAI3_RAT]
P09527	Ras-related protein Rab-7a OS=Rattus norvegicus (Rat) OX=10116 GN=Rab7a PE=1 SV=2 - [RAB7A_RAT]
P0C5H9	Mesencephalic astrocyte-derived neurotrophic factor OS=Rattus norvegicus (Rat) OX=10116 GN=Manf PE=1 SV=1 - [MANF_RAT]
P14408	Fumarate hydratase, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Fh PE=1 SV=2 - [FUMH_RAT]
P15800	Laminin subunit beta-2 OS=Rattus norvegicus (Rat) OX=10116 GN=Lamb2 PE=1 SV=1 - [LAMB2_RAT]
P17046	Lysosome-associated membrane glycoprotein 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Lamp2 PE=1 SV=3 - [LAMP2_RAT]
P18437	Non-histone chromosomal protein HMG-17 OS=Rattus norvegicus (Rat) OX=10116 GN=Hmgn2 PE=1 SV=2 - [HMGN2_RAT]
P19234	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Ndufv2 PE=1 SV=2 - [NDUV2_RAT]
P21575	Dynamin-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Dnm1 PE=1 SV=2 - [DYN1_RAT]
P24268	Cathepsin D OS=Rattus norvegicus (Rat) OX=10116 GN=Ctsd PE=1 SV=1 - [CATD_RAT]
P31399	ATP synthase subunit d, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Atp5pd PE=1 SV=3 - [ATP5H_RAT]
P34064	Proteasome subunit alpha type-5 OS=Rattus norvegicus (Rat) OX=10116 GN=Psma5 PE=1 SV=1 - [PSA5_RAT]
P36201	Cysteine-rich protein 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Crip2 PE=2 SV=1 - [CRIP2_RAT]
P39069	Adenylate kinase isoenzyme 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Ak1 PE=1 SV=3 - [KAD1_RAT]
P47967	Galectin-5 OS=Rattus norvegicus (Rat) OX=10116 GN=Lgals5 PE=1 SV=2 - [LEG5_RAT]
P49134	Integrin beta-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Itgb1 PE=2 SV=1 - [ITB1_RAT]
P52296	Importin subunit beta-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Kpnb1 PE=1 SV=1 - [IMB1_RAT]
P55314	Complement component C8 beta chain OS=Rattus norvegicus (Rat) OX=10116 GN=C8b PE=2 SV=2 - [CO8B_RAT]
P61959	Small ubiquitin-related modifier 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Sumo2 PE=1 SV=1 - [SUMO2_RAT]
P62329	Thymosin beta-4 OS=Rattus norvegicus (Rat) OX=10116 GN=Tmsb4x PE=1 SV=2 - [TYB4_RAT]
P63036	DnaJ homolog subfamily A member 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Dnaja1 PE=1 SV=1 - [DNJA1_RAT]
P63255	Cysteine-rich protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Crip1 PE=1 SV=2 - [CRIP1_RAT]
P68101	Eukaryotic translation initiation factor 2 subunit 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Eif2s1 PE=1 SV=2 - [IF2A_RAT]
P85515	Alpha-centractin OS=Rattus norvegicus (Rat) OX=10116 GN=Actr1a PE=1 SV=1 - [ACTZ_RAT]
P97536	Cullin-associated NEDD8-dissociated protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Cand1 PE=1 SV=1 - [CAND1_RAT]
Q01205	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Dist PE=1 SV=2 - [ODO2_RAT]
Q07009	Calpain-2 catalytic subunit OS=Rattus norvegicus (Rat) OX=10116 GN=Capn2 PE=1 SV=3 - [CAN2_RAT]
Q2Q0I9	Fibronectin type III domain-containing protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Fndc1 PE=1 SV=2 - [FNDC1_RAT]
Q32PX7	Far upstream element-binding protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Fubp1 PE=1 SV=1 - [FUBP1_RAT]

Q4FZT9	26S proteasome non-ATPase regulatory subunit 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Psm2 PE=1 SV=1 - [PSMD2_RAT]
Q4G061	Eukaryotic translation initiation factor 3 subunit B OS=Rattus norvegicus (Rat) OX=10116 GN=Eif3b PE=1 SV=1 - [EIF3B_RAT]
Q4G075	Leukocyte elastase inhibitor A OS=Rattus norvegicus (Rat) OX=10116 GN=Serp1a PE=1 SV=1 - [ILEUA_RAT]
Q566E4	Heterogeneous nuclear ribonucleoprotein R OS=Rattus norvegicus (Rat) OX=10116 GN=Hnrnp PE=1 SV=1 - [Q566E4_RAT]
Q5M7W5	Microtubule-associated protein 4 OS=Rattus norvegicus (Rat) OX=10116 GN=Map4 PE=1 SV=1 - [MAP4_RAT]
Q5M860	Rho GDP dissociation inhibitor beta OS=Rattus norvegicus (Rat) OX=10116 GN=Arhgdib PE=1 SV=1 - [Q5M860_RAT]
Q5U2V4	Phospholipase B-like 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Plbd1 PE=2 SV=1 - [PLBL1_RAT]
Q5XI78	2-oxoglutarate dehydrogenase, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Ogdh PE=1 SV=1 - [ODO1_RAT]
Q5XIP9	Transmembrane protein 43 OS=Rattus norvegicus (Rat) OX=10116 GN=Tmem43 PE=2 SV=1 - [TMM43_RAT]
Q60587	Trifunctional enzyme subunit beta, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Hadhb PE=1 SV=1 - [ECHB_RAT]
Q62658	Peptidyl-prolyl cis-trans isomerase FKBP1A OS=Rattus norvegicus (Rat) OX=10116 GN=Fkbp1a PE=1 SV=3 - [FKB1A_RAT]
Q62715	Neutrophil antibiotic peptide NP-2 OS=Rattus norvegicus (Rat) OX=10116 GN=Defa PE=1 SV=1 - [DEF2_RAT]
Q62786	Prostaglandin F2 receptor negative regulator OS=Rattus norvegicus (Rat) OX=10116 GN=Ptgfrn PE=1 SV=1 - [FPRP_RAT]
Q63270	Cytoplasmic aconitate hydratase OS=Rattus norvegicus (Rat) OX=10116 GN=Aco1 PE=1 SV=1 - [ACOC_RAT]
Q63803	Guanine nucleotide-binding protein G(s) subunit alpha isoforms XLas OS=Rattus norvegicus (Rat) OX=10116 GN=Gnas PE=1 SV=3 - [GNAS1_RAT]
Q63945	Protein SET OS=Rattus norvegicus (Rat) OX=10116 GN=Set PE=2 SV=2 - [SET_RAT]
Q6AXQ0	SUMO-activating enzyme subunit 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Sae1 PE=2 SV=1 - [SAE1_RAT]
Q6AY20	Cation-dependent mannose-6-phosphate receptor OS=Rattus norvegicus (Rat) OX=10116 GN=M6pr PE=1 SV=1 - [MPRD_RAT]
Q6AYS3	Carboxypeptidase OS=Rattus norvegicus (Rat) OX=10116 GN=Ctsa PE=1 SV=1 - [Q6AYS3_RAT]
Q6IG04	Keratin, type II cytoskeletal 72 OS=Rattus norvegicus (Rat) OX=10116 GN=Krt72 PE=3 SV=2 - [K2C72_RAT]
Q6JE36	Protein NDRG1 OS=Rattus norvegicus (Rat) OX=10116 GN=Ndr1 PE=1 SV=1 - [NDRG1_RAT]
Q6MG61	Chloride intracellular channel protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Clc1 PE=1 SV=1 - [CLIC1_RAT]
Q6P734	Plasma protease C1 inhibitor OS=Rattus norvegicus (Rat) OX=10116 GN=Serp1 PE=2 SV=1 - [IC1_RAT]
Q6P742	Proteolipid protein 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Plp2 PE=2 SV=1 - [PLP2_RAT]
Q6P747	Heterochromatin protein 1-binding protein 3 OS=Rattus norvegicus (Rat) OX=10116 GN=Hp1bp3 PE=1 SV=1 - [HP1B3_RAT]
Q6RUV5	Ras-related C3 botulinum toxin substrate 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Rac1 PE=1 SV=1 - [RAC1_RAT]
Q6VBQ5	Myeloid-associated differentiation marker OS=Rattus norvegicus (Rat) OX=10116 GN=Myadm PE=1 SV=1 - [MYADM_RAT]
Q75WE7	von Willebrand factor A domain-containing protein 5A OS=Rattus norvegicus (Rat) OX=10116 GN=Vwa5a PE=2 SV=1 - [VWA5A_RAT]
Q8CG45	Aflatoxin B1 aldehyde reductase member 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Akr7a2 PE=1 SV=2 - [ARK72_RAT]

Q91ZN1	Coronin-1A OS=Rattus norvegicus (Rat) OX=10116 GN=Coro1a PE=1 SV=3 - [COR1A_RAT]
Q920P6	Adenosine deaminase OS=Rattus norvegicus (Rat) OX=10116 GN=Ada PE=1 SV=3 - [ADA_RAT]
Q99NA5	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Idh3a PE=1 SV=1 - [IDH3A_RAT]
Q9ES21	Phosphatidylinositol phosphatase SAC1 OS=Rattus norvegicus (Rat) OX=10116 GN=Sacm1l PE=1 SV=1 - [SAC1_RAT]
Q9JHL4	Drebrin-like protein OS=Rattus norvegicus (Rat) OX=10116 GN=Dbnl PE=1 SV=1 - [DBNL_RAT]
Q9JLJ3	4-trimethylaminobutyraldehyde dehydrogenase OS=Rattus norvegicus (Rat) OX=10116 GN=Aldh9a1 PE=1 SV=1 - [AL9A1_RAT]
Q9R1T1	Barrier-to-autointegration factor OS=Rattus norvegicus (Rat) OX=10116 GN=Banf1 PE=1 SV=1 - [BAF_RAT]
Q9WVB1	Ras-related protein Rab-6A OS=Rattus norvegicus (Rat) OX=10116 GN=Rab6a PE=1 SV=2 - [RAB6A_RAT]
Q9Z0V6	Thioredoxin-dependent peroxide reductase, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Prdx3 PE=1 SV=2 - [PRDX3_RAT]

Table S3. All M exclusive proteins list.

Protein_ID	Description
A0A0G2JUD2	Inorganic pyrophosphatase 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Sar1a PE=1 SV=1 - [A0A0G2JUD2_RAT]
A0A0G2JVZ6	Integrin_alpha2 domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Itgav PE=1 SV=1 - [A0A0G2JVZ6_RAT]
A0A0G2JZA1	Roundabout guidance receptor 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Robo2 PE=4 SV=1 - [A0A0G2JZA1_RAT]
A0A0G2JZZ4	Sep15_SelM domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Selenom PE=1 SV=1 - [A0A0G2JZZ4_RAT]
A0A0G2K7F0	FA complementation group M OS=Rattus norvegicus (Rat) OX=10116 GN=Fancm PE=4 SV=1 - [A0A0G2K7F0_RAT]
A0A0G2K8W9	Spectrin beta chain OS=Rattus norvegicus (Rat) OX=10116 GN=Sptbn1 PE=1 SV=1 - [A0A0G2K8W9_RAT]
B5DFB0	Fe2OG dioxygenase domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=P3h3 PE=2 SV=1 - [B5DFB0_RAT]
D3ZGT6	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (Proline 4-hydroxylase), alpha II polypeptide (Predicted), isoform CRA_a OS=Rattus norvegicus (Rat) OX=10116 GN=P4ha2 PE=1 SV=1 - [D3ZGT6_RAT]
D3ZHG3	Protein tyrosine kinase 7 OS=Rattus norvegicus (Rat) OX=10116 GN=Ptk7 PE=3 SV=3 - [D3ZHG3_RAT]
D3ZPR0	Importin N-terminal domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Cse1l PE=1 SV=1 - [D3ZPR0_RAT]
D3ZSL2	Costars domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Abrac1 PE=1 SV=1 - [D3ZSL2_RAT]
D4AAZ6	60S ribosomal protein L37a OS=Rattus norvegicus (Rat) OX=10116 GN=Rpl37a PE=1 SV=1 - [RL37A_RAT]
E9PT66	CPSF_A domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Sf3b3 PE=1 SV=2 - [E9PT66_RAT]
F1LTY5	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000048167 PE=1 SV=3 - [F1LTY5_RAT]
G3V6G1	Immunoglobulin joining chain OS=Rattus norvegicus (Rat) OX=10116 GN=Jchain PE=1 SV=1 - [G3V6G1_RAT]
M0RAB8	Ig-like domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG00000048452 PE=4 SV=2 - [M0RAB8_RAT]
P05708	Hexokinase-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Hk1 PE=1 SV=4 - [HXX1_RAT]
P08592	Amyloid-beta A4 protein OS=Rattus norvegicus (Rat) OX=10116 GN=App PE=1 SV=2 - [A4_RAT]
P18421	Proteasome subunit beta type-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Psmb1 PE=1 SV=3 - [PSB1_RAT]
P24050	40S ribosomal protein S5 OS=Rattus norvegicus (Rat) OX=10116 GN=Rps5 PE=1 SV=3 - [RS5_RAT]
P27615	Lysosome membrane protein 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Scarb2 PE=1 SV=2 - [SCRB2_RAT]
P41542	General vesicular transport factor p115 OS=Rattus norvegicus (Rat) OX=10116 GN=Uso1 PE=1 SV=1 - [USO1_RAT]
P45479	Palmitoyl-protein thioesterase 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Ppt1 PE=1 SV=1 - [PPT1_RAT]
P60901	Proteasome subunit alpha type-6 OS=Rattus norvegicus (Rat) OX=10116 GN=Psma6 PE=1 SV=1 - [PSA6_RAT]
P70490	Lactadherin OS=Rattus norvegicus (Rat) OX=10116 GN=Mfge8 PE=2 SV=1 - [MFGM_RAT]
Q3T1J1	Eukaryotic translation initiation factor 5A-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Eif5a PE=1 SV=3 - [IF5A1_RAT]
Q4G058	RCG42593, isoform CRA_a OS=Rattus norvegicus (Rat) OX=10116 GN=Polr1d PE=1 SV=1 - [Q4G058_RAT]

Q4KLL4	Transmembrane 9 superfamily member 4 OS=Rattus norvegicus (Rat) OX=10116 GN=Tm9sf4 PE=2 SV=1 - [TM9S4_RAT]
Q4KM38	RRM domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Srsf10 PE=1 SV=1 - [Q4KM38_RAT]
Q4V797	IRG-type G domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=RGD1309362 PE=1 SV=1 - [Q4V797_RAT]
Q5M8C3	SERPIN domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Serpina4 PE=1 SV=1 - [Q5M8C3_RAT]
Q5XI04	PHB domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Stom PE=1 SV=1 - [Q5XI04_RAT]
Q63357	Unconventional myosin-IId OS=Rattus norvegicus (Rat) OX=10116 GN=Myo1d PE=1 SV=3 - [MYO1D_RAT]
Q641X3	Beta-hexosaminidase subunit alpha OS=Rattus norvegicus (Rat) OX=10116 GN=Hexa PE=2 SV=1 - [HEXA_RAT]
Q641Z6	EH domain-containing protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Ehd1 PE=1 SV=1 - [EHD1_RAT]
Q64375	Endoplasmic reticulum protein SC65 OS=Rattus norvegicus (Rat) OX=10116 GN=P3h4 PE=2 SV=3 - [SC65_RAT]
Q6AY58	B-cell receptor-associated protein 31 OS=Rattus norvegicus (Rat) OX=10116 GN=Bcap31 PE=1 SV=1 - [Q6AY58_RAT]
Q6IRJ7	Annexin OS=Rattus norvegicus (Rat) OX=10116 GN=Anxa7 PE=1 SV=1 - [Q6IRJ7_RAT]
Q8CG08	Collagen triple helix repeat-containing protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Cthrc1 PE=1 SV=1 - [CTHR1_RAT]
Q925G0	RNA-binding protein 3 OS=Rattus norvegicus (Rat) OX=10116 GN=Rbm3 PE=1 SV=2 - [RBM3_RAT]
Q9JHY2	Sideroflexin-3 OS=Rattus norvegicus (Rat) OX=10116 GN=Sfxn3 PE=2 SV=1 - [SFXN3_RAT]

Table S4. All OM differentially abundant proteins list.

Protein_ID	Description	Average_Group_p_M	Average_Group_OM	P_value_T_test	Fold Change
A0A0G2JTX2	PRA1 family protein OS=Rattus norvegicus (Rat) OX=10116 GN=Prpf2 PE=1 SV=1 - [A0A0G2JTX2_RAT]	0	747093,3812	0,017296995	747093,3812
A0A0G2K6L6	Ribosomal_S10 domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG000000 59138 PE=3 SV=1 - [A0A0G2K6L6_RAT]	0	2255123,365	0,042444907	2255123,365
A0A0G2K896	Similar to RIKEN cDNA 1300017J02 OS=Rattus norvegicus (Rat) OX=10116 GN=RGD1310507 PE=1 SV=1 - [A0A0G2K896_RAT]	378230,6532	2545291,632	0,018602656	6,729469466
A0A140UHY3	EWS RNA-binding protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Ewsr1 PE=1 SV=1 - [A0A140UHY3_RAT]	0	1863084,555	0,043079351	1863084,555
D3ZCR3	High mobility group protein B1 pseudogene OS=Rattus norvegicus (Rat) OX=10116 GN=LOC108349189 PE=4 SV=2 - [D3ZCR3_RAT]	860069,6564	6338215,646	0,012942021	7,369421301
D3ZN79	Uncharacterized protein OS=Rattus norvegicus (Rat) OX=10116 GN=ENSRNOG000000 30364 PE=3 SV=1 - [D3ZN79_RAT]	0	4921706,978	0,009318352	4921706,978
F1M3H8	Hypothetical protein LOC681410 OS=Rattus norvegicus (Rat) OX=10116 GN=LOC681410 PE=1 SV=3 - [F1M3H8_RAT]	0	1680008,803	0,020448413	1680008,803
F7FHF3	SERPIN domain- containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Serpinf2 PE=1 SV=1 - [F7FHF3_RAT]	0	771474,4399	0,012270751	771474,4399
G3V9R8	Heterogeneous nuclear ribonucleoprotein C OS=Rattus norvegicus (Rat) OX=10116 GN=Hnrnpc PE=1 SV=2 - [HNRPC_RAT]	250583,3438	1425496,726	0,015952598	5,688713001

O35796	Complement component 1 Q subcomponent-binding protein, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=C1qbp PE=1 SV=2 - [C1QBP_RAT]	0	1416700,683	0,016702834	1416700,683
O88767	Parkinson disease protein 7 homolog OS=Rattus norvegicus (Rat) OX=10116 GN=Park7 PE=1 SV=1 - [PARK7_RAT]	428626,5882	4335658,455	0,00082327	10,11523451
P00173	Cytochrome b5 OS=Rattus norvegicus (Rat) OX=10116 GN=Cyb5a PE=1 SV=2 - [CYB5_RAT]	523282,4876	3393783,534	0,038209864	6,48556681
P00697	Lysozyme C-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Lyz1 PE=1 SV=2 - [LYSC1_RAT]	0	5172127,501	0,035299095	5172127,501
P04906	Glutathione S-transferase P OS=Rattus norvegicus (Rat) OX=10116 GN=Gstp1 PE=1 SV=2 - [GSTP1_RAT]	2061182,148	4787342,171	0,048115651	2,322619656
P06399	Fibrinogen alpha chain OS=Rattus norvegicus (Rat) OX=10116 GN=Fga PE=1 SV=3 - [FIBA_RAT]	11013065,97	29115938,21	0,046163104	2,643763171
P07632	Superoxide dismutase [Cu-Zn] OS=Rattus norvegicus (Rat) OX=10116 GN=Sod1 PE=1 SV=2 - [SODC_RAT]	1938236,002	6725072,694	0,033292608	3,469687225
P08721	Osteopontin OS=Rattus norvegicus (Rat) OX=10116 GN=Spp1 PE=1 SV=2 - [OSTP_RAT]	364074,7454	4173910,759	0,015123072	11,46443364
P08753	Guanine nucleotide-binding protein G(i) subunit alpha OS=Rattus norvegicus (Rat) OX=10116 GN=Gnai3 PE=1 SV=3 - [GNAI3_RAT]	0	3591397,152	0,004835001	3591397,152
P09527	Ras-related protein Rab-7a OS=Rattus norvegicus (Rat) OX=10116 GN=Rab7a PE=1 SV=2 - [RAB7A_RAT]	0	2663970,927	0,004451915	2663970,927

P0DP29	Calmodulin-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Calm1 PE=1 SV=1 - [CALM1_RAT]	1598929,545	9791563,994	0,008478587	6,123824 545
P14562	Lysosome-associated membrane glycoprotein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Lamp1 PE=1 SV=1 - [LAMP1_RAT]	1033401,994	3439948,206	0,039543769	3,328760 953
P16617	Phosphoglycerate kinase 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Pgk1 PE=1 SV=2 - [PGK1_RAT]	562339,0032	3143175,417	0,005850633	5,589467 206
P17046	Lysosome-associated membrane glycoprotein 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Lamp2 PE=1 SV=3 - [LAMP2_RAT]	0	2043491,499	0,021104587	2043491, 499
P18437	Non-histone chromosomal protein HMG-17 OS=Rattus norvegicus (Rat) OX=10116 GN=Hmgn2 PE=1 SV=2 - [HMGN2_RAT]	0	848573,4931	0,02954119	848573,4 931
P24268	Cathepsin D OS=Rattus norvegicus (Rat) OX=10116 GN=Ctsd PE=1 SV=1 - [CATD_RAT]	0	1845475,614	0,022460222	1845475, 614
P25113	Phosphoglycerate mutase 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Pgam1 PE=1 SV=4 - [PGAM1_RAT]	336936,8126	3032379,4	0,02563257	8,999845 924
P26772	10 kDa heat shock protein, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Hspe1 PE=1 SV=3 - [CH10_RAT]	392970,4562	1882572,958	0,024045102	4,790622 114
P39069	Adenylate kinase isoenzyme 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Ak1 PE=1 SV=3 - [KAD1_RAT]	0	1195498,63	0,02626615	1195498, 63
P49134	Integrin beta-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Itgb1 PE=2 SV=1 - [ITB1_RAT]	0	972950,2057	0,041049473	972950,2 057

P52296	Importin subunit beta-1 OS=Rattus norvegicus (Rat) OX=10116 GN=Kpnb1 PE=1 SV=1 - [IMB1_RAT]	0	1603647,386	0,015834945	1603647, 386
P61107	Ras-related protein Rab-14 OS=Rattus norvegicus (Rat) OX=10116 GN=Rab14 PE=1 SV=3 - [RAB14_RAT]	406260,9234	3822041,209	0,008255767	9,407848 476
P62832	60S ribosomal protein L23 OS=Rattus norvegicus (Rat) OX=10116 GN=Rpl23 PE=2 SV=1 - [RL23_RAT]	519178,5376	3189266,626	0,016260698	6,142909 221
P62859	40S ribosomal protein S28 OS=Rattus norvegicus (Rat) OX=10116 GN=Rps28 PE=1 SV=1 - [RS28_RAT]	295405,8188	5811675,568	0,003853558	19,67353 112
P85515	Alpha-centractin OS=Rattus norvegicus (Rat) OX=10116 GN=Actr1a PE=1 SV=1 - [ACTZ_RAT]	0	1618081,082	0,015360007	1618081, 082
P85972	Vinculin OS=Rattus norvegicus (Rat) OX=10116 GN=Vcl PE=1 SV=1 - [VINC_RAT]	1596467,43	5531198,725	0,005666678	3,464648 65
Q02874	Core histone macro- H2A.1 OS=Rattus norvegicus (Rat) OX=10116 GN=Macroh2a1 PE=1 SV=4 - [H2AY_RAT]	733717,0032	2742309,593	0,048098251	3,737557 643
Q5M7W5	Microtubule-associated protein 4 OS=Rattus norvegicus (Rat) OX=10116 GN=Map4 PE=1 SV=1 - [MAP4_RAT]	0	824230,7969	0,018432604	824230,7 969
Q5XI73	Rho GDP-dissociation inhibitor 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Arhgdia PE=1 SV=1 - [GDIR1_RAT]	615552,2876	3847670,886	0,011353893	6,250762 061
Q5XII0	Mammalian ependymin-related protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Epdr1 PE=1 SV=1 - [EPDR1_RAT]	276607,6718	1289328,975	0,049121538	4,661219 142
Q62826	Heterogeneous nuclear ribonucleoprotein M	160568,4063	1405240,531	0,020698322	8,751662 697

	OS=Rattus norvegicus (Rat) OX=10116 GN=Hnrrnpm PE=1 SV=4 - [HNRPM_RAT]				
Q62902	Protein ERGIC-53 OS=Rattus norvegicus (Rat) OX=10116 GN=Lman1 PE=1 SV=1 - [LMAN1_RAT]	1511218,43	5002741,641	0,016197168	3,310402 746
Q63803	Guanine nucleotide- binding protein G(s) subunit alpha isoforms XLas OS=Rattus norvegicus (Rat) OX=10116 GN=Gnas PE=1 SV=3 - [GNAS1_RAT]	0	3810020,038	0,007324839	3810020, 038
Q63910	GLOBIN domain- containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Hba-a3 PE=1 SV=2 - [Q63910_RAT]	1812510,207	6280829,941	0,019619975	3,465265 97
Q6AY20	Cation-dependent mannose-6-phosphate receptor OS=Rattus norvegicus (Rat) OX=10116 GN=M6pr PE=1 SV=1 - [MPRD_RAT]	0	1144732,663	0,039531234	1144732, 663
Q6MG61	Chloride intracellular channel protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Clc1 PE=1 SV=1 - [CLIC1_RAT]	0	2805672,387	0,007891086	2805672, 387
Q6P742	Proteolipid protein 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Plp2 PE=2 SV=1 - [PLP2_RAT]	0	1877945,621	0,015174963	1877945, 621
Q6P747	Heterochromatin protein 1-binding protein 3 OS=Rattus norvegicus (Rat) OX=10116 GN=Hp1bp3 PE=1 SV=1 - [HP1B3_RAT]	0	1426369,735	0,039449215	1426369, 735
Q9WVB1	Ras-related protein Rab-6A OS=Rattus norvegicus (Rat) OX=10116 GN=Rab6a PE=1 SV=2 - [RAB6A_RAT]	0	4888514,452	0,014931775	4888514, 452
Q9WVJ6	TGc domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Tgm2 PE=1 SV=1 - [Q9WVJ6_RAT]	428409,025	2340765,606	0,015271348	5,463856 898

Table S5. All M differentially abundant proteins list

Protein_ID	Description	Average_Group_M	Average_Group_OM	P_value_T_t_est	Fold Change
A0A0G2JVZ6	Integrin_alpha2 domain-containing protein OS=Rattus norvegicus (Rat) OX=10116 GN=Itgav PE=1 SV=1 - [A0A0G2JVZ6_RAT]	3426483,693	0	0,010927327	- 3426483,693
A0A0G2JXD0	60S ribosomal protein L9 pseudogene OS=Rattus norvegicus (Rat) OX=10116 GN=LOC103692829 PE=4 SV=1 - [A0A0G2JXD0_RAT]	6169901,021	2029533,956	0,021504298	- 3,04005804
D3Z9E1	Elastin microfibril interfacier 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Emilin1 PE=1 SV=1 - [D3Z9E1_RAT]	5393880,117	3135185,261	0,025724172	- 1,720434255
D3ZAF5	Periostin OS=Rattus norvegicus (Rat) OX=10116 GN=Postn PE=1 SV=1 - [D3ZAF5_RAT]	242097623,5	61441724,55	0,02585852	- 3,940280409
D3ZVB7	Osteoglycin OS=Rattus norvegicus (Rat) OX=10116 GN=Ogn PE=1 SV=1 - [D3ZVB7_RAT]	82236951,08	18516812,9	0,014187066	- 4,441204408
G3V624	Coronin OS=Rattus norvegicus (Rat) OX=10116 GN=Coro1c PE=1 SV=1 - [G3V624_RAT]	5165071,941	257393,1836	0,034467386	- 20,06685596
G3V852	RCG55135, isoform CRA_b OS=Rattus norvegicus (Rat) OX=10116 GN=Tln1 PE=1 SV=1 - [G3V852_RAT]	8068581,499	2162141,726	0,032238784	- 3,731754215

G3V928	Prolow-density lipoprotein receptor-related protein 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Lrp1 PE=1 SV=1 - [LRP1_RAT]	4100663,551	1087708,791	0,008916586	- 3,7700012 96
O35763	Moesin OS=Rattus norvegicus (Rat) OX=10116 GN=Msn PE=1 SV=3 - [MOES_RAT]	8558623,139	2878085,422	0,019116858	- 2,9737210 28
P00406	Cytochrome c oxidase subunit 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Mtco2 PE=1 SV=3 - [COX2_RAT]	10026082,34	2395183,87	0,049462547	- 4,1859343 1
P00507	Aspartate aminotransferase, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Got2 PE=1 SV=2 - [AATM_RAT]	6978454,569	2723941,599	0,02263613	- 2,5618958 09
P02767	Transthyretin OS=Rattus norvegicus (Rat) OX=10116 GN=Ttr PE=1 SV=1 - [TTHY_RAT]	21786152,73	5974321,544	0,022702719	- 3,6466321 01
P04256	Heterogeneous nuclear ribonucleoprotein A1 OS=Rattus norvegicus (Rat) OX=10116 GN=Hnrnpa1 PE=1 SV=3 - [ROA1_RAT]	5905771,22	798314,5828	0,006716509	- 7,3977994 99
P04785	Protein disulfide- isomerase OS=Rattus norvegicus (Rat) OX=10116 GN=P4hb PE=1 SV=2 - [PDIA1_RAT]	34011332,26	9504332,783	0,007830206	- 3,5785081 43

P04937	Fibronectin OS=Rattus norvegicus (Rat) OX=10116 GN=Fn1 PE=1 SV=2 - [FINC_RAT]	13256431,04	6102496,172	0,046545482	- 2,1722964 94
P05197	Elongation factor 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Eef2 PE=1 SV=4 - [EF2_RAT]	29126243,48	8773078,284	7,42E-05	- 3,3199570 93
P08010	Glutathione S- transferase Mu 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Gstm2 PE=1 SV=2 - [GSTM2_RAT]	11559522,17	3113094,063	0,000689595	- 3,7131939 92
P10719	ATP synthase subunit beta, mitochondrial OS=Rattus norvegicus (Rat) OX=10116 GN=Atp5f1b PE=1 SV=2 - [ATPB_RAT]	31886262,24	14567979,7	0,00147995	- 2,1887909 58
P11442	Clathrin heavy chain 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Cltc PE=1 SV=3 - [CLH1_RAT]	24227589,11	9004193,235	0,016570338	- 2,6907007 08
P11598	Protein disulfide- isomerase A3 OS=Rattus norvegicus (Rat) OX=10116 GN=Pdia3 PE=1 SV=2 - [PDIA3_RAT]	25432302,62	14931101,68	0,044293769	- 1,7033105 24
P12346	Serotransferrin OS=Rattus norvegicus (Rat) OX=10116 GN=Tf PE=1 SV=3 - [TRFE_RAT]	136083377,8	63962217,13	0,028295678	- 2,1275587 98
P13084	Nucleophosmin OS=Rattus norvegicus (Rat) OX=10116 GN=Npm1 PE=1 SV=1 - [NPM_RAT]	18056562,74	2283206,189	0,032226221	- 7,9084240 5

P13832	Myosin regulatory light chain RLC-A OS=Rattus norvegicus (Rat) OX=10116 GN=Rlc-a PE=2 SV=2 - [MRLCA_RAT]	9507277,301	4709818,966	0,008091241	-2,0186078
P19804	Nucleoside diphosphate kinase B OS=Rattus norvegicus (Rat) OX=10116 GN=Nme2 PE=1 SV=1 - [NDKB_RAT]	15050609,01	7631584,013	0,01928934	- 1,9721474 58
P19944	60S acidic ribosomal protein P1 OS=Rattus norvegicus (Rat) OX=10116 GN=Rplp1 PE=3 SV=1 - [RLA1_RAT]	7585784,248	2256924,174	0,025979754	- 3,3611161 31
P19945	60S acidic ribosomal protein P0 OS=Rattus norvegicus (Rat) OX=10116 GN=Rplp0 PE=1 SV=2 - [RLA0_RAT]	6479748,23	3820068,26	0,014483386	- 1,6962388 6
P21531	60S ribosomal protein L3 OS=Rattus norvegicus (Rat) OX=10116 GN=Rpl3 PE=1 SV=3 - [RL3_RAT]	12202010,35	3328134,807	0,047323565	- 3,6663209 46
P24368	Peptidyl-prolyl cis- trans isomerase B OS=Rattus norvegicus (Rat) OX=10116 GN=Ppib PE=1 SV=3 - [PPIB_RAT]	21665588,36	9904318,017	0,00082396	- 2,1874891 66
P27139	Carbonic anhydrase 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Ca2 PE=1 SV=2 - [CAH2_RAT]	12983880,87	4565608,439	0,019849674	- 2,8438445 91
P30427	Plectin OS=Rattus norvegicus (Rat)	19206743,25	5693461,035	0,033479893	- 3,3734740 85

	OX=10116 GN=Plec PE=1 SV=2 - [PLEC_RAT]				
P35565	Calnexin OS=Rattus norvegicus (Rat) OX=10116 GN=Canx PE=1 SV=1 - [CALX_RAT]	4482759,314	2435422,414	0,011440105	- 1,8406496 09
P46462	Transitional endoplasmic reticulum ATPase OS=Rattus norvegicus (Rat) OX=10116 GN=Vcp PE=1 SV=3 - [TERA_RAT]	11173096,74	2660682,025	0,042245185	- 4,1993355 97
P47942	Dihydropyrimidina se-related protein 2 OS=Rattus norvegicus (Rat) OX=10116 GN=Dpysl2 PE=1 SV=1 - [DPYL2_RAT]	11271685,8	5188573,043	0,008559613	- 2,1724057 28
P48037	Annexin A6 OS=Rattus norvegicus (Rat) OX=10116 GN=Anxa6 PE=1 SV=2 - [ANXA6_RAT]	18132183,97	4700600,114	0,027541979	- 3,8574189 53
P50123	Glutamyl aminopeptidase OS=Rattus norvegicus (Rat) OX=10116 GN=Enpep PE=1 SV=2 - [AMPE_RAT]	8503119,169	3365634,994	0,003390499	- 2,5264531 61
P60901	Proteasome subunit alpha type-6 OS=Rattus norvegicus (Rat) OX=10116 GN=Psma6 PE=1 SV=1 - [PSA6_RAT]	4296022,781	0	0,005691264	- 4296022,7 81
P61751	ADP-ribosylation factor 4 OS=Rattus norvegicus (Rat) OX=10116 GN=Arf4 PE=2 SV=2 - [ARF4_RAT]	16528164,81	2665719,126	0,04967168	- 6,2002649 32

P62083	40S ribosomal protein S7 OS=Rattus norvegicus (Rat) OX=10116 GN=Rps7 PE=1 SV=1 - [RS7_RAT]	34075639,99	3431896,741	0,041682926	- 9,9290982 7
P63245	Receptor of activated protein C kinase 1 OS=Rattus norvegicus (Rat) OX=10116 GN=Rack1 PE=1 SV=3 - [RACK1_RAT]	16208480,38	5846962,578	0,000580054	- 2,7721197 39
P69897	Tubulin beta-5 chain OS=Rattus norvegicus (Rat) OX=10116 GN=Tubb5 PE=1 SV=1 - [TBB5_RAT]	64757570,79	24912447,61	0,02985362	-2,5994062
P84083	ADP-ribosylation factor 5 OS=Rattus norvegicus (Rat) OX=10116 GN=Arf5 PE=1 SV=2 - [ARF5_RAT]	16528164,81	2087540,093	0,044280946	- 7,9175316 77

Table S6. All proteins presented in the STRING network image.

queryItem	stringId	preferredName	Annotation
D3ZN79	10116.ENSRNOP00000046281	LOC686074	Similar to 60S ribosomal protein L35
F1M3H8	10116.ENSRNOP00000057942	LOC681410	Hypothetical protein LOC681410
F7FHF3	10116.ENSRNOP00000004322	Serpinf2	Serpin peptidase inhibitor
G3V9R8	10116.ENSRNOP00000057257	Hnrpc	Heterogeneous nuclear ribonucleoprotein C
P00697	10116.ENSRNOP00000007747	Lyz2	Lysozyme C-1
P06399	10116.ENSRNOP00000060007	Fga	Fibrinogen alpha chain
P07632	10116.ENSRNOP00000002885	Sod1	Superoxide dismutase [Cu-Zn]
P08721	10116.ENSRNOP00000062358	Spp1	Osteopontin
P09527	10116.ENSRNOP00000016432	Rab7a	Ras-related protein Rab-7a
P14562	10116.ENSRNOP00000026580	Lamp1	Lysosome-associated membrane glycoprotein 1
P16617	10116.ENSRNOP00000003390	Pgk1	Phosphoglycerate kinase 1
P17046	10116.ENSRNOP00000000177	Lamp2	Lysosome-associated membrane glycoprotein 2
P24268	10116.ENSRNOP00000027407	Ctsd	Cathepsin D
P25113	10116.ENSRNOP00000065690	Pgam1	Phosphoglycerate mutase 1
P26772	10116.ENSRNOP00000020066	Hspe1	10 kDa heat shock protein, mitochondrial
P49134	10116.ENSRNOP00000014785	Itgb1	Integrin beta-1
P52296	10116.ENSRNOP00000012376	Kpnb1	Importin subunit beta-1
P61107	10116.ENSRNOP00000025649	Rab14	Ras-related protein Rab-14
P62832	10116.ENSRNOP00000005471	Rpl23	Ribosomal protein L23
P62859	10116.ENSRNOP00000060568	Rps28	Ribosomal protein S28
P85515	10116.ENSRNOP00000026792	Actr1a	Alpha-centractin
P85972	10116.ENSRNOP00000015179	Vcl	Vinculin
Q02874	10116.ENSRNOP00000049143	H2afy	Core histone macro-H2A.1
Q62826	10116.ENSRNOP00000032108	HnrnpM	Heterogeneous nuclear ribonucleoprotein M
Q6P747	10116.ENSRNOP00000019696	Hp1bp3	Heterochromatin protein 1-binding protein 3
Q9WVB1	10116.ENSRNOP00000025104	Rab6a	Ras-related protein Rab-6A

Table S7. All KEGG Pathway terms for OM differentially proteins list.

Term	Number of proteins	Benjamini
Tuberculosis (rno05152)	5	0.4597996200515788
Phagosome (rno04145)	5	0.4597996200515788
Lysosome (rno04142)	4	0.4853528999348182
Platelet activation (rno04611)	4	0.4853528999348182
Renin secretion (rno04924)	3	0.4853528999348182
Alcoholism (rno05034)	4	0.4853528999348182
Pertussis (rno05133)	3	0.4853528999348182
Gastric acid secretion (rno04971)	3	0.4853528999348182
Salivary secretion (rno04970)	3	0.4853528999348182
Estrogen signaling pathway (rno04915)	3	0.5089650040099455
Rap1 signaling pathway (rno04015)	4	0.5089650040099455
Circadian entrainment (rno04713)	3	0.5089650040099455
Melanogenesis (rno04916)	3	0.5089650040099455
Glucagon signaling pathway (rno04922)	3	0.5089650040099455
Amoebiasis (rno05146)	3	0.5781961007258999
Leukocyte transendothelial migration (rno04670)	3	0.5841368401429031

Table S8. All GO BP and MF terms for OM differentially proteins list.

Molecular function (MF)		
Term	Number of proteins	Benjamini
GO:0019904~protein domain specific binding	11	1,76E+10
GO:0019899~enzyme binding	8	0.008166325903532284
GO:0005515~protein binding	14	0.016159800051104303
GO:0019901~protein kinase binding	7	0.05721153007378836
GO:0003924~GTPase activity	5	0.0797128119245966
GO:0044822~poly(A) RNA binding	10	0.1018849006889884
GO:0005525~GTP binding	6	0.1018849006889884
GO:0031492~nucleosomal DNA binding	3	0.15255682207533813
GO:0003729~mRNA binding	4	0.1663214069071901
GO:0019003~GDP binding	3	0.22205365331246074
GO:0098641~cadherin binding involved in cell-cell adhesion	4	0.38505812948176
GO:0042802~identical protein binding	6	0.49913504214646987
GO:0031489~myosin V binding	2	0.7330474535659048
GO:0000166~nucleotide binding	4	0.7473098720616113
GO:0005537~mannose binding	2	0.7473098720616113
GO:0031683~G-protein beta/gamma-subunit complex binding	2	0.7473098720616113
GO:0004857~enzyme inhibitor activity	2	0.7599248252829
GO:0046872~metal ion binding	8	0.8357027231918286
GO:0051879~Hsp90 protein binding	2	0.8357027231918286
GO:0017048~Rho GTPase binding	2	0.8357027231918286
GO:0048365~Rac GTPase binding	2	0.8848127509385675
Biological Process (BP)		
Term	Number of proteins	Benjamini
GO:0070527~platelet aggregation	4	0.08262068646032715
GO:0051881~regulation of mitochondrial membrane potential	3	0.5201831787558018
GO:0031667~response to nutrient levels	4	0.5201831787558018
GO:0006749~glutathione metabolic process	3	1.0
GO:1902177~positive regulation of oxidative stress-induced intrinsic apoptotic signaling pathway	2	1.0
GO:0007160~cell-matrix adhesion	3	1.0
GO:0006914~autophagy	3	1.0
GO:0034498~early endosome to Golgi transport	2	1.0

GO:0032930~positive regulation of superoxide anion generation	2	1.0
GO:0001649~osteoblast differentiation	3	1.0
GO:0070935~3'-UTR-mediated mRNA stabilization	2	1.0
GO:0006954~inflammatory response	4	1.0
GO:0050821~protein stabilization	3	1.0
GO:0042493~response to drug	5	1.0
GO:0046716~muscle cell cellular homeostasis	2	1.0
GO:0000302~response to reactive oxygen species	2	1.0
GO:1900182~positive regulation of protein localization to nucleus	2	1.0
GO:0001894~tissue homeostasis	2	1.0
GO:0010033~response to organic substance	3	1.0
GO:0048675~axon extension	2	1.0
GO:0006094~gluconeogenesis	2	1.0
GO:0001895~retina homeostasis	2	1.0
GO:0032355~response to estradiol	3	1.0
GO:0006096~glycolytic process	2	1.0

Table S9. All GO CC terms for OM differentially proteins list.

Term	Amount of proteins	Benjamini
GO:0070062~extracellular exosome	37	2,02E-04
GO:0016020~membrane	19	3,01E+12
GO:0045121~membrane raft	8	3,01E+12
GO:0005737~cytoplasm	28	0.0010188147324258297
GO:0005829~cytosol	15	0.0010188147324258297
GO:0005764~lysosome	7	0.0011255576575965546
GO:0031982~vesicle	6	0.001530155997446664
GO:0097208~alveolar lamellar body	3	0.004240527974088332
GO:0048471~perinuclear region of cytoplasm	9	0.004240527974088332
GO:0005770~late endosome	5	0.005664486323055029
GO:0005615~extracellular space	12	0.006580587358463024
GO:0005765~lysosomal membrane	5	0.03692328284102456
GO:0042383~sarcolemma	4	0.05154126173420486
GO:0031012~extracellular matrix	5	0.05316179009974875
GO:0030017~sarcomere	3	0.05316179009974875
GO:0045335~phagocytic vesicle	3	0.060969242270797454
GO:0031410~cytoplasmic vesicle	5	0.06312878969394427
GO:0043005~neuron projection	5	0.1865357809075666
GO:0005577~fibrinogen complex	2	0.1865357809075666
GO:0009986~cell surface	6	0.19054984103033096
GO:0042470~melanosome	3	0.2097284403874833
GO:0005768~endosome	4	0.23281338936631052
GO:0005913~cell-cell adherens junction	4	0.243860069656727
GO:0005886~plasma membrane	17	0.25923933339822625
GO:0072562~blood microparticle	3	0.28922341569580007
GO:0030140~trans-Golgi network transport vesicle	2	0.28922341569580007
GO:0000785~chromatin	3	0.29030629951394865
GO:0000139~Golgi membrane	4	0.29030629951394865
GO:0005739~mitochondrion	9	0.3238731555605389
GO:0030670~phagocytic vesicle membrane	2	0.38056869568644935
GO:0005802~trans-Golgi network	3	0.3818488688392234
GO:0005759~mitochondrial matrix	3	0.3868677248693755
GO:0043234~protein complex	5	0.41834099437581496
GO:0005795~Golgi stack	2	0.4202787010990554
GO:0005925~focal adhesion	4	0.4494641514225866
GO:0005834~heterotrimeric G-protein complex	2	0.4494641514225866
GO:0043209~myelin sheath	3	0.4595957160371428

APÊNCIDE B – MATERIAL E MÉTODO ESTUDO 1

Estudo 1 – Obesity affects the proteome profile of periodontal ligament submitted to mechanical forces induced by orthodontic tooth movement in rats.

OBJETIVOS ESPECÍFICOS

Avaliar por meio de análise proteômica a composição proteica do ligamento periodontal sob condição de movimentação ortodôntica associada ou não à obesidade, em ratos;

Validar a presença e definir a localização de três proteínas relevantes à remodelação periodontal, previamente selecionadas a partir da análise proteômica, no ligamento periodontal de dentes de ratos sob movimentação ortodôntica em condição de obesidade ou não através de análise imuno- histoquímica.

MATERIAIS E MÉTODOS

Desenho experimental

Este estudo foi realizado nos mesmos animais que um estudo publicado anteriormente por nosso grupo, que incluiu resultados lipídicos, séricos, histológicos, microtomográficos e de expressão gênica¹. O estudo foi submetido e aprovado na Comissão de Ética no Uso de Animais da Faculdade de Odontologia de Araraquara (FOAr Unesp) (CEUA: 16/2015). Foram utilizados 10 ratos *Holtzman* adultos, com peso médio de 300 g, que foram mantidos no biotério da Faculdade de Odontologia de Araraquara - UNESP. Os animais foram acomodados em gaiolas de polipropileno e receberam alimentação granulada (Labina/Purina) e água *ad libitum*. Sendo assim, 10 animais foram distribuídos em 2 grupos experimentais, contendo 5 animais por grupo, conforme detalhados abaixo:

Grupo M: submetidos ao movimento ortodôntico;

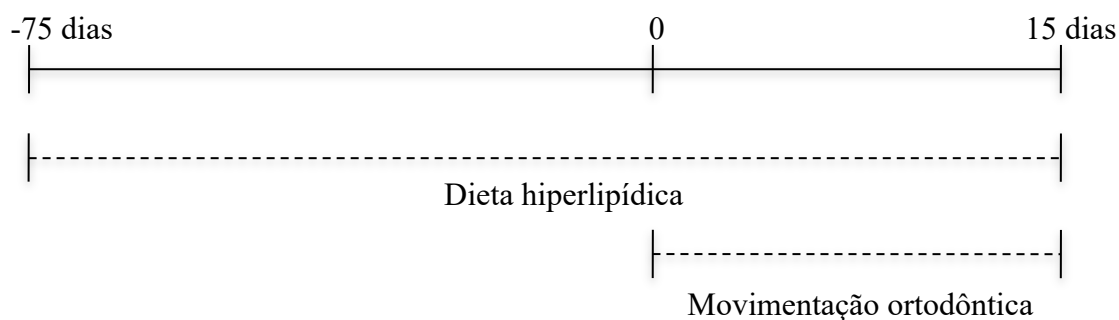
Grupo OM: submetidos à indução de obesidade e movimento ortodôntico.

Indução da obesidade

Os animais que foram submetidos à indução de obesidade (grupo OM) receberam dieta hiperlipídica que apresentava aproximadamente 3,82 kcal/g. Sua tabela nutricional consiste em 40 g de carboidratos, 19 g de lipídios, 13 g de proteínas, 4 g de fibras e 73 mg de sódio por 100 g de ração. A dieta hiperlipídica constituiu de

uma ração especial preparada manualmente, composta de ração para roedores, amendoim, bolacha maizena, e chocolate ao leite em uma proporção de 3:2:1:1². A ração padrão (Labina/Purina, Ribeirão Preto, Brasil), por sua vez, contém aproximadamente 2,25 kcal/g e sua tabela nutricional consiste em 48 g carboidratos, 4 g de lipídios, 22 g de proteínas, 8 g de fibras e 200 mg de sódio por 100 g de ração. A ração foi substituída diariamente e iniciou-se 75 dias antes do tempo zero (quando foram instalados os aparelhos) conforme *timeline* apresentado na Figura 1.

Figura 1 - Descrição do modelo experimental



Fonte: Elaboração própria.

Instalação do aparelho ortodôntico

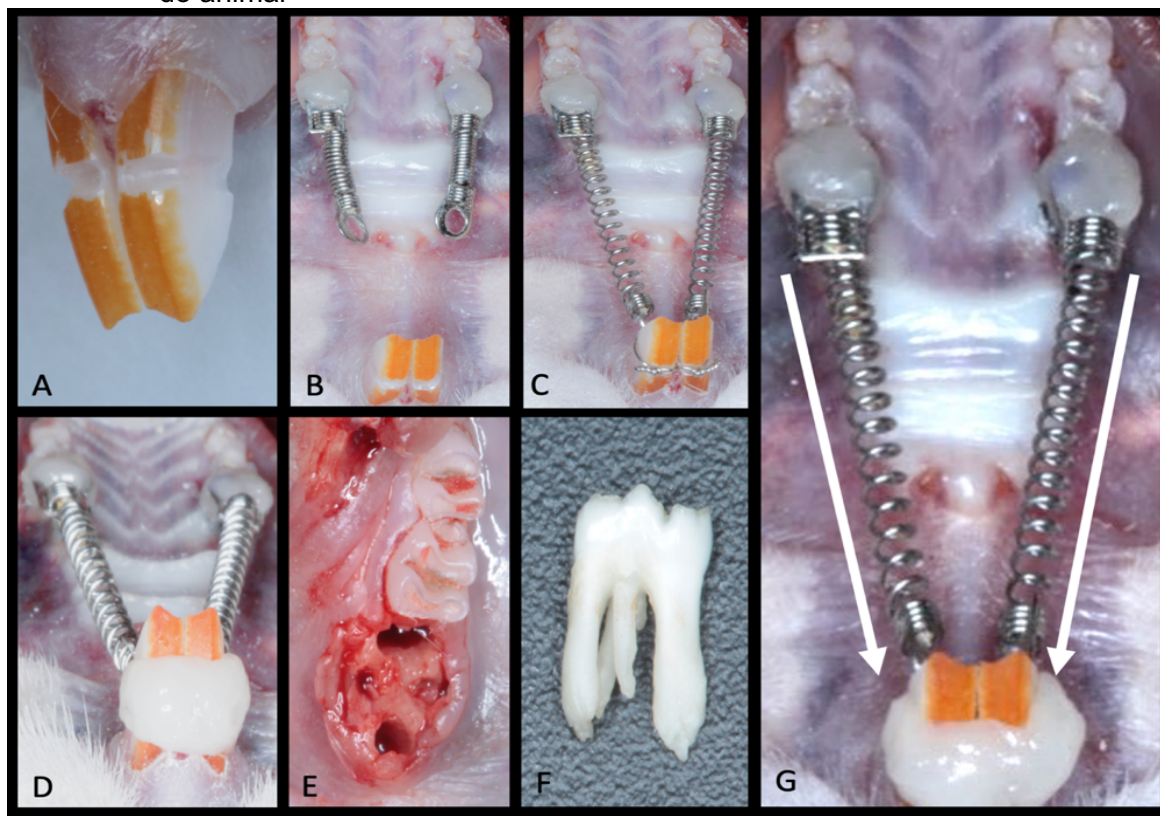
Foi utilizado um modelo de movimento dentário em ratos e um tipo de aparelho previamente estabelecidos por nosso grupo^{1,3,4} (Figura 2). Após os animais serem submetidos à anestesia geral (0,08 mL de ketamina e 0,04 mL de cloridrato de xilasina por 100g de peso corporal, intramuscular na pata posterior) e serem posicionados em mesa operatória, uma mola fechada de níquel-titânio (*Sentalloy*[®], GAC, *Dentsply*) foi conectada entre o primeiro molar superior e incisivo central superior com fio de amarrilho (CrNi, 0.20 mm, código 55.01.208, Morelli). Para colocação da mola nos incisivos centrais superiores, sulcos nas superfícies mesial, distal e vestibular desses dentes foram preparados para prevenir que o fio de amarrilho se soltasse devido à curvatura lingual e à erupção contínua dos incisivos. Após os fios serem amarrados, uma camada de resina composta foi colocada acima do fio para prevenir seu deslocamento e irritação pulpar devido à exposição dentinária. Já para colocação da

mola nos primeiros molares superiores, o fio de amarrilho foi colocado na oclusal dos dentes e resina composta foi colocada sobre o fio de amarrilho para que ele não se soltasse. Para que não houvesse danos à oclusão do animal devido ao fio e à resina, o dente antagonista (primeiro molar inferior) foi extraído. A mola de níquel-titânio foi utilizada para promover uma liberação de força constante de 0.25 N durante o curso do experimento. A instalação do aparelho ortodôntico foi realizada no tempo zero.

Após a colocação do aparelho ortodôntico pode haver um desconforto que foi minimizado com administração de cetoprofeno (Ketoflex®; Mundo Animal, São Paulo, Brasil) por via sub-cutânea de 12/12 horas por 3 dias.

Além disso, a ração foi triturada para que os animais dos grupos com aparelho ortodôntico possam mastigar/ingerir a ração sem dificuldades.

Figura 2 - Aparelho ortodôntico instalado entre o incisivo central e o primeiro molar superior do animal



Sulcos nas superfícies mesial, distal e vestibular preparados nos incisivos (A), colocação da mola na oclusal dos primeiros molares com resina composta (B), colocação da mola no sulco realizado na incisal do incisivo com fio de amarrilho e resina composta (C e D), extração do primeiro molar inferior (E e F), mola em posição e seta indicando a direção da força exercida (G).

Fonte: Arquivo pessoal da autora.

Sacrifício dos animais

Após 15 dias do tempo zero, todos os animais foram sacrificados por overdose anestésica. A seleção deste período considerou a representação do período em que já ocorre significativo movimento dentário. Após o sacrifício, a maxila de cada animal foi removida e separada em duas hemimaxilas para posterior redução e distribuição aleatória para realização de imuno-histoquímica e microdissecção a laser para análise proteômica.

Preparo das amostras para análise proteômica

As hemimaxilas foram colocadas em cassetes plásticos, imersas e mantidas no fixador formol tamponado a 10% (Fisher Diagnostics, Midletown, VA, EUA) por 24 horas a 4°C. Após esse período, as amostras passaram por três lavagens em solução de tampão fosfato 1x (PBS, pH 7,4, Applied Biosystems, Foster City, CA, EUA) por 30 minutos cada a 4°C e foram descalcificadas em solução de ácido etilenodiaminotetracético (EDTA, Merck/Millipore, Burlington, MA, EUA) a 20% em tampão fosfato a 0,01M, pH 7.4 e mantidas a 4°C sob agitação com três trocas diárias da solução por um período de 30 dias.

As hemimaxilas foram reduzidas mantendo-se a região dos molares, lavadas em PBS por 30 minutos a 4°C, desidratadas em 3 trocas de etanol absoluto (Sigma-Aldrich, San Luis, MO, EUA) por 30 minutos a 4°C, incubadas em 2 trocas de xilol (Arcturus Bioscience, San Francisco, CA, EUA) por 20 minutos em temperatura ambiente e incluídas em parafina (Merck/Millipore, Burlington, MA, EUA) a 56°C. Cortes longitudinais dos primeiros molares foram seccionados com 5 µm de espessura na direção méso-distal, passados em H₂O MilliQ tratada com DEPC (dietilpirocarbonato) a 42°C e colocados em lâminas de vidro com membrana de polietileno naftaleno (PEN membrane glass slides, Applied Biosystem, Foster City, CA, EUA) para captura por microdissecção a laser (LCM).

As lâminas contendo os cortes (10 cortes) foram armazenadas a - 20°C até serem processadas para a microdissecção a laser.

Preparo das amostras para análise imuno-histoquímica

Após o sacrifício dos animais, 5 hemimaxilas por grupo foram removidas e fixadas em paraformaldeído 4% (Sigma-Adlrich, San Luis, Missouri, EUA) por 48 horas. Após fixação, as hemimaxilas foram desmineralizadas em solução de EDTA

7% por 90 dias para posterior inclusão em parafina. Cortes histológicos seriados de 5 µm de espessura foram obtidos, montados em lâminas silanizadas (Perfecta, São Paulo, SP, Brasil) em banho-maria de água destilada e secas em posição vertical para facilitar a adesão entre o corte e a lâmina. Esta adesão foi intensificada deixando as lâminas à 58°C por 2 horas na estufa para posterior realização de análise imuno-histoquímica.

Captura por microdissecção a laser (LCM)

As lâminas contendo os cortes histológicos foram desparafinizadas em 2 trocas de xilol (Arcturus Bioscience, San Francisco, CA, EUA) por 2 minutos, os cortes foram desidratados em concentrações crescentes de etanol, secos em temperatura ambiente por 5 minutos e imediatamente microdissecados. A área de captura incluiu o ligamento periodontal (PDL), delimitada utilizando como referência a junção cimento-esmalte (JCE) e a crista óssea alveolar no espaço interdental e por toda a região de furca, evitando as áreas adjacentes ao osso alveolar e ao cimento para excluir células do tecido ósseo, osteoblastos e osteoclastos, e cementoblastos.

O PDL foi capturado utilizando uma combinação de “laser” infravermelho (IR) e “laser” cortante ultravioleta (UV) do equipamento de microdissecção Arcturus XT (Applied Biosystem, Foster City, CA, EUA), usando as seguintes configurações para a captura do PDL sobre um aumento de 10x: velocidade de corte UV de 100 a 200 mm/s; potência do laser IR de 65 a 70mW, duração de 20 ms, tamanho do foco de 20µm. Foram microdissecados 8-10 cortes histológicos por amostra e a área total de captura foi calculada e usada para normalizar a quantidade de proteína/tecido para a cromatografia líquida e espectrometria de massa de alta resolução (LC-MS/MS).

Os “caps” contendo os tecidos microdissecados foram adaptados a um microtubo de 0,5 mL e ficou armazenado a -80° C.

Preparo das amostras microdissecadas para cromatografia líquida e espectrometria de massa de alta resolução (LC-MS/MS)

Para o preparo das amostras para a análise por LC-MS/MS os “caps” contendo os tecidos microdissecados foram descongelados em gelo. As amostras foram desnaturadas por incubação em 30 µl de ureia 8 M (Sigma-Aldrich, San Luis, MO, USA) durante 30 minutos à temperatura ambiente. As proteínas extraídas foram reduzidas por incubação com ditioneitol 0,5 M (DTT, Sigma-Aldrich, San Luis, MO,

EUA) a 56°C durante 25 minutos e alquiladas por incubação com iodoacetamida 0,14 M (Sigma-Aldrich, San Luis, MO, EUA) à temperatura ambiente durante 30 minutos, e nova incubação em ditioneitol 0,5 M durante 15 minutos à temperatura ambiente. As amostras em solução foram então diluídas em bicarbonato de amônio 50 mM (Sigma-Aldrich, San Luis, MO, EUA) para uma concentração final de 1,6 M de ureia, e 0,1 M de cloreto de cálcio (Sigma-Aldrich, San Luis, MO, EUA) foi adicionado à mistura de proteínas. As amostras foram digeridas por meio de incubação com 2 µg de tripsina (Promega, Madison, WI, EUA) durante a noite, à 37°C.

Todos os passos de incubação descritas acima foram realizados com o “cap” acoplado ao microtubo colocado numa posição invertida para permitir que os reagentes entrem em contato com os tecidos capturados. Antes de adicionar os reagentes os conjuntos de microtubo/“cap” foram centrifugados para levar a mistura de proteínas para baixo. Depois da digestão, os “caps” foram descartados e as misturas de peptídeos foram equilibradas ao pH 2,0 por adição de solução de ácido fórmico 0,1%, pH 2,0 (Sigma-Aldrich, San Luis, MO, EUA)⁵. Em seguida as amostras ficaram mantidas em -20°C até a análise por LC-MS/MS no Biomass, laboratório de espectrometria de massas LC-MS/MS localizado no CEFAP no prédio do ICB IV da Universidade de São Paulo em São Paulo.

Análise por cromatografia líquida e espectrometria de massa de alta resolução (LC-MS/ MS)

A análise proteômica foi realizada para avaliar o perfil da síntese protéica de proteínas relacionadas à remodelação tecidual no ligamento periodontal. As análises foram realizadas no LTQ-Orbitrap Velos ETD (Thermo Fisher Scientific, Waltham, MA, EUA) acoplado ao Easy nanoLC II (Thermo Fisher Scientific, Waltham, MA, EUA). Os peptídeos foram separados por um gradiente de 2-95% acetonitrila em 0,1% de ácido fórmico usando uma coluna analítica C18 PicoFrit (C18 PepMAp, 75 µm id x 10 cm, tamanho de partícula 3,5 µm, tamanho de poro 100 Å; New Objective, Ringoes, NJ, EUA), em um quociente de vazão de 300 nL/minuto por 105 minutos. O LTQ-Orbitrap Velos foi operado no modo ion-positivo com aquisição dependente dos dados. A varredura completa foi obtida no Orbitrap com um valor alvo de controle de ganho automático (AGC) de 10^{-6} íons e um tempo máximo de preenchimento de 500 ms. Cada varredura de íons precursores foi adquirida com uma resolução de 60.000

FWHM na faixa de massa de 400-1500 m/z. Os peptídeos foram fragmentados por CID MS/MS usando uma energia de colisão normativa de 35. Os 20 peptídeos mais abundantes foram selecionados para MS/MS e dinamicamente excluídos por uma duração de 30 segundos. As amostras foram analisadas em duas replicatas técnicas que foram combinadas para realizar a pesquisa no banco de dados. Todos os arquivos raw foram acessados no software Xcalibur (Thermo Fisher Scientific, Waltham, Massachusetts, EUA) e processados utilizando o Proteome Discover (versão 1.4.0.288, Thermo Finnigan, São Jose, CA, EUA).

O espectro gerado pelo MS/MS (msf) dos arquivos raw foram pesquisados novamente no banco de dados UniProt *Rattus_norvegicus* (26-02-2020), assumindo digestão de tripsina com parâmetros definidos para um máximo de duas clivagens ausentes, com uma tolerância de 20,0 ppm de parent íon para pesquisa de MS e uma tolerância de massa de fragmento de íon de 0,6 Da. A oxidação de metionina (+ 16 Da) foi definida como uma modificação dinâmica, e a carbamidometilação em resíduos de cisteína (+ 57 Da) foi definida como uma modificação estática. Proteínas com pelo menos dois valores válidos em pelo menos duas amostras foram consideradas para análise posterior. O teste t-student foi aplicado para testar as diferenças nas intensidades de proteína entre os grupos de amostras M e OM. Os índices de proteína foram calculados a partir da média das intensidades proteicas espectrais normalizadas.

Os dados de espectrometria de massa foram depositados no Consórcio ProteomeXchange através do repositório parceiro PRIDE⁶ com o identificador PXD033647.

A Análise de Componentes Principais (PCA) foi realizada para determinar a variabilidade da amostra do PDL entre os perfis de proteoma de ambos os grupos. Foi gerado o gráfico de volcano plot para representar as proteínas diferencialmente abundantes nos PDLs de cada grupo. As análises de PCA e volcano plot foram realizadas utilizando o software R.

Na análise de PCA, os valores brutos foram convertidos para a escala log₂ seguida pelo uso da função "prpcomp" do software estatístico R para determinar os valores dos componentes principais, e o pacote "ggfortify" (de "ggplot2") para representação gráfica. O volcano plot foi construído usando a função "plot" no software estatístico R. Este consiste em valores de p convertidos para a escala "-log₁₀" no eixo Y e valores de log₂ (fold change) no eixo X. Os valores de fold change foram

calculados usando os seguintes critérios: (1) Quando a média dos valores brutos de M for zero, o fold-change foi igual à média de OM; (2) Quando a média dos valores brutos de OM for zero, o fold-change foi igual a $-(\text{média de M})$; (3) Quando o resultado da equação $(\text{média de OM} / \text{média de M})$ for < 1 , e o fold-change for igual a $-(\text{média de M} / \text{média de OM})$; caso contrário, o valor de fold-change foi $(\text{média de OM} / \text{média de M})$. Quando $\log_2$ (fold change) foi maior que zero, a proteína é mais abundante em OM, caso contrário, é mais abundante em M. O agrupamento de amostras e a organização gráfica do heat map foram realizados convertendo o NSC em z-scores e submetendo-os ao software EXPANDER.

Para análise de enriquecimento gênico (GO) e de vias metabólicas KEGG (Enciclopédia de Genes e Genoma de Kioto) foi utilizada a plataforma de Banco de dados para anotação, visualização e descoberta integrada (DAVID versão 6.8, realizada 26-11-2020; <https://David.ncicrf.gov>), onde os termos enriquecidos foram determinados através do teste estatístico de t-student seguido de correção múltipla Benjamini-Hochberg ($p \leq 0,05$). Em seguida, foi realizada análise de interação entre proteínas diferencialmente abundantes no grupo OM a fim de se revelar os princípios da organização e funções celulares, este foi realizado com uso do software STRING versão 11.0 (realizada em 26-11-2020; <https://string-db.org>), utilizando alta confiança (0,700).

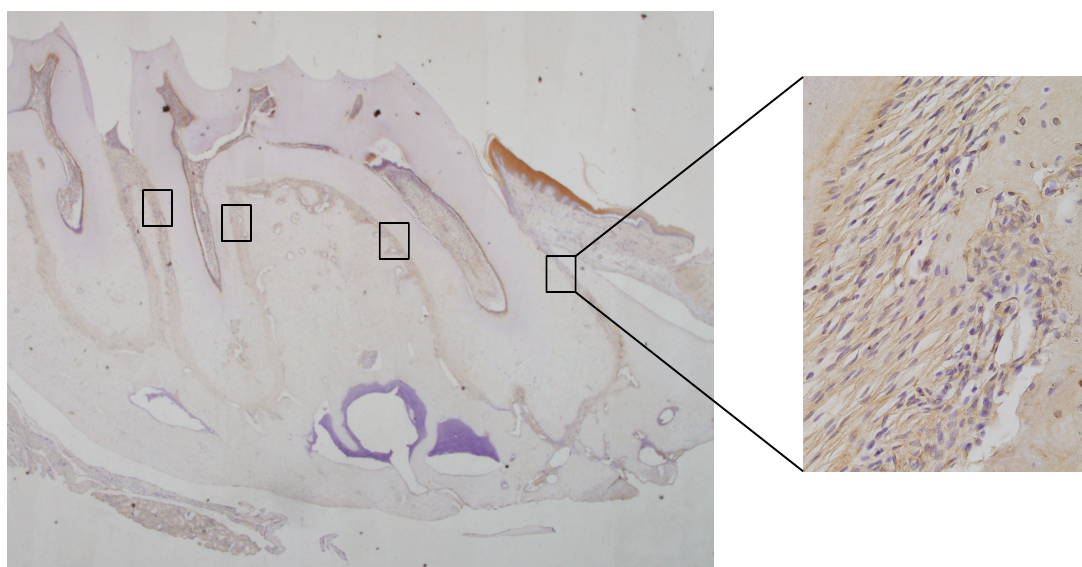
Análise imuno-histoquímica

A análise imuno-histoquímica foi realizada para se confirmar a detecção e localização da expressão proteica de 3 proteínas selecionadas na análise proteômica por espectrometria de massas. A desparafinização foi feita por meio de duas incubações de 5 minutos cada em xilol, seguidas por três lavagens em etanol absoluto. Em seguida, foi feita a re-hidratação gradual até a água destilada. E então, recuperação antigênica utilizando Rodent (Biocare Medical; Pacheco, CA, EUA). Em seguida os cortes foram submetidos ao bloqueio dos epítomos inespecíficos através da aplicação de peróxido de hidrogênio 5% por 40 minutos e de proteína bovina albumina (BSA) a 5% por 30 minutos. Em seguida, os cortes foram incubados por 16 horas nos anticorpos primários Vinculina/VCL (1:50, LS-B5770, LifeSpan BioSciences, Seattle, WA EUA), Osteopontina/SPP1 (1:100, LS-B425, LifeSpan BioSciences, Seattle, Washington, EUA) e Catepsina D/CTSD (1:50, LS-B9108, LifeSpan BioSciences, Seattle, Washington, EUA). Posteriormente, os cortes foram tratados

com o sistema DAB-streptavidina (Dako, Glostrup, Dinamarca). O sistema de revelação utilizado foi o DAB (Dako, Glostrup, Dinamarca). Os cortes então foram contra-corados com solução de hematoxilina de Carrazi para visualização dos núcleos celulares.

As imagens foram obtidas através de uma câmera acoplada a um microscópio de luz (Leica DMLS – Leica Reichet Diastar Products & Jung, Wetzlar, Alemanha) no aumento de 400x. As áreas de interesse foram selecionadas e fotografadas por uma câmera digital (Leica DFC 300 FX - Leica Reichet Diastar Products & Jung, Wetzlar, Alemanha), e as imagens capturadas foram salvas em formato TIFF (Formato de Arquivo de Imagem com Tags). A intensidade e localização da coloração foram registradas por um examinador cego para os grupos experimentais utilizando um software de sistema de imagem apropriado (Image J 1.37b National Institutes of Health, EUA) (Figura 3).

Figura 3 - Imagem representativa da área de ligamento periodontal do primeiro molar superior, analisada com aumento de 400x para análise de imuno-histoquímica



Fonte: Arquivo pessoal da autora.

Análise estatística

Todos os dados obtidos foram tabulados e submetidos a um teste de normalidade (Shapiro-Wilk). As análises estatísticas foram realizadas com o auxílio do software GraphPad Prism (Graph-Pad Prism 9 Software; Inc, La Jolla, San Diego, CA,

EUA). Em todos os procedimentos, foi adotado um nível de significância de 5% para a tomada da decisão quanto à validade da hipótese testada. A partir disso, foi utilizado o teste t-student para comparação entre os grupos.

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APÊNCIDE C – MATERIAL E MÉTODO ESTUDO 2

Estudo 2 – Influence of the monoclonal anti-sclerostin antibody on osteoporotic female rats' periodontal tissues during orthodontic tooth movement.

OBJETIVOS ESPECÍFICOS

Avaliar o deslocamento dentário, densidade óssea alveolar e volume ósseo alveolar em dentes submetidos ou não à movimentação ortodôntica, em ratas saudáveis ou osteoporóticas e que receberam ou não tratamento subcutâneo com anticorpo monoclonal anti-esclerostina (Scl-Ab), por meio de microtomografia computadorizada;

Avaliar os tecidos periodontais de dentes submetidos ou não à movimentação ortodôntica, em ratas saudáveis ou osteoporóticas e que receberam ou não Scl-Ab, por meio de análise histológica;

Avaliar a expressão gênica de mediadores inflamatórios nos tecidos gengivais de dentes submetidos ou não a movimentação ortodôntica, em ratas saudáveis ou osteoporóticas e que receberam ou não Scl-Ab;

Avaliar a presença de colágeno birrefringente no ligamento periodontal dos primeiros molares nos lados de pressão e tensão.

MATERIAIS E MÉTODOS

Desenho experimental

O estudo foi submetido e aprovado na Comissão de Ética no Uso de Animais da Faculdade de Odontologia de Araraquara (FOAr Unesp) (CEUA: 38/2020). Foram utilizadas 48 ratas fêmeas (*Rattus Norvegicus*, variação *albinus*, Holtzman), de aproximadamente 13 semanas de idade, com massa corporal entre 250-300 gramas, que foram mantidas no Biotério da Faculdade de Odontologia da UNESP de Araraquara (FOAr-UNESP), alimentadas com ração sólida e com acesso a água *ad libitum*, antes e durante todo o período experimental, em ambiente com água, luz e temperatura controladas.

Para o cálculo amostral, foi utilizado o teste ANOVA. Este foi baseado nos resultados da análise de microtomografia computadorizada (micro-CT) realizada em um trabalho anterior do grupo¹. Foi determinada uma diferença mínima entre as médias para se ter diferença estatística de 8,00 μm com desvio padrão de 3,87 μm .

Levando em consideração um poder de teste de 0,80 ($1 - \beta = 0,80$) e nível de significância de $\alpha = 0,05$, considerando uma possível perda relativa de animais de 14%. Sendo assim, 48 animais foram distribuídos em 6 grupos experimentais (totalizando 8 animais por grupo) conforme detalhado abaixo:

Grupo C: animais saudáveis sem nenhuma intervenção;

Grupo OM: animais saudáveis que foram submetidos ao movimento ortodôntico;

Grupo OVX: animais osteoporóticos;

Grupo OVX+OM: animais osteoporóticos que foram submetidos ao movimento ortodôntico;

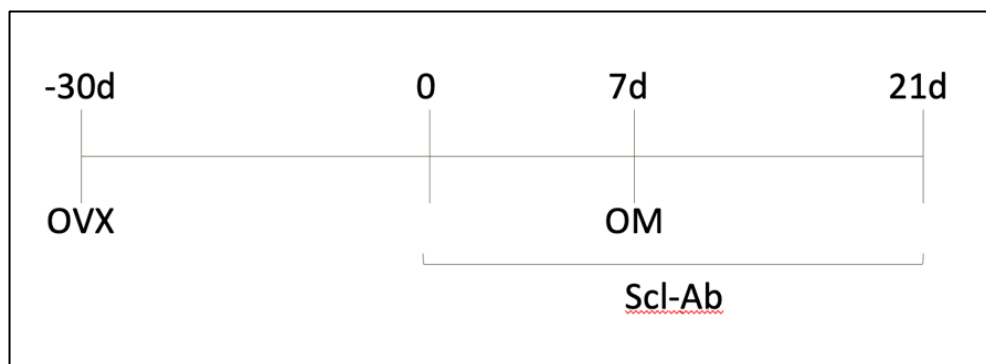
Grupo OVX+Scl-Ab: animais osteoporóticos que receberam anti-esclerostina;

Grupo OVX+Scl-Ab+OM: animais osteoporóticos que receberam anti-esclerostina e foram submetidos ao movimento ortodôntico.

Delineamento experimental e sacrifício dos animais

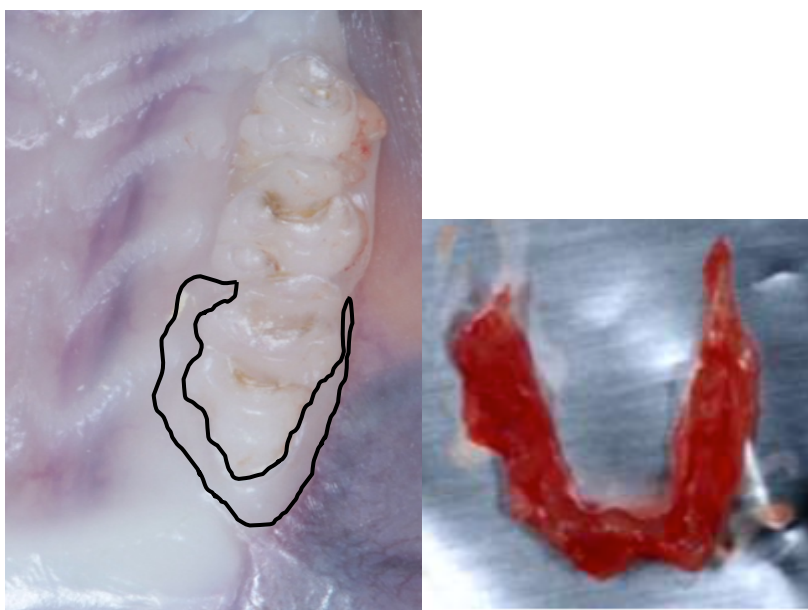
Após 21 dias do tempo zero (Figura 1), todos os animais foram sacrificados por overdose anestésica. Após o sacrifício, a maxila de cada animal foi removida e separada em duas hemimaxilas para posterior redução e distribuição aleatória para realização de microtomografia computadorizada e extração de RNA total para PCR tempo real. Para as peças destinadas à extração de RNA foi removido um colar gengival ao redor dos primeiros molares superiores utilizando lâmina de bisturi 15C (Figura 2) e imediatamente esse tecido gengival removido foi congelado em nitrogênio líquido e posteriormente armazenados em freezer -80 °C.

Figura 1 - Desenho experimental



Fonte: Elaboração própria.

Figura 2 - Colar gengival removido do primeiro molar superior



Fonte: Arquivo pessoal da autora.

Indução da osteoporose/cirurgia de ovariectomia (OVX)

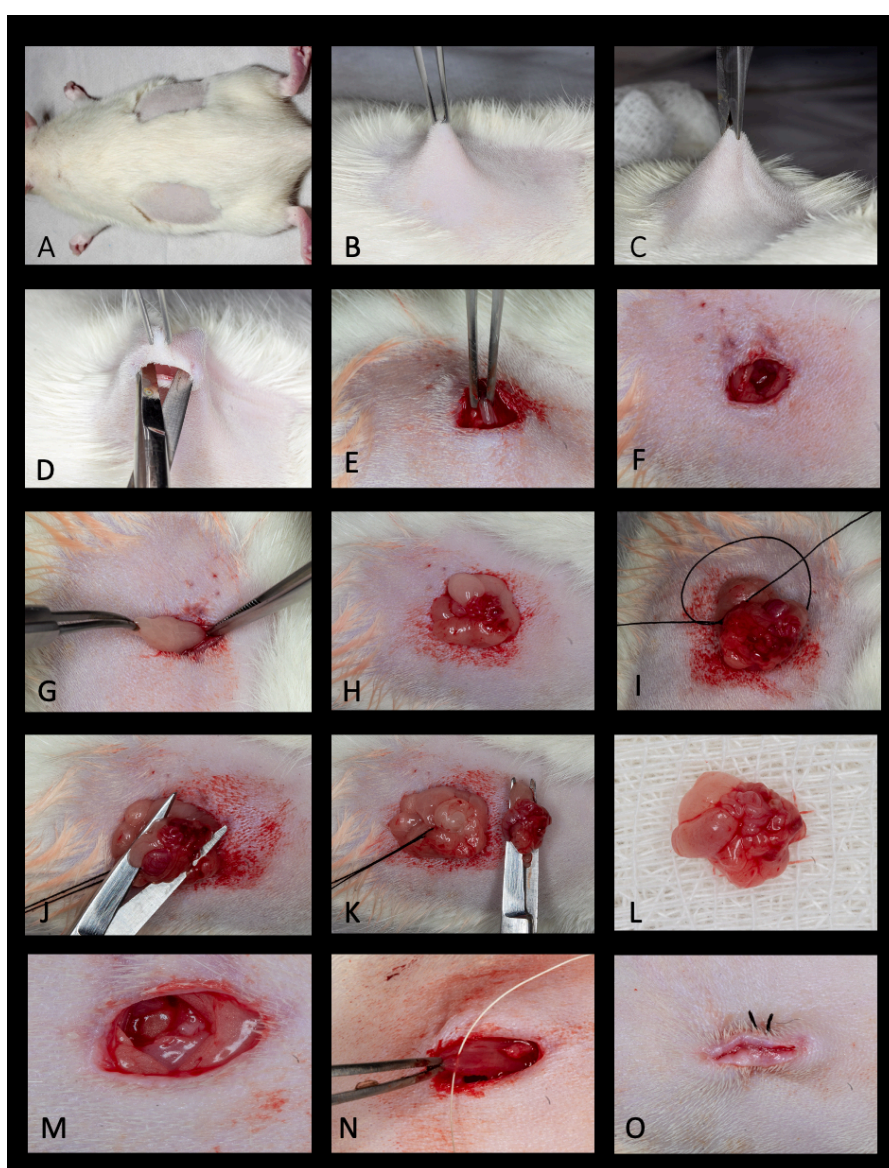
Para indução da osteoporose, todas as ratas foram submetidas à anestesia intramuscular na pata posterior por uma combinação de Quetamina com Xilazina, na proporção de 0,08 ml/100g de massa corporal (Cloridrato de Quetamina – Francotar – Virbac do Brasil Ind. Com. Ltda.) e 0,04 ml/100g de massa corporal (Cloridrato de Xilazina - Virbaxyl 2% - Virbac do Brasil Ind. E Com. Ltda.), respectivamente.

Posteriormente foi executado um acesso cirúrgico dorsal para obtenção de acesso aos ovários dos animais. Nos grupos de animais saudáveis apenas o acesso cirúrgico foi executado, enquanto no grupo de animais osteoporóticos os ovários foram removidos. Após anestesia, resumidamente, o procedimento consiste em (1) remover os pelos da área cirúrgica (tricotomia), (2) limpar a área cirúrgica com álcool 70%, (3) pinçar a pele com uma pinça e realizar uma pequena incisão com tesoura em 90° na região entre o final da costela e o início do fêmur, nos dois lados, (4) divulsionar a pele com a tesoura, (5) pinçar o músculo e realizar uma pequena incisão com tesoura em 90° e divulsionar, (6) inserir um fio separando o ovário das trompas e cornos uterinos e amarrar forte, (7) remover o ovário com a tesoura (Figura 3). Após este procedimento, o tecido foi suturado por planos internamente com fio reabsorvível 5.0 (Vicryl Ethicon®; Johnson & Johnson, São José dos Campos, Brasil) e externamente

com fio de seda 4.0 (Ethicon®; Johnson & Johnson, São José dos Campos, Brasil) (Figura B3).

Os animais receberam penicilina associada à estreptomicina na dosagem 0,1 ml/100g de massa corporal (Pentabiótico®; Wyeth-Whitehall Ltda, São Paulo, Brasil) por via intramuscular em dose única, e 0,03 ml/100 g de massa corporal de cetoprofeno (Ketoflex®; Mundo Animal, São Paulo, Brasil) por via sub-cutânea de 12/12 horas por 3 dias, para cuidados pós- operatórios.

Figura 3 - Procedimento de cirurgia de ovariectomia (OVX), nos grupos osteoporóticos



Acesso a área cirúrgica após tricotomia (A), incisão e divulsão do tecido epitelial (B, C e D), incisão do tecido muscular (E e F), exposição do ovário (G e H), separação e remoção do ovário (I, J, K, L e M), sutura do plano muscular (N) e sutura do plano epitelial (O).

Fonte: Arquivo pessoal da autora.

Instalação do aparelho ortodôntico

Foi realizado exatamente da mesma forma descrita no estudo 1. A instalação do aparelho ortodôntico foi realizada 7 dias após o início da administração do anticorpo monoclonal anti-esclerostina (Scl-Ab) e foi mantido até o final do experimento, totalizando 15 dias de aparelho (Figura 1).

Aplicação do anticorpo monoclonal anti-esclerostina (Scl-Ab)

Após 30 dias da cirurgia de ovariectomia, os animais dos grupos OVX+Scl-Ab e OVX+Scl-Ab+OM foram tratados com injeção subcutânea de anticorpo monoclonal anti-esclerostina (Evenity®, romosozumabe, Scl-Ab VI, Amgen Inc., Thousand Oaks, CA, EUA) na dose de 25 mg/kg de massa corporal. A administração deste foi realizada 2 vezes na semana até o fim do período experimental, totalizando 3 semanas (Figura B1). Os demais grupos experimentais (C, OM, OVX e OVX+OM) receberam injeção subcutânea de PBS 1x na mesma frequência dos animais que receberam Scl-Ab. A dose e tempo de aplicação foram determinados com base na literatura^{2, 3}.

Massa corporal

A massa corporal dos animais de todos os grupos foi registrada semanalmente utilizando uma balança de pesagem apropriada.

Peso dos úteros

Após a eutanásia, para comprovação do estabelecimento da osteoporose, o útero de todos os animais de todos os grupos foi coletado e pesado em balança de precisão.

Microtomografia computadorizada (micro-CT)

Para confirmação do estabelecimento da osteoporose induzida por ovariectomia, os animais de todos os grupos foram avaliados quanto ao volume (BV/TV) e densidade óssea (BMD) e também quanto ao número (Tb.N), espessamento (Tb.Th) e separação (Tb.Sp) de trabéculas ósseas por meio de micro-CT in vivo, previamente à cirurgia (dia -30) e 30 dias após esta (dia 0).

Para isso, os animais foram submetidos à anestesia geral intramuscular na pata posterior por uma combinação de Quetamina com Xilazina, na proporção de 0,08 ml/100g de massa corporal (Cloridrato de Quetamina – Francotar – Virbac do Brasil

Ind. Com. Ltda.) e 0,04 ml/100g massa corporal (Cloridrato de Xilazina - Virbaxyl 2% - Virbac do Brasil Ind. E Com. Ltda.), respectivamente, e então foram colocados em posição de decúbito lateral e tiveram seu fêmur e tíbia direitos escaneados através do aparelho de microtomografia computadorizada (μ CT Skyscan 1176, Aartselaar, Kontich, Bélgica).

O gerador de raios- x foi operado a 50 kVp, com corrente do feixe em 497 μ A, tempo de integração de 360 ms, filtro de alumínio de 0,5 mm, tamanho do voxel de 17,48 μm^3 e com resolução de imagem de 12,45 μm . As imagens foram reconstruídas, com o auxílio de um software específico (NRecon versão 1.6.1.5 – Bruker, Kontich, Bélgica) em todas as três dimensões espaciais, em seguida foram orientadas utilizando o *Data Viewer* software (Data Viewer versão 1.4.3.1 - Bruker, Kontich, Bélgica) (Figura 4) e então analisadas utilizando *software* CTan (CT Analyser versão 1.10.1.0 - Bruker, Kontich, Bélgica) (Figura 5).

A fim de analisar se o modelo de indução osteoporose por ovariectomia impactará na qualidade óssea dos animais, os parâmetros de qualidade óssea do osso medular da metáfise femoral e tibial foram avaliados. Isto foi realizado utilizando uma ferramenta específica (*custom processing*) do *software* CTan (Figura B5). Além disso, a densidade de osso alveolar foi medida com o auxílio de phantoms com densidades previamente definidas, fornecidos pela própria empresa Bruker (Kontich, Bélgica).

Após o sacrifício dos animais, 8 hemimaxilas de ratas por grupo foram removidas e fixadas em paraformaldeído 4% por 48 horas, e mantidas em álcool 70% em temperatura ambiente até serem escaneadas com o auxílio do microtomógrafo computadorizado (μ CT Skyscan 1176, Aartselaar, Kontich, Bélgica).

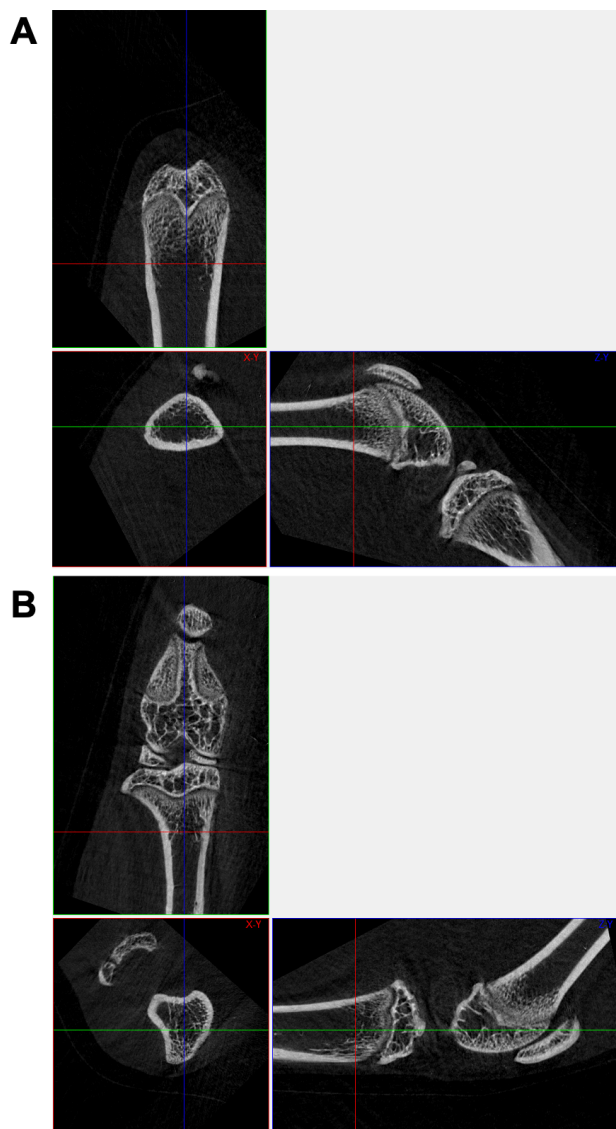
O gerador de raios-x foi operado a 50 kVp, com corrente do feixe em 497 μ A, filtro de alumínio de 0,5 mm e com uma resolução de imagem de 18 μm . As imagens foram reconstruídas, e em seguida todas as imagens das hemimaxilas foram orientadas e salvas em cortes axiais (2000 x 1336) (Figura 6) e então foram analisadas utilizando os mesmos softwares citados acima.

Foram medidos: volume de osso alveolar (BV) e o volume tecidual (TV), para isso a região de interesse (ROI) foi delimitada do ápice das raízes do primeiro e segundo molar à crista alveolar, com a exclusão das raízes e do espaço periodontal, compreendendo assim apenas o osso alveolar ao redor do primeiro molar (Figura 7). Isto foi realizado utilizando uma ferramenta específica (*custom processing*) do

software CTan. Além disso, a densidade de osso alveolar foi medida com o auxílio de phantoms com densidades previamente definidas, fornecidos pela própria empresa Bruker (Kontich, Bélgica).

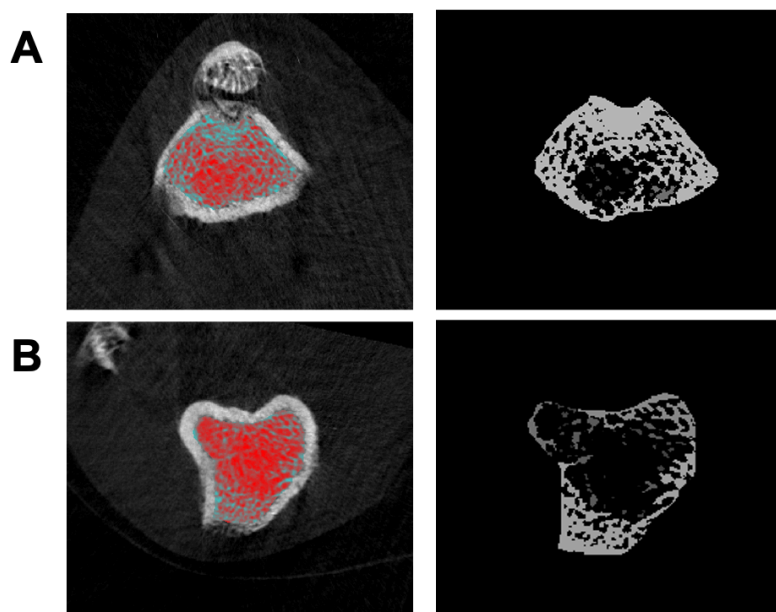
Para avaliação da mensuração da movimentação dentária, uma linha passando pela JCE distal do terceiro molar e JCE mesial do primeiro molar foi traçada e a distância linear entre a distal dos primeiros molares e mesial dos segundos molares maxilares foi mensurada realizando uma linha em cima dessa linha previamente posicionada, utilizando o software Data Viewer (Figura 8).

Figura 4 - Orientação dos cortes do fêmur (A) e tibia (B) para análise de micro-CT no Software DataViewer



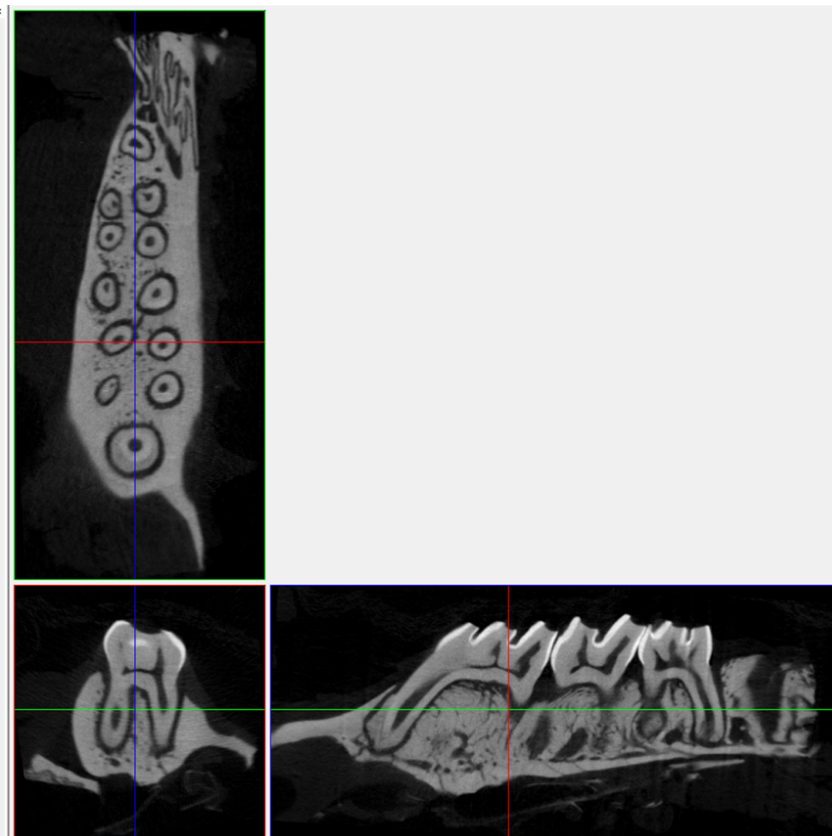
Fonte: Arquivo pessoal da autora.

Figura 5 - Delimitação da região de interesse (ROI) do fêmur (A) e da tíbia (B) no software CTAn



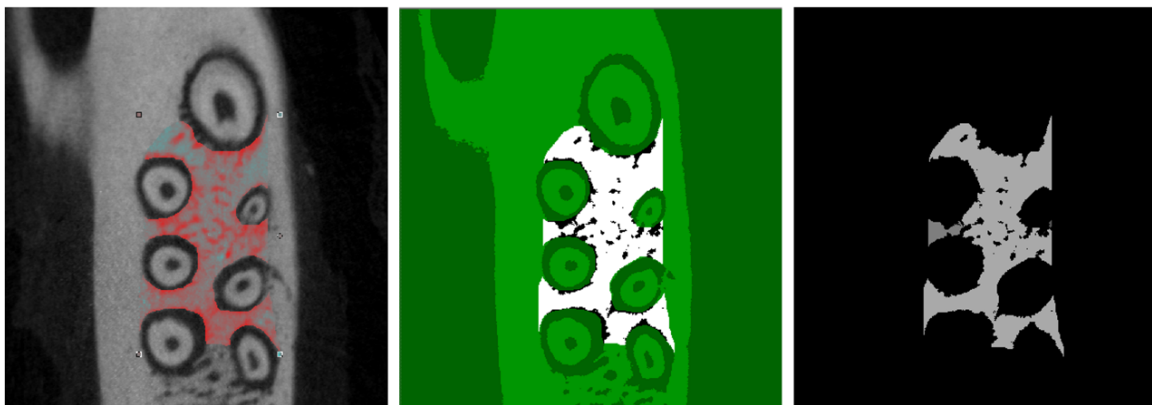
Fonte: Arquivo pessoal da autora.

Figura 6 - Orientação dos cortes das maxilas para análise de micro-CT no software DataViewer



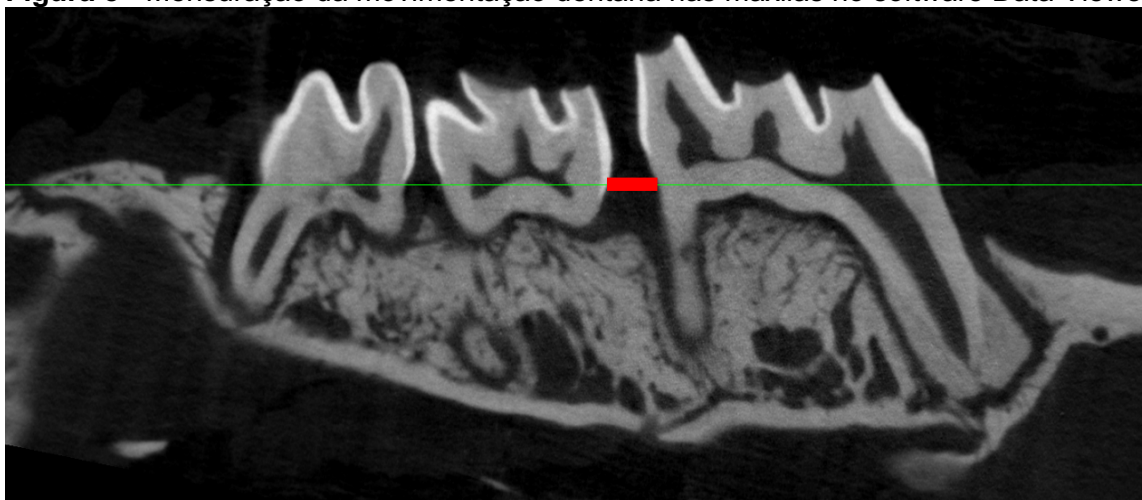
Fonte: Arquivo pessoal da autora.

Figura 7 - Delimitação da região de interesse (ROI) em maxilas no software CTAn



Fonte: Arquivo pessoal da autora.

Figura 8 - Mensuração da movimentação dentária nas maxilas no software Data Viewer



Fonte: Arquivo pessoal da autora.

Reação de polimerase em cadeia quantitativa em tempo real (RT-qPCR):

O RNA total dos tecidos gengivais foi extraído com o kit RNeasy Mini (catálogo no 74134, Qiagen, Hilden, Alemanha), segundo as instruções do fabricante. Este kit se baseia no método de colunas de purificação e inclui tratamento com DNase (RNase-FreeDNase Set - catálogo no 79254, Qiagehn, Hilden, Alemanha) de modo a eliminar contaminação com DNA genômico, que pode afetar os resultados da reação de qPCR em tempo real. A quantidade e pureza do RNA foram determinadas no aparelho Synergy H1 Hybrid Reader (Biotek, Winooski, VT, EUA) por meio da avaliação das absorbâncias a 260 nm e da relação entre as absorbâncias a 260/280 nm, respectivamente. O DNA complementar foi sintetizado pela transcrição reversa de 500 ng do total de RNA dos tecidos gengivais, seguindo o protocolo do fabricante

(High-Capacity cDNA Reverse Transcription Kit – catálogo no 4368814, Applied Biosystems, Waltham, MA, EUA).

A reação de PCR foi realizada através do termociclador StepOne Plus Real-Time PCR System (Applied Biosystems, Waltham, MA, EUA). 1 uL do produto da reação de RT foi utilizado em um volume total de reação de PCR de 10 uL. Este volume incluiu, além do produto da reação de RT, água livre de nucleases, TaqMan® Fast Advanced Master Mix (Applied Biosystems, Waltham, MA, EUA) e TaqMan® GeneExpression Assays (Applied Biosystems, Waltham, MA, EUA) para os genes alvo de rato: RANKL (Rn00589289- M1), osteopontina (Spp1) (Rn01449972-M1), Tnfa (Rn01525859_g1) e Il1b (Rn00580432_m1). As condições pré- otimizadas de ciclagem utilizadas foram: 95°C por 10 minutos, seguidos de 40 ciclos de desnaturação à 95°C por 15 segundos e de anelamento/extensão à 60°C por 1 minuto.

A expressão gênica tanto dos genes-alvo quanto do gene constitutivo GAPDH foi quantificada a partir do ΔCT . A expressão do gene alvo foi normalizada para a expressão do gene Gapdh (Rn01775763- G).

Análise ao microscópio de polarização

Para análise do conteúdo de colágeno do ligamento periodontal, foi utilizada a técnica picrosírius associado à análise ao microscópio com luz polarizada. Para isso foram utilizados 02 cortes/animal. Em cada corte foram analisadas três regiões da raiz distal dos primeiros molares: cervical, média e apical, tanto no lado de pressão e como no lado de tensão, totalizando assim seis regiões por animal.

Após a desparafinização, os cortes foram hidratados em séries decrescentes de etanol até água destilada. Subsequentemente, os cortes foram imersos em solução de 0,1% de Sirius-Red por 1 hora. Após lavagem, os cortes foram desidratados, diafanizados e montados com meio resinoso (PermOUNT, Fisher Scientific, New Jersey, USA). Os cortes corados com picrosírius foram analisados com os filtros de polarização acoplados ao microscópio de luz Olympus (modelo BX-51) com a objetiva de 40x.

A contagem de colágeno birrefringente foi realizada através de um software específico de imagem (ImageJ®, <http://rsbweb.nih.gov/ij>). Os dados de cada tonalidade (vermelho, laranja, amarelo e verde) foram determinados como percentagem do número total de pixels de colágeno que, por sua vez, foi expressa como uma percentagem do número total de pixels da imagem.

Análise estatística

Todos os dados obtidos foram tabulados e submetidos a um teste de normalidade (Shapiro-Wilk). A partir disso, as análises estatísticas foram realizadas com o auxílio do software GraphPad Prism (Graph-Pad Prism 9 Software; Inc, La Jolla, San Diego, CA, EUA). Foi utilizado o teste One-Way ANOVA seguido de Tukey para comparação entre os grupos. Em todos os procedimentos foi adotado um nível de significância de 5% para a tomada da decisão quanto à validade da hipótese testada.

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ANEXO A – CAPA DO ARTIGO DO ESTUDO 1

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Obesity affects the proteome profile of periodontal ligament submitted to mechanical forces induced by orthodontic tooth movement in rats

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ARTICLE INFO

Keywords:
 Proteomics
 Obesity
 Orthodontic tooth movement
 Vinculin
 Osteopontin
 Cathepsin D

ABSTRACT

The prevalence of obesity has increased significantly worldwide. Therefore, this study aimed to evaluate the influence of obesity on the proteomic profile of periodontal ligament (PDL) tissues of rat first maxillary molars (1 M) submitted to orthodontic tooth movement (OTM). Ten Holtzman rats were distributed into two groups ($n = 5$): the M group (OTM), and the OM group (obesity induction plus OTM). Obesity was induced by a high-fat diet for the entire experimental periods. After that period, the animals were euthanized and the hemimaxillae removed and processed for laser capture microdissection of the PDL tissues of the 1 M. Peptide extracts were obtained and analyzed by LC-MS/MS. Data are available via ProteomeXchange with identifier PXD033647. Out of the 109 proteins with differential abundance, 49 were identified in the OM group, including Vinculin, Cathepsin D, and Osteopontin, which were selected for in situ localization by immunohistochemistry analysis (IHC). Overall, Gene Ontology (GO) analysis indicated that enriched proteins were related to the GO component cellular category. IHC validated the trends for selected proteins. Our study highlights the differences in the PDL proteome profiling of healthy and obese subjects undergoing OTM. These findings may provide valuable information needed to better understand the mechanisms involved in tissue remodeling in obese patients submitted to orthodontic treatment.

Significance: The prevalence of obesity is increasing worldwide. Emerging findings in the field of dentistry suggest that obesity influences the tissues around the teeth, especially those in the periodontal ligament. Therefore, evaluation of the effect of obesity on periodontal tissues remodeling during orthodontic tooth movement is a relevant research topic.

To our knowledge, this is the first study to evaluate proteomic changes in periodontal ligament tissue in response to the association between orthodontic tooth movement and obesity. Our study identified a novel protein profile associated with obesity by using laser microdissection and proteomic analysis, providing new information to increase understanding of the mechanisms involved in obese patients undergoing orthodontic treatment which can lead to a more personalized orthodontic treatment approach.

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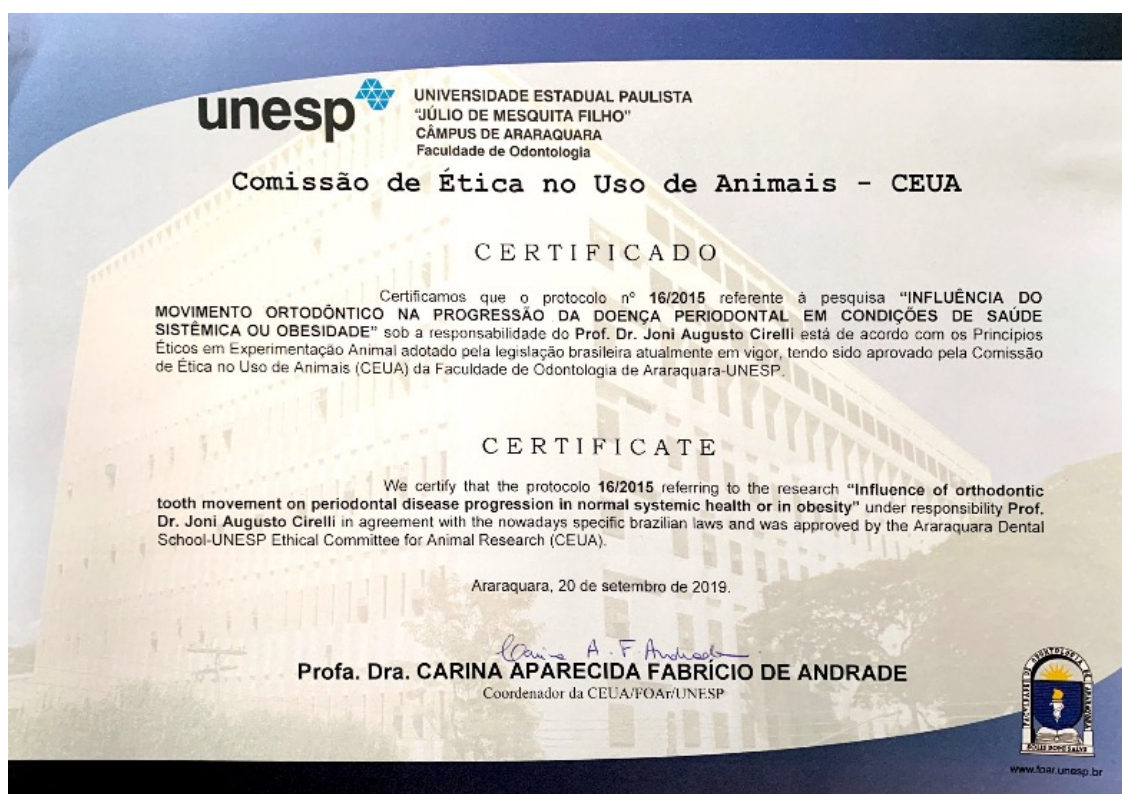
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ANEXO B - COMITÊ DE ÉTICA ESTUDO 1



ANEXO C - Comitê de Ética Estudo 2



UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara



FACULDADE DE ODONTOLOGIA

CERTIFICADO

Certificamos que a proposta intitulada ***"Influência da osteoporose e do anticorpo monoclonal anti-esclerostina nos tecidos periodontais de ratas sob movimentação ortodôntica"*** registrada com o nº 38/2020, sob a responsabilidade da Prof.^a Dr.^a Joni Augusto Cirelli – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA FACULDADE DE ODONTOLOGIA DE ARARAQUARA** em reunião de 24/02/2021.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	Abril/2022
Espécie/linhagem/raça	Rattus Novergicus, variação albinus, Holtzman
Nº de animais	48
Peso/Idade	250 – 300 gramas/13 semanas
Sexo	Fêmea
Origem	Biotério Central da FOAr (Campus-Araraquara)


Profa. Dra. CARINA APARECIDA FABRÍCIO DE ANDRADE
Coordenadora da CEUA

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**Não autorizo a publicação deste trabalho até 25 de agosto de 2024
(Direitos de publicação reservado ao autor)**

Araraquara, 25 de agosto de 2022.

Camila Chierici Marcantonio