LUCAS SOBRAL DE ROSSI

DESENVOLVIMENTO DE DOENÇA PERIODONTAL NA SÍNDROME METABÓLICA MURINA: EFEITO DO TRATAMENTO COM ASPIRINA

Londrina – PR

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para a obtenção do título de Mestre.

Orientador: Prof. Dr. Phileno Pinge Filho

Coorientadora: Profa. Dra. Marli Cardoso Martins Pinge

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito para a obtenção do título de Mestre.

BANCA EXAMINADORA

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Prof. Dr. Phileno Pinge Filho

Universidade Estadual de Londrina

_____

Prof. Dr. Fábio Henrique Kwasniewski

Universidade Estadual de Londrina

_____

Profa. Dra. Giovana Gomes de Carvalho Ishiuchi

Universidade Estadual de Londrina

Londrina, 26 de outubro de 2023

DEDICATÓRIA

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“O importante é não deixar de questionar. A curiosidade tem sua própria razão de existir. Não podemos deixar de nos maravilhar ao contemplar os mistérios da eternidade, da vida, da estrutura maravilhosa da realidade, tente apenas compreender um pouco deste mistério todos os dias."

Albert Einstein

DE ROSSI, Lucas Sobral. **Desenvolvimento de doença periodontal na síndrome metabólica murina: efeito do tratamento com aspirina.** Exame de qualificação apresentado ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Mestre.

RESUMO

A doença periodontal (PD) é uma condição inflamatória crônica multifatorial associada à formação de biofilmes bacterianos na superfície dos dentes, afetando os tecidos de suporte. Essa doença está relacionada a condições sistêmicas como diabetes tipo 2, hipertensão e obesidade abdominal, características clássicas da síndrome metabólica (MetS). Embora os usos e efeitos da aspirina (ASA) sejam bem conhecidos, seus efeitos no contexto da PD e MetS ainda não estão totalmente esclarecidos. Portanto, este estudo teve como objetivo avaliar o impacto da ASA em um modelo de camundongo com MetS e PD simultâneas. Para isso, camundongos Swiss fêmeas recém-nascidos receberam glutamato monossódico (MSG) (4 mg kg-1 dia, s.c.) nos primeiros 5 dias de vida para induzir a MetS, enquanto o grupo de controle (SAL) recebeu uma solução salina equimolar. No 60º dia, a PD foi induzida em ambos os grupos MSG e SAL. A MetS foi caracterizada usando o índice de Lee, níveis de glicose no sangue e parâmetros cardiovasculares. As maxilas foram avaliadas por microtomografia e análise histopatológica, revelando perda significativa de osso após a indução da PD. Os animais MSG apresentaram índices de Lee elevados, maior massa de tecido adiposo, hipertensão e níveis elevados de glicose no sangue em comparação com o grupo SAL. Independentemente da MetS, os animais submetidos à indução da PD exibiram níveis reduzidos de osso em comparação com seus respectivos controles. A reabsorção óssea alveolar foi significativamente maior nos grupos MSG em relação aos seus controles. Os animais SAL tratados com ASA apresentaram menor perda óssea do que os equivalentes do grupo MSG. Os níveis de óxido nítrico (NO) gengival, determinados pela reação de Griess/Cádmio, foram mais elevados em animais com PD. Uma correlação robusta de Pearson foi estabelecida, associando a perda óssea aos níveis de NO gengival. Esses resultados sugerem um mecanismo protetor da ASA contra a perda óssea em animais não obesos com PD, um efeito ausente em animais MSG. Consequentemente, este estudo fornece novas perspectivas sobre a intricada relação entre obesidade e PD em camundongos.

Palavras-Chave: Periodontite, Síndrome Metabólica, Microtomografia, Aspirina Óxido nítrico.

DE ROSSI, Lucas Sobral. **Periodontal disease development in murine metabolic syndrome: effect of ASA treatment.** Dissertation presented to the Graduate Program in Experimental Pathology at the State University of Londrina as a partial requirement for obtaining a master's degree.

ABSTRACT

Periodontal disease (PD) is a multifactorial chronic inflammatory condition associated to the formation of bacterial biofilms on the tooth surface, impacting the supporting tissues. This disease is associated with systemic conditions such as type 2 diabetes, hypertension, and abdominal obesity, classic characteristics present in metabolic syndrome (MetS). While the utilities and effects of Aspirin (ASA) are well-known, its effects within the context of PD and MetS remain incomplete. Therefore, this study aimed to assess the impact of ASA in a mouse model of MetS with concurrent PD. To achieve this, newborn Swiss mice received monosodium glutamate (MSG) (4 mg kg-1 day, s.c.) during the initial 5 days of life to induce MetS, while the control group (SAL) received an equimolar saline solution. On the 60th day, PD was induced in both the MSG and SAL groups. MetS was characterized using the Lee index, blood glucose levels, and cardiovascular parameters. The maxillae were evaluated through microtomography and histopathological analysis, revealing significant bone loss post-PD induction. MSG animals exhibited elevated Lee indices, greater adipose tissue mass, hypertension, and blood glucose levels compared to the SAL group. Regardless of MetS, animals subjected to PD induction displayed reduced bone levels compared to their respective controls. Alveolar bone resorption was notably higher in MSG groups than in their controls. SAL animals treated with ASA exhibited lower bone loss than MSG counterparts. Gingival nitric oxide (NO) levels, determined by the Griess/Cadmium reaction, were higher in animals with PD. A robust Pearson correlation was established, associating bone loss with gingival NO levels. These findings suggest a protective mechanism of ASA against bone loss in non-obese animals with PD, an effect absent in MSG animals. Consequently, this study provides novel insights into the intricate relationship between obesity and PD in mice.

Keywords: Periodontitis, Metabolic Syndrome, Microtomography. Nitric oxide.

LISTA DE ABREVIAÇÕES E SIGLAS

AdipoR1	Receptor de Adiponectina 1
AdipoR2	Receptor de Adiponectina 2
COX	Ciclooxigenase
DNA	Ácido desoxirribonucleico
DP	Doença periodontal
IL-1	Interleucina 1
IL-6	Interleucina 6
IL-11	Interleucina 11
IL-17	Interleucina 17
IMC	Índice de massa corporal
iNOS	Óxido nítrico-sintase induzida
LDL	Colesterol de baixa densidade
MCP-1	Proteína quimiotática de monócitos – E1
MCS-F	Fator estimulador de colônia de macrófagos
MEC	Matriz Extra Celular
mRNA	Ácido ribonucleico mensageiro
NET	Armadilhas extracelulares de neutrófilos
NF- κB	Factor nuclear kappa B
NO	Óxido nítrico
PGE_2	Prostaglandina E2
PUFAs	Ácidos graxos poli-insaturados
RANK	Receptor ativador do fator nuclear kappa B
ROS	Espécies reativas de oxigênio
SMet	Síndrome metabólica
TNF-a	Fator de Necrose Tumoral Alfa

SUMÁRIO

1. INTRODUÇÃO

1.1 O PERIODONTO E A DOENÇA PERIODONTAL (DP)

A periodontite é uma doença de cunho inflamatório que tem como principal característica a perda dos tecidos de sustentação do elemento dentário. Essa doença pode acometer todas as faixas etárias e estudos recentes indicam que a prevalência de doença periodontal global aumenta com a idade (Nazir; Al-Ansari; Al-Khalifa et al., 2020). A DP apresenta consequências significativas na qualidade de vida do paciente acometido pela doença. Desde perdas de dentes decorrentes da extensa reabsorção óssea, dor e desconforto contribuem para dificuldade na mastigação e fala, além de ter um impacto estético (Slots, 2017). Além disso, as implicações sistêmicas da periodontite podem estar relacionadas a outras condições sistêmicas, como diabetes, hipertensão e doenças das artérias coronárias, resultando em aumento do risco geral para a saúde do indivíduo (Del Pinto; Pietropaoli; Munoz-Aguilera et al., 2020; Preshaw; Alba; Herrera et al., 2012; Sanz; Marco Del Castillo; Jepsen et al., 2020).

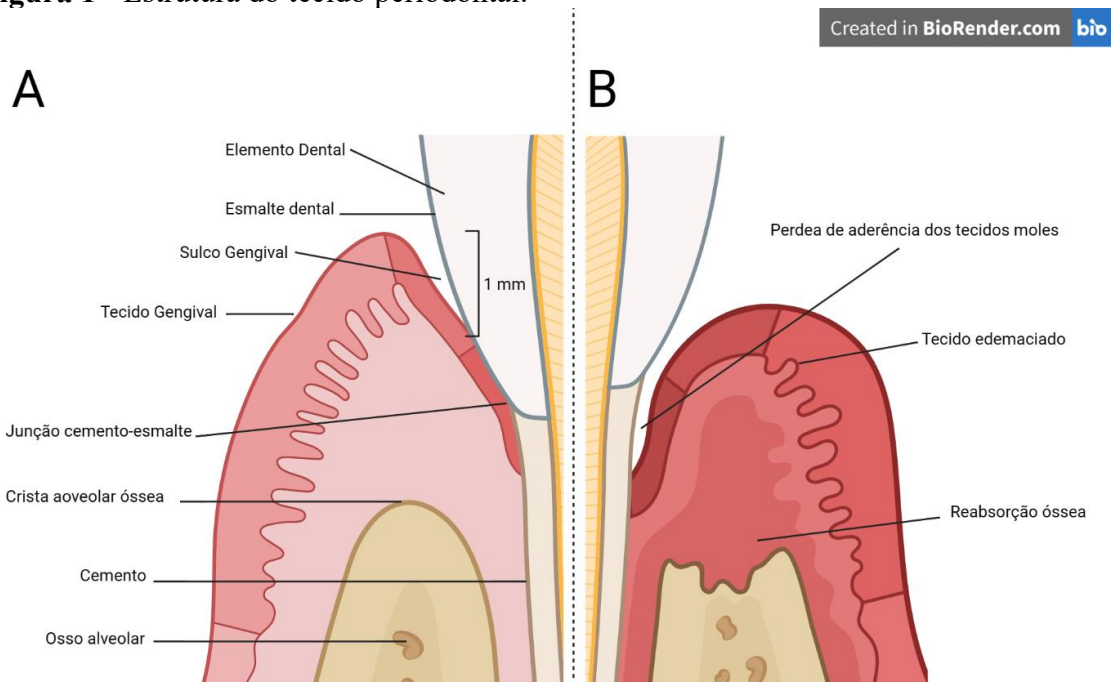
A mucosa oral é formada pela união de um tecido conjuntivo com um tecido epitelial pavimentoso estratificado. Essa junção é disposta em áreas onde certas regiões como palato duro, gengiva e dorso da língua são queratinizadas, enquanto mucosa jugal (mucosa da bochecha), lábios e ventre da língua não possuem tal revestimento (Wang; Tang; Pang et al., 2019). Do ponto de vista anatômico, as estruturas mais relevantes relacionadas ao processo de desenvolvimento da DP são o espaço biológico periodontal e os tecidos de inserção dental. O espaço biológico periodontal é uma área adjacente ao dente, composta por três componentes estruturais: o sulco gengival, o epitélio juncional e a inserção conjuntiva (Fig.1). Cada uma dessas partes possui, em média, uma profundidade de aproximadamente um milímetro. Essas características são de extrema importância uma vez que servem como referência para determinar a normalidade do periodonto saudável (Vacek; Gher; Assad et al., 1994).

O sulco gengival (Fig.1) é um pequeno espaço que existe entre a margem gengival e a superfície dentária, esta região é constantemente irrigada por líquido crevicular composto por anticorpos e fatores pró-inflamatórios, que a nível imunológico, controlam a microbiota bucal mantendo-a e em equilíbrio (Eley; Cox, 2003; Subbarao; Nattuthurai; Sundararajan et al., 2019).

Nesse ambiente, e principalmente sobre a superfície dental, existe uma microbiota considerada saudável que conta com uma multitude de espécies distintas (Zaura; Keijser; Huse et al., 2009). Estas bactérias residentes se organizam em um biofilme complexo onde ocorre competição por recursos como açúcares e oxigênio (Zijnge; Van Leeuwen; Degener et al., 2010).

O sistema imunológico do hospedeiro tem um papel fundamental no controle deste biofilme uma vez que o encontro dos dois se dá principalmente no sulco gengival mencionado anteriormente, além da frequência e qualidade da higienização bucal e da frequência e quantidade de açúcares presentes na alimentação que desempenham um papel importante no metabolismo bacteriano influenciando seu crescimento e produção de compostos metabólicos que alteram o microambiente do sulco gengival (Paes Leme; Koo; Bellato et al., 2006; Rode Sde; Gimenez; Montoya et al., 2012).

Figura 1 - Estrutura do tecido periodontal.



Fonte: O próprio autor.
Representação do tecido periodontal saudável (A) com suas características principais: gengiva na cor rosa, espaço biológico do sulco gengival de aproximadamente 1 milímetro (mm) e crista óssea conservada; (B) tecido apresentando periodontite, com gengiva edemaciada e eritematosa, espaços biológicos prejudicados devido a perda de aderência e reabsorção óssea alveolar.

Estas características tornam a cavidade bucal um nicho ecológico complexo uma vez que está sujeito a constantes mudanças de pH, temperatura e descamação de mucosas além da reposta imunológica do hospedeiro. Estes fatores são interdependentes e quando um deles se torna deficiente, todo o equilíbrio bucal é perdido.

Uma higienização deficiente juntamente com a ingestão de açúcares em curtos intervalos de tempo ou uma alteração na resposta imunológica tornam o ambiente propicio para uma disbiose desse biofilme resultando no crescimento de determinadas populações bacterianas. O biofilme alterado aumenta em espessura, espécies bacterianas dependentes de oxigênio dão lugar a espécies predominantemente Gram-negativas e anaeróbicas. Essa troca de padrão microbiológico resulta no aumento de bactérias residentes que constituem o chamado "Complexo Vermelho" (Mohanty; Asopa; Joseph et al., 2019), uma categorização que agrega um grupo específicos de bactérias altamente patogênicas aos tecidos periodontais, entre elas a *Treponema denticola* e *Porphyromonas gingivalis (*Hajishengallis; Lamont, 2012*)*. Essas espécies apresentam grande capacidade de lesão aos tecidos adjacentes seja pela presença de LPS (Vitkov; Muñoz; Schoen et al., 2021), que é um importante fator gerador de uma resposta imunológica ou por estimulação da produção de Metaloproteinases por macrófagos e fibroblastos residentes do tecido gengival (Birkedal-Hansen, 1993; Page; Offenbacher; Schroeder et al., 1997) além do aumento na produção de citocinas como IL-1, IL-6, IL-11, IL-17 e TNF-α (Garaicoa-Pazmino; Fretwurst; Squarize et al., 2019; Graves, 2008). Esta resposta inflamatória exacerbada é a principal responsável por causar danos ao periodonto.

Já é estabelecido pela literatura que a liberação de NETs por neutrófilos, na tentativa de conter as espécies do biofilme, desempenha papel na lesão dos tecidos periodontais (Wang; Zhou; Ren et al., 2021) além de um desequilíbrio na homeostase do tecido ósseo resultando em maior reabsorção deste tecido por osteoclastos ativados principalmente por RANKL, MCS-F e

osteoprotegerina presentes no ambiente periodontal inflamado (Hienz; Paliwal; Ivanovski, 2015; Kitaura; Marahleh; Ohori et al., 2020).

Outro ponto importante, é a produção de óxido nítrico (NO) no tecido gengival. O NO é uma molécula solúvel em lipídios, altamente instável, produzida pela conversão da L-arginina pelas enzimas óxido nítrico sintase (NOS) se apresentando em três formas, duas delas constitutivas e uma terceira, iNOS, é induzida e se mostra presente principalmente em macrófagos ativados em tecidos inflamados. Tratando-se de periodontite, a célula que se mostra a maior responsável pela produção de NO é o macrófago (Corradin; Fasel; Buchmüller-Rouiller et al., 1993; Lappin; Kjeldsen; Sander et al., 2000). A produção elevada de NO proporciona vasodilatação e facilita a diapedese de neutrófilos além de favorecer a formação de ânions superóxidos (O_2^-) que podem gerar lesão de DNA, oxidar LDL e impedir a respiração mitocondrial (Guzik; Korbut; Adamek-Guzik, 2003).

Estes problemas funcionais, físicos e psicossociais destacam a importância da prevenção, diagnóstico e tratamento adequados da DP visando minimizar seu impacto negativo e promover uma melhor qualidade de vida.

1.2 A Obesidade e a Síndrome Metabólica (SMet)

Aproximadamente 39% dos indivíduos acima de 18 anos apresentam sobrepeso, e desses, cerca de 13% são classificados como obesos, além disso, estima-se que até o ano de 2020, aproximadamente 39 milhões de crianças com menos de 5 anos de idade estavam em situação de sobrepeso ou obesidade (Obesity and overweight, 2021). O acúmulo excessivo de tecido adiposo, principalmente na região abdominal, causa um desequilíbrio na proporção peso x altura do indivíduo. O IMC consiste em uma relação entre o peso corporal em quilogramas e a altura em metros ao quadrado (Kg/m^2) (Gonzalez-Muniesa; Martinez-Gonzalez; Hu et al., 2017). Com base no valor obtido, a obesidade pode ser colocada em uma escala de modo a facilitar o seu tratamento (Khanna; Peltzer; Kahar et al., 2022)

Os adipócitos são células altamente especializadas que compõem o tecido adiposo e desempenham um papel central no armazenamento de lipídios no organismo, principalmente na forma de triglicerídeos (Duncan; Ahmadian; Jaworski et al., 2007). Além da função de armazenamento de energia, os adipócitos também têm uma relevância fisiológica adicional. Essas células também apresentam papel importante na produção e liberação de citocinas pró-inflamatórias, como IL-1 e IL-6 (Blaszczak; Jalilvand; Hsueh, 2021). Essas citocinas indicam a capacidade dos adipócitos de desempenhar funções imunológicas no tecido adiposo. A iNOS, uma enzima responsável pela produção de NO, é encontrada no tecido adiposo de indivíduos obesos, acompanhada por um infiltrado de macrófagos dando a este tecido uma característica de padrão inflamatório (Kawai; Autieri; Scalia, 2021; Weisberg; Mccann; Desai et al., 2003).

A adiponectina é um hormônio secretado por adipócitos. A interação deste hormônio com seus receptores AdipoR1 e AdipoR2 regula o balanço energético celular aumentando a captação de glicose (Pajvani; Hawkins; Combs et al., 2004; Wang; Xu; Knight et al., 2002). Porém, em situações de acumulo de tecido adiposo, a expressão de adiponectina se mostra reduzida (Arita; Kihara; Ouchi et al., 1999), o que se relaciona com a resistência à insulina presente em indivíduos obesos (Gluvic; Zaric; Resanovic et al., 2017).

A hipertensão também é um fator preocupante da obesidade e no sobrepeso com adipose abdominal. Obesidade pode modificar o sistema renina-angiotensina-aldosterona levando a reabsorção de sódio pelos rins resultando em maior volume sanguíneo e maior pressão arterial (Huby; Antonova; Groenendyk et al., 2015). A hipertensão por consequência acaba gerando uma carga maior no coração e juntamente com a resistência arterial periférica causada por

inflamações crônicas e fadiga muscular no órgão devido a hiperinsulinemia geram um grande índice de cardiopatias em pacientes obesos (Butler; Kalogeropoulos; Georgiopoulou et al., 2011; Westermeier; Navarro-Marquez; López-Crisosto et al., 2015). Todas essas características somadas formam a chamada síndrome metabólica (SMet).

Tratando-se de uma síndrome, a SMet é uma condição de saúde composta por uma série de manifestações clínicas que, tanto isoladamente quanto em conjunto, aumentam as chances do desenvolvimento de doenças cardiometabólicas. Os componentes da SMet são a obesidade, adipose abdominal, resistência insulínica (hiperglicemia) e hipertensão (Piché; Tchernof; Després, 2020; Samson; Garber, 2014). Devido a sua complexidade, ainda existem muitas perguntas a serem respondidas em torno da SMet e seus efeitos no organismo. Um exemplo disso é o fato de que ainda não é completamente elucidado se as manifestações da SMet são causadas isoladamente pelas patologias que a compõe ou se essas patologias apresentam novos sinais e sintomas quando estão presentes ao mesmo tempo no individuo (Rochlani; Pothineni; Kovelamudi et al., 2017).

A interação SMet e DP é objeto de estuda de uma série de revisões visto que as duas enfermidades são condições de caráter inflamatório e tem o potencial de influenciar o prognóstico uma da outra (Daudt; Musskopf; Mendez et al., 2018; Gobin; Tian; Liu et al., 2020; Thanakun; Watanabe; Thaweboon et al., 2014). Nesbitt e colaboradores levantam a hipótese de que a presença de DP é um fator que pode contribuir para o desenvolvimento de MetS (Nesbitt; Reynolds; Shiau et al., 2010) enquanto que Morita e colaboradores encontraram uma associação entre a presença de DP e os componentes da MetS (Morita; Yamazaki; Mita et al., 2010). A DP também apresenta maiores taxas de destruição óssea quando presente juntamente com a MetS (Li; Lu; Zhang et al., 2015). A DP também apresenta maior destruição óssea quando relacionada à hemoglobina glicada (Izuora; Ezeanolue; Schlauch et al., 2015; Saito; Shimazaki; Kiyohara et al., 2004), além de ter o potencial de alterar a glicemia de indivíduos diabéticos mesmo que estes não apresentem MetS (Longo; Artese; Rabelo et al., 2014).

1.3 ÁCIDO ACETILSALICÍLICO

O ácido acetilsalicílico, do inglês acetilsalicilic acid (ASA), é um fármaco inibidor não seletivo e irreversível de enzimas COX, deste modo modulando a produção de prostaglandinas e tromboxanos (Fullerton; O'brien; Gilroy, 2014). Atua também na redução do recrutamento de neutrófilos polimorfonucleares, pois estimula a síntese de 15-epi-lipoxina A_4, um metabólito do ácido araquidônico com função pró-resolução e anti-inflamatória, aumentando a síntese do oxido nítrico inibindo as proteínas responsáveis pela interação neutrófilo e endotélio, diminuindo assim a diapedese (Morris; Stables; Hobbs et al., 2009). Já foi demonstrado que o uso de baixas doses de ASA inibem a ativação plaquetária , de modo que é utilizada por indivíduos com SMet como um método de tratamento para alterações cardiovasculares (Shields; Hennekens, 2004; Soodi; Vanwormer; Rezkalla, 2020), além de apresentar efeitos anti-inflamatórios nestes indivíduos, atuando na diminuição da expressão de mediadores pró-inflamatórios como o MCP-1, TNF, IL-6 (Muhlestein, 2010). A ASA se mostrou uma potencial forma de tratamento para periodontite, uma vez que administrada com PUFAs, promoveu melhoras no quadro inflamatório da PD (Neprelyuk; Zhad'ko; Romanenko et al., 2023). De fato, já é descrito que a ASA desencadeia a síntese de mediadores pró-resolutivos mais potentes, chamados de resolvinas, protectinas e lipoxinas, que têm uma meia-vida mais longa no sangue (Serhan, 1997; Serhan; Fredman; Yang et al., 2011; Van Dyke; Hasturk; Kantarci et al., 2015). Esses mediadores pró-resolutivos promovem a resolução da inflamação, reduzindo a infiltração de neutrófilos (Serhan; Jain; Marleau et al., 2003), afetam também a produção de TNF-α

diminuindo o tráfego de leucócitos para o local afetado (Pouliot; Serhan, 1999) além de regular a síntese de citocinas/quimiocinas (Serhan; Gotlinger; Hong et al., 2006), atenuando a produção sistêmica de proteína c-reativa e interleucina (IL)-1 (Hasturk; Kantarci; Goguet-Surmenian et al., 2007), diminuindo a produção de RANKL (El Kholy; Freire; Chen et al., 2018) e regulando citocinas pró-inflamatórias secretadas por macrófagos (Gilligan; Gartung; Sulciner et al., 2019).

2. JUSTIFICATIVA

Levando em consideração as possíveis interações entre a síndrome metabólica (SMet) e doença periodontal (DP), além do uso crônico de aspirina (ASA) por pacientes que apresentam SMet, este trabalho tem a intenção de avaliar o desenvolvimento da DP durante o tratamento com ASA em camundongos normais e com SMet.

3. OBJETIVOS

3.1 OBJETIVOS GERAIS

Avaliar o efeito do tratamento com ASA sobre o desenvolvimento da doença periodontal em animais Swiss portadores de Smet ou não.

3.2 OBJETIVOS ESPECÍFICOS

Avaliar o efeito da SMet no desenvolvimento da DP por meio de análises de microtomografia computadorizada (micro CT) e obtenção de medidas dos níveis ósseos.

Realizar análise hematológica antes e depois da indução da doença periodontal.

Quantificar a produção de óxido nítrico (NO) nos tecidos gengivais afetados pela doença periodontal.

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5. APROVAÇÃO CEUA – UEL

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 005/2022

Londrina, 21 de janeiro de 2022.

Prezado (a) professor (a),

Certificamos que o projeto intitulado: **"Desenvolvimento de doença periodontal na síndrome metabólica murina: efeito do tratamento prévio com aspirina sobre marcadores inflamatórios e parâmetros cardiovasculares"** protocolo CEUA nº **063.2021** sob a responsabilidade de **Phileno Pinge Filho**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), 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 da Universidade Estadual de Londrina (CEUA/UEL) no dia 21/01/2022.

Este projeto tem por objetivo caracterizar os marcadores inflamatórios e cardiovasculares na doença periodontal em camundongos com síndrome metabólica. **Grau de invasividade: GI 2.**

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	22/01/2022 a 03/01/2025
Espécie/ linhagem/ raça	Camundongo heterogênico/ Swiss
Nº de animais	**450 a 500**
Peso/ Idade	-
Sexo	Fêmeas
Origem	Prole feminina oriunda das matrizes do projeto CEUA **051.2021 "Sepse polimicrobiana na síndrome metabólica murina: efeito da aspirina sobre a formação de armadilhas extracelulares de neutrófilos (NETs), marcadores inflamatórios e parâmetros cardiovasculares"** - Biotério Central da Universidade Estadual de Londrina.
Amostras a serem coletadas	Sangue, coração e maxila.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações.

Em cumprimento às exigências do Conselho Nacional de Controle de Experimentação Animal (CONCEA), em até 30 dias da finalização do projeto de pesquisa ou extensão envolvendo o uso de animais (verificar período de vigência expresso neste ofício), é necessário encaminhar relatório da descrição de uso de animais para ceua@uel.br, conforme modelo disponível no site da CEUA: http://www.uel.br/comites/ceua/pages/relatorio-de-projetos.php.

Coloco-me à disposição para quaisquer esclarecimentos que se fizerem necessários. Sem mais para o momento, subscrevo-me, cordialmente,

Maria Fernanda R. Graciano

Profª Drª Maria Fernanda Rodrigues Graciano
Vice-coordenadora da CEUA/UEL

Ilmo.(a) Sr.(a)
Prof. (a) Dr. (a) Phileno Pinge Filho
Responsável pelo projeto
C/C para a Chefia do Departamento de Ciências Patológicas/CCB
C/C para a Direção do Centro de Ciências Biológicas/CCB
C/C para o Biotério Central/ CCB

ANEXO A

Este trabalho foi realizado na Universidade Estadual de Londrina e deu origem a um artigo científico que se intitula: “Metabolic syndrome promotes resistance to aspirin in mitigating bone loss in murine periodontal disease”

Life Sciences

Metabolic syndrome promotes resistance to aspirin in mitigating bone loss in murine periodontal disease

--Manuscript Draft--

Manuscript Number:	LFS-D-24-02366
Article Type:	Research paper
Keywords:	Periodontitis; Ligature model; Obesity; Bone loss; Nitric oxide
Corresponding Author:	Phileno Pinge-Filho, Ph.D State University of Londrina Londrina, Paraná BRAZIL
First Author:	Lucas Sobral de Rossi
Order of Authors:	Lucas Sobral de Rossi
	Raquel Pires Nakama
	Lucas Felipe Dos Santos
	Leonardo Berto-Pereira
	Aparecida Donizette Malvezi
	Maria Isabel Lovo-Martins
	Ana Paula Canizares Cardoso
	Luiz Claúdio Tozoni-Filho
	Eduardo Inocente Jussiani
	Andressa de Freitas Mendes Dionísio
	Marli Cardoso Martins-Pinge
	Phileno Pinge-Filho, Ph.D
Abstract:	Aims: This study aimed to assess the impact of aspirin (ASA) in a mouse model presenting metabolic syndrome (MetS) with concurrent periodontal disease (PD). Main methods: Newborn female Swiss mice were administered monosodium glutamate (MSG) (4 mg/g) during the initial 5 days of life to induce MetS (MetS group), while the control group (SAL) was administered an equimolar saline solution. On the 60th day, PD was induced in both the MetS and SAL groups. Half of the animals were treated daily with ASA via gavage (40 mg/kg). MetS was characterized using the Lee index, blood glucose levels, and cardiovascular parameters. The maxillae were evaluated through microtomography and histopathological analysis, revealing significant bone loss post-PD induction. Key findings: Despite MetS, animals subjected to PD induction displayed reduced bone levels compared to their respective controls. Alveolar bone resorption was notably higher in MetS groups than in their controls. SAL animals treated with ASA exhibited lower bone loss than MetS counterparts. Gingival nitric oxide (NO) levels, determined by the cadmium/Griess test, were higher in animals with PD. A robust Pearson correlation was established, associating bone loss with gingival NO levels. The MetS animals were also resistant to the decrease in NO levels caused by ASA under PD conditions. Significance: These findings suggest a protective mechanism of ASA against bone loss in non-MetS animals with PD, an effect absent in MetS animals. Consequently, this study provides novel insights into the intricate relationship between MetS and PD in mice.
Suggested Reviewers:	Gaetano Isola, PhD University of Catania gaetano.isola@unict.it Dr. Isola has experise in periodontal helath and systemic diseases.
	Patricia Weidlich, PhD

	Universidade Federal do Rio Grande do Sul Faculdade de Odontologia patricia.weidlich@ufrgs.br Dr. Weidlich has expertise in the association between metabolic syndrome and periodontitis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this article.

Phileno Pinge Filho

Cover Letter

Londrina, April 4, 2024

Dr. Farah Sheikh
Editor in Chief
Life Sciences

I am very pleased to submit this manuscript describing our recent investigation into the relationship between metabolic syndrome (MetS) and concurrent periodontal disease (PD) to ***Life Sciences*** for your consideration. Entitled "**Metabolic syndrome promotes resistance to aspirin in mitigating bone loss in murine periodontal disease",** this manuscript is authored by Lucas Sobral de Rossi, Raquel Pires Nakama, Lucas Felipe Dos Santos, Leonardo Berto Pereira, Aparecida Donizette Malvezi, Maria Isabel Lovo-Martins, Ana Paula Cardoso Canizares, Luiz Claúdio Tozoni Filho, Eduardo Inocente Jussiani, Andressa de Freitas Mendes Dionísio, Marli Cardoso Martins-Pinge, and Phileno Pinge-Filho.

Clinical investigations of PD pose challenges due to its intricate nature, which involves complex interactions among genetic and behavioral factors, along with oral biofilms. Human-based studies on PD face difficulties such as the assessment of disease severity, ethical concerns, and variations in individual susceptibility to periodontitis progression. The utilization of experimental animal data can provide models reflecting biological trends, thereby serving as a preliminary step before applying findings to humans. Our focus was directed on studying the progression of periodontal disease (PD) in mice undergoing aspirin (ASA) treatment, encompassing both normal mice and those with MetS. We found that ASA has potential in the treatment of damage caused by PD. However, in patients with MetS, ASA alone may not confer therapeutic benefits. These

findings complement existing knowledge regarding PD and its treatment approaches in the literature.

I declare that the coauthors have meticulously reviewed the manuscript and approved its submission to Life Sciences. Furthermore, our manuscript has not been previously published, nor is it under consideration by any other journal. We have read and comprehended your journal's policies and believe that neither the manuscript nor the study violates any of these policies. Finally, there are no conflicts of interest to declare.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

Phileno Pinge-Filho Ph.D.

Departamento de Imunologia, Parasitologia e Patologia Geral, Centro de Ciências Biológicas, Universidade Estadual de Londrina

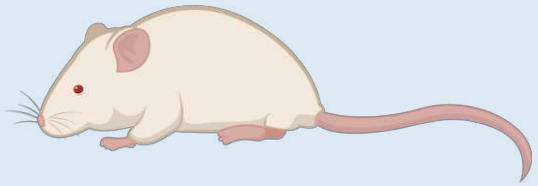
CEP: 86051-970, Londrina, Paraná, Brasil. Email: pingefilho@uel.br,

Tel: + 55 43 3371-4978

Graphical Abstract

ASA: Aspirin
MSG:Monosodium glutamate
PD: Periodontal disease
SAL: Saline

Saline Group

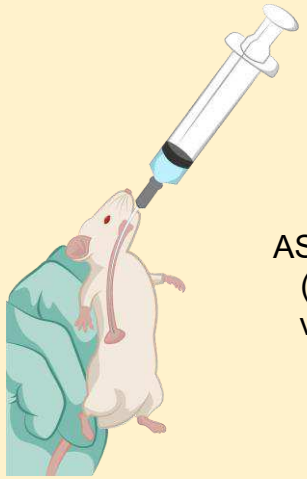


Female Swiss mice were administered a saline solution during their first five days of life

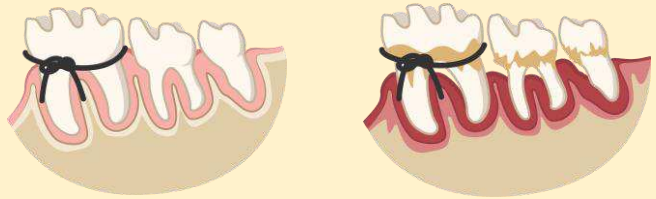
Metabolic Syndrome Group

Female Swiss mice were treated with 4mg/g of MSG during their first five days of life

MetS Induction



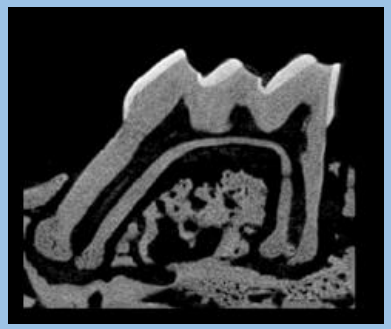
ASA treatment (40 mg/kg) via gavage



15 days of Periodontitis induction. Ligature on superior first molar

PD induction and ASA treatment

SAL + PD

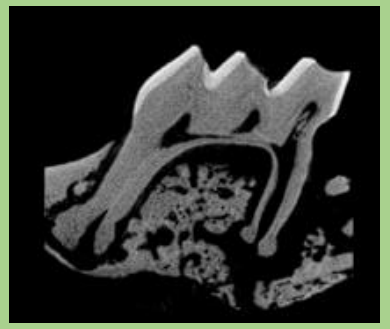


Bone loss in mesial and distal alveolar bone crest

SAL + PD + ASA

ASA treatment **reduced** alveolar bone loss in SAL animals

MSG + PD + ASA



Bone loss in mesial and distal alveolar bone crest

MSG + PD

ASA treatment has **no effect on** alveolar bone level

Micro-CT Analysis

Research article

Metabolic syndrome promotes resistance to aspirin in mitigating bone loss in murine periodontal disease

Lucas Sobral de Rossi [a], Raquel Pires Nakama [a], Lucas Felipe Dos Santos [b], Leonardo Berto Pereira [a], Aparecida Donizette Malvezi [a], Maria Isabel Lovo-Martins [a], Ana Paula Cardoso Canizares [a], Luiz Claúdio Tozoni Filho [a], Eduardo Inocente Jussiani [c], Andressa de Freitas Mendes Dionísio [d], Marli Cardoso Martins-Pinge [d], Phileno Pinge-Filho [a,b*]

[a] *Department of Immunology, Parasitology and General Pathology, State University of Londrina, Londrina, Paraná, Brazil.*

[b] *Department of Microbiology, State University of Londrina, Londrina, Paraná, Brazil.*

[c] *Department of Physics, State University of Londrina, Londrina, Paraná, Brazil.*

[d] *Department of Physiological Sciences, State University of Londrina, Londrina, Paraná, Brazil.*

*Correspondence: Phileno Pinge-Filho, Laboratory of Experimental Immunopathology, Department of Immunology, Parasitology and General Pathology, Londrina, Paraná, Brazil. Celso Garcia Cid Highway, PR-445, Km 380, Londrina, PR, 86057-970. Tel: + 55 43 3371-4978. Email: pingefilho@uel.br

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Abstract

Aims: This study aimed to assess the impact of aspirin (ASA) in a mouse model presenting metabolic syndrome (MetS) with concurrent periodontal disease (PD).

Main methods: Newborn female Swiss mice were administered monosodium glutamate (MSG) (4 mg/g) during the initial 5 days of life to induce MetS (MetS group), while the control group (SAL) was administered an equimolar saline solution. On the 60^{th} day, PD was induced in both the MetS and SAL groups. Half of the animals were treated daily with ASA via gavage (40 mg/kg). MetS was characterized using the Lee index, blood glucose levels, and cardiovascular parameters. The maxillae were evaluated through microtomography and histopathological analysis, revealing significant bone loss post-PD induction.

Key findings: Despite MetS, animals subjected to PD induction displayed reduced bone levels compared to their respective controls. Alveolar bone resorption was notably higher in MetS groups than in their controls. SAL animals treated with ASA exhibited lower bone loss than MetS counterparts. Gingival nitric oxide (NO) levels, determined by the cadmium/Griess test, were higher in animals with PD. A robust Pearson correlation was established, associating bone loss with gingival NO levels. The MetS animals were also resistant to the decrease in NO levels caused by ASA under PD conditions.

Significance: These findings suggest a protective mechanism of ASA against bone loss in non-MetS animals with PD, an effect absent in MetS animals. Consequently, this study provides novel insights into the intricate relationship between MetS and PD in mice.

Keywords: Periodontitis; Ligature model; Obesity; Bone loss; Nitric oxide.

1. Introduction

Periodontal disease (PD) is a common biofilm-induced inflammatory condition that affects the integrity of the tissues surrounding and supporting the teeth [1]. It is linked to the interplay between imbalanced oral microbiota and host immunity and has been associated with numerous systemic conditions and diseases [2]. Periodontal health requires a controlled inflammatory state that can maintain host-microbe homeostasis in the periodontium [3].

The periodontium is comprised of four principal components: namely, the gingiva, periodontal ligament, alveolar bone, and cementum [4]. The gingival sulcus receives continuous irrigation by crevicular fluid containing antibodies and pro-inflammatory factors, which immunologically control the biofilm, keeping its balance [5]. However, the introduction of foreign substances into the sulcus space can lead to inflammation and PD [5].

Clinical investigations of PD pose challenges due to its intricate nature, which involves complex interactions among genetic and behavioral factors, along with oral biofilms. Human-based studies on PD face difficulties such as the assessment of disease severity, ethical concerns, and variations in individual susceptibility to periodontitis progression [6]. The utilization of experimental animal data can provide models reflecting biological trends, thereby serving as a preliminary step before applying findings to humans [7].

Considered the second most influential risk factor after smoking, obesity has been linked to the inflammatory degradation of periodontal tissues [8]. Histopathological alterations have been observed in the periodontium of Zucker rats with hereditary obesity. When subjected to ligature-induced periodontitis, obese animals exhibited a higher degree of alveolar bone resorption compared to their non-

obese counterparts [9]. Meta-analyses and systematic reviews provide evidence of an association between obesity and periodontitis in humans [10]. Most of these data describe a correlation between PD and metabolic syndrome (MetS), of which obesity, insulin resistance, dyslipidemia, and hypertension are components.

To investigate the origins of obesity and its pathological implications, various experimental models involving obese animals have been employed. One such model consists of the induction of obesity through monosodium glutamate (MSG) treatment, which partially mimics clinical MetS. MSG-treated rats and mice exhibited body fat accumulation, increased arterial pressure, hyperleptinemia, hyperinsulinemia, insulin resistance, and hypertriglyceridemia [11-15].

The association between obesity and a chronic inflammatory response is widely recognized, marked by abnormal production of cytokines and activation of inflammatory signaling pathways. This is accompanied by an upregulation in iNOS expression across several tissues. [16]. In prior research, we showed an increase in iNOS expression in heart tissues from obese rats, highlighting the participation of this inducible pathway in MSG-induced obesity [17].

Aspirin, or acetylsalicylic acid (ASA), is known for its anti-inflammatory and analgesic properties, which can be beneficial in addressing periodontal disease. Studies have demonstrated that ASA has potential application in preventing and treating osteopenia [18]. Low-dose ASA has been shown to modulate the inflammatory process within periodontal tissues by reducing pro-inflammatory mediators such as PGE_2 and IL-1β, while also increasing pro-resolving mediators such as aspirin-triggered lipoxin (ATL) [19].

Therefore, in the present study, we aimed to evaluate, for the first time, the development of PD during ASA treatment in both normal mice and those with MetS.

2. Materials and methods

2.1. Animals

Male and female Swiss mice, at 7 weeks of age, were acquired from the Animal Care Unit of the Biological Sciences Centre of the State University of Londrina (Brazil). Nine colonies were maintained at the Animal Facility within the Department of Immunology, Parasitology and General Pathology, under a 12-h light/dark cycle at 23 °C. The mice were provided *ad libitum* access to standard laboratory chow (Nuvilab-CR1, Quimtia-Nuvital, Colombo, Paraná, Brazil), in compliance with research ethics guidelines.

The establishment of the MetS model and experimental groups began by subjecting newborn mice to subcutaneous injections of monosodium glutamate (MSG) (Sigma, St. Louis, USA) at a concentration of 4 mg/g of body weight (MetS group) or an equimolar saline solution (SAL group) from the first to the fifth day of life. At 30 days of age, the mice were separated by sex, and only female mice were selected for further experimentation. The animals were then divided into the following groups: control (CTL, n = 12-31 per group) and MetS (n = 8-30 per group). At 60 days of age, obesity was characterized using the Lee Index for each animal through the formula: $\sqrt[3]{\text{body weight}}/\text{naso-anal length} \times 1000$, in addition to abdominal circumference and the weight of retroperitoneal and perigonadal fats. Cardiovascular parameters were measured through the non-invasive CODA system (Kent Scientific, Torrington, CT) based on the pressure-volume obtained from the mouse tail. Additionally, a subgroup of animals underwent PD induction, which involved the placement of a ligature around the first molar and maintaining it for 15 days. Some groups within this subset received ASA

treatment at 40 mg/kg (ASA was first diluted in DMSO and then in water, resulting in 2% DMSO) daily via gavage for the same duration. At 75 days of age, samples of the maxillary bone were collected for micro-CT analysis. All animals were anesthetized with ketamine (1.25 mg/mL) and xylazine (0.5 mg/mL) and subsequently euthanized by cervical dislocation. The time points involved in the induction of MetS and the subsequent comprehensive assessments are shown in Figure 1.

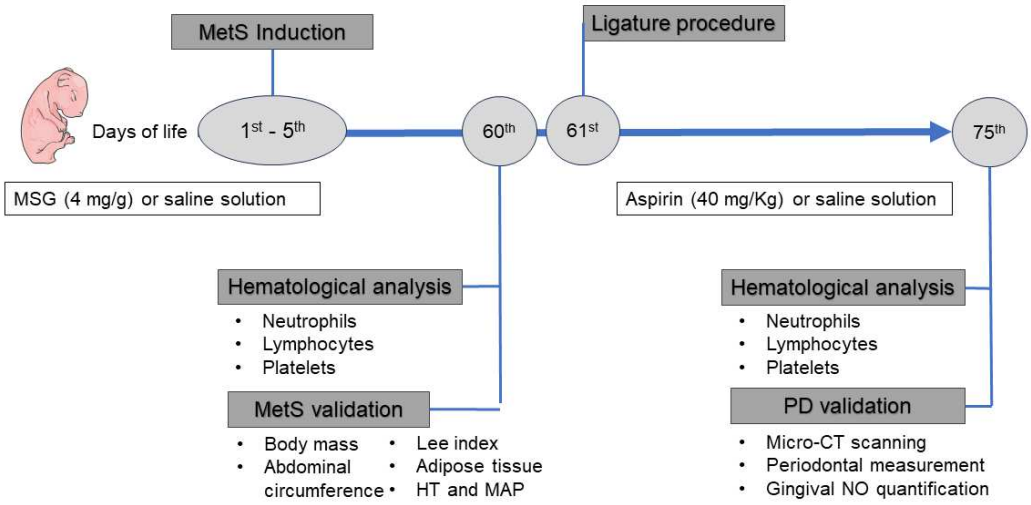

Figure 1. Experimental design**.** The experimental protocol encompassed the induction of Metabolic Syndrome (MetS) in Swiss mice. MetS was induced from the first day of life until the fifth day by administering monosodium glutamate (MSG) at 4 mg/g body weight. For comparison, the control group was administered an equimolar saline solution. At 60 days of age, hematological analysis, measurement of cardiovascular parameters such as heart rate (HR) and mean arterial pressure (MAP), and quantification of adipose tissue mass were conducted. Part of the animals underwent a ligature procedure around the first molar, maintained for 15 days. Some groups within this subset received ASA treatment at 40 mg/kg daily via gavage for the same duration.

Upon reaching 75 days of age, the animals were euthanized, and samples of the maxillary bone and bone tissues were obtained for further analysis.

2.2. PD induction via ligature

At 60 days of age, some of the animals underwent the induction of PD through a model described in 1966 by Rovin *et al*., which is still commonly used nowadays [20, 21]. This procedure involved placing a 6-0 nylon ligature around the upper first molar of the anesthetized animal (Figure S1). To ensure accuracy and consistency in the ligature application, the animal was carefully positioned within an apparatus to facilitate mouth opening [22].

Both obese (MetS group) and non-obese (SAL group) mice were divided into eight distinct experimental groups based on their respective conditions. These groups were categorized as follows: obese mice treated with vehicle and placed in a control group (MetS + CTL + Vehicle); obese mice treated with vehicle and subjected to periodontal disease (MetS + PD + Vehicle); obese mice treated orally with aspirin (ASA, 40 mg/kg/day) for 15 days until the 75th day and placed in a control group (MetS + CTL + ASA); obese mice treated with ASA and subjected to periodontal disease (MetS + PD + ASA). The same groupings were replicated for mice that received saline solution after birth: saline-treated mice placed in a control group (SAL + CTL + Vehicle); saline-treated mice subjected to periodontal disease (SAL + PD + Vehicle); saline-treated mice administered ASA and placed in a control group (SAL + CTL + ASA); and saline-treated mice treated with ASA and subjected to periodontal disease (SAL + PD + ASA).

2.3. Micro-CT analysis

At 75 days of age, maxillary samples were obtained and washed three times in 75% alcohol before being subjected to Micro-CT scanning. The equipment used was a Bruker 1172 scanner with a microfocus X-ray tube model L7901-01 featuring a tungsten anode and air cooling manufactured by Hamamatsu Photonic. Detection was carried out through an 11 Mp CCD camera, model C4742-55-12HRF, also manufactured by Hamamatsu Photonic. Each camera pixel measures 5.9 × 5.9 micrometers, resulting in an effective area of 15.5 × 23.6 millimeters. The microtomography was operated at an accelerated potential of 44 kV with a beam current of 226 microamperes. The X-ray source was combined with a 2-D detector, operating with an exposure time of 950 microseconds and rotation steps of 0.25 degrees. The obtained images were analyzed via software (Data Viewer V 1.7. Bruker Micro-CT), with the maxilla models positioned uniformly so that the occlusal side of the teeth was parallel to the x- axis. Three measurements were then taken, one at each root of the tooth ligated with the suture, to determine the enamel-cement junction to alveolar bone crest (CEJ-ABC) distance in micrometers (Figure S2). These measurements were transformed into a mean value for each sample.

2.4. Hematological parameters of MetS mice

At 60 and 75 days, blood samples were collected from the mice groups under anesthesia via cardiac puncture using a 26-gauge needle and a 1-mL syringe. The blood was transferred to a microtube containing 30 µL of EDTA for hematological analysis. Neutrophils, lymphocytes, platelets, monocytes, reticulocytes, and leucocytes were counted, and hematocrit levels were determined using standard methods [23, 24].

2.5. NO quantification

At 75 days of age, the animals were euthanized, and their maxillae were removed. The gingival tissue was manually separated from the hard tissue using a scalpel, weighed, homogenized, than stored at -80 ºC. Nitric oxide (NO) concentration in gingival tissue samples was determined using the nitrite concentration method based on the cadmium-Griess technique with specific modifications [25, 26].

2.6. Glucose measurement

Blood glucose levels were measured at 60 days of age. Lidocaine ointment 50 mg/g (AstraZeneca do Brasil Ltda - Cotia - SP) was topically applied to the tail end as a method of analgesia. Peripheral blood samples (1-5 µL) were then collected from the tail, and glucose levels were determined using a glucose monitor (Accu–Check Active–Roche Diagnosis®).

2.7. Statistical analysis

Data was subjected to statistical analysis using the GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA). Results were presented as mean ± standard error of the mean (SEM). Normality of data was assessed using the Shapiro-Wilk test. ANOVA followed by Tukey's test was used for comparison among more than two groups. Pearson’s correlation was performed, and a correlation coefficient (R) greater than 0.7 was considered a strong correlation. Results with p-values less than 0.05 were considered statistically significant.

3. Results

3.1. MSG-induced MetS

On the 60th day of life, the obesity induced by MSG was validated through the weight of retroperitoneal (RP) and perigonadal (PG) adipose tissue and Lee's obesity index. As expected, Lee's index and metabolic parameters, such as RT and PG adipose tissue weight, and basal glucose concentrations, were higher in the MetS group compared to SAL groups. Moreover, cardiovascular parameters, including heart rate (HR) and mean arterial pressure (MAP), were also higher in the MetS group compared to the SAL group (Figure 2).

Interestingly, comparing the SAL group to the MetS group, no significant difference in body mass was observed ($p = 0.866$) (Figure S3A). However, the MetS group exhibited greater abdominal circumference than the SAL group [MetS = (9.493 ± 0.10) cm vs SAL = (7.695 ± 0.10) cm, $p \leq 0.0001$] (Figure 2B). The MetS group also displayed a higher Lee index (Figure 2C), indicative of a higher obesity level, compared to the SAL group [MetS = (392.1 ± 16.73) g/cm^3 vs SAL = (326.9 ± 8.82) g/cm^3, $p = 0.0004$]. Additionally, it was possible to observe that the MSG-treated group exhibited higher blood glucose levels (Figure 2D) [MetS = (169.4 ± 3.90) mg/dL vs SAL = (130.7 ± 2.66) mg/dL, $p \leq 0.0001$].

Analyses of PG (Figure 2E) and RT (Figure 2F) adipose tissue mass demonstrated that the MetS group exhibited significantly higher values compared to the SAL group [MetS = (1367 ± 58.14) mg vs SAL = (430.7 ± 53.16) mg, $p \leq 0.0001$]; [MetS = (701.7 ± 172.5) mg vs SAL = (167.3 ± 20.5) mg, $p < 0.0001$], respectively. Furthermore, HR (Figure 2G) and MAP (Figure 2H) were elevated in the MetS group compared to the SAL group [MetS = (630.4 ± 11.27) bpm vs SAL = (472.0 ± 16.04)

bpm, p ≤ 0.0001]; [MetS = (109.6 ± 2.29) mmHg vs SAL = (92.08 ± 1.45) mmHg, p ≤ 0.0001], respectively. These data indicate that neonatal treatment with MSG induces MetS in female mice, as described previously for male mice [14, 15].

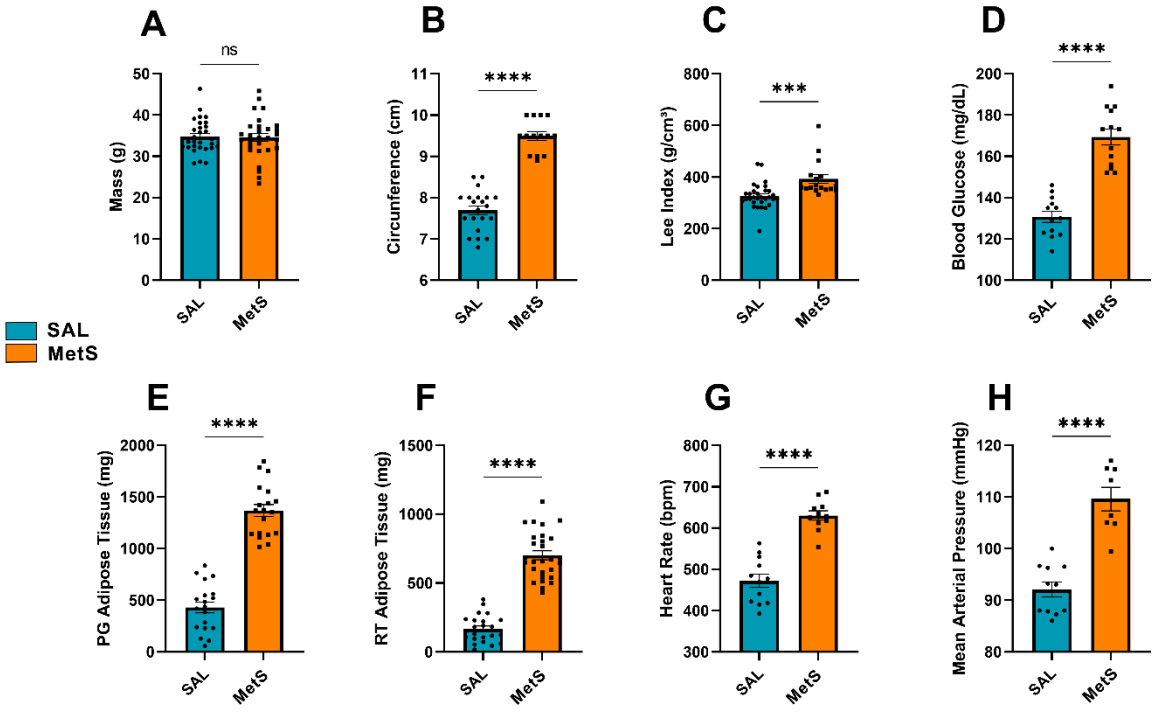

Figure 2. Validation of MSG-induced MetS. Swiss mice, 60 days old, previously treated with either saline solution or MSG, were euthanized. The SAL group served as the control (n = 12 - 31 per group), while the MetS consisted of n = 8 - 30 mice per group. Measurements were obtained for (A) body mass, expressed in grams (g); (B) abdominal circumference, expressed in centimeters (cm); (C) Lee index, expressed in grams per cubic centimeter (g/cm³); (D) glucose levels, expressed in milligrams per deciliter (mg/dL); (E) perigonadal adipose tissue (PG); and (F) retroperitoneal adipose tissue (RP), both expressed in milligrams (mg). Cardiovascular parameters such as (G) heart rate and (H) mean arterial pressure were expressed in beats per minute (bpm) and millimeters of mercury (mmHg), respectively. Data are presented as

mean ± SEM.**p < 0.01, ***p < 0.001, ****p < 0.0001 when comparing the SAL group to MetS group, as determined by unpaired Student's t-test.

3.2. MetS mice developed neutropenia

To evaluate the effects of MetS in the hematological parameters, including neutrophils (n = 9 - 12), lymphocytes (n = 10), and platelets (n = 23), cell counting was performed. The MetS group presented neutropenia (Figure 3A) compared to the SAL group [MetS = (854.7 ± 51.41) mm^3 vs SAL = (1089 ± 82.16) mm^3, p = 0.0202]. However, no significant differences were observed in the number of lymphocytes (Figure 3B) and platelets (Figure 3C) between the MetS and SAL groups (p = 0.7293 and p = 0.1628, respectively). Additionally, leukocytes, reticulocytes, and monocytes were counted, but no statistically significant difference was observed, as shown in Figure S3.

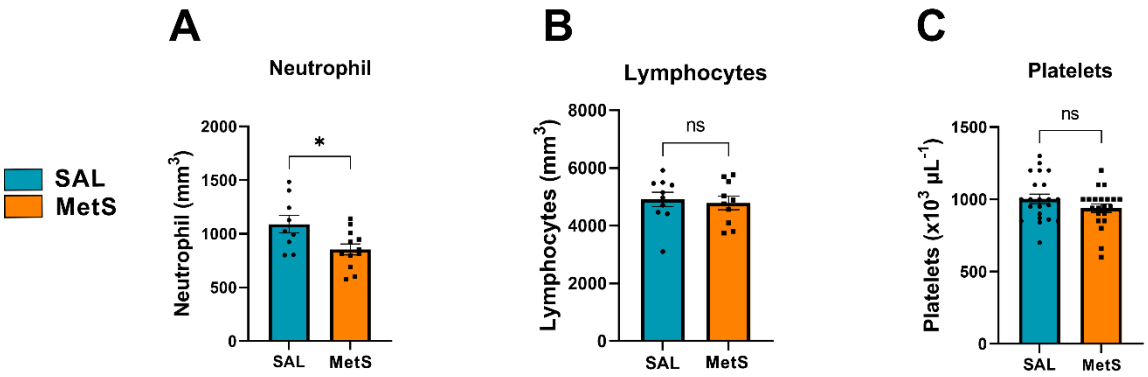

Figure 3. Hematological parameters in control and obese mice. Peripheral blood analysis was performed at 60 days of age in both the control and obese groups. (A) Neutrophil counts are expressed as the number of cells per cubic millimeter (mm^3); (B) Lymphocyte levels are expressed as the number of cells per cubic millimeter (mm^3); (C) Platelet levels are expressed as millions per microliter ($\times 10^3$ μL^{-1}). Error bars

represent the mean ± SEM of the diverse groups. * $p < 0.05$; determined by unpaired Student's t-test.

3.3. Validation of periodontitis model

The mean distance from the enamel-cement junction to the alveolar bone crest (CEJ-ABC) was measured three times at three points around the first upper molar: the mesiobuccal root, the distobuccal root, and the palate root. The locations of measurement are illustrated in Figure S2. Greater CEJ-ABC distance indicates more significant bone reabsorption.

Micro-computed tomography (Micro-CT) analysis generated 2D images for each sample (n = 4). Analysis of the 2D images revealed a higher bone loss in the (SAL + PD + Vehicle) group compared to the corresponding control group [SAL + PD + Vehicle = (483.51 ± 30.21) μm vs SAL + CTL + Vehicle = (201.18 ± 7.80) μm, $p = 0.0005$] (Figure 4A). Similar results were observed for the MetS groups (Figure 4A) [MetS + PD + Vehicle = (608.95 ± 45.07) μm vs MetS + CTL + Vehicle = (204.46 ± 26.03) μm, $p < 0.0001$]. Comparing SAL + PD + Vehicle to MetS + PD + Vehicle, a statistical difference was observed [SAL + PD + Vehicle = (483.51 ± 30.21) μm vs MetS + PD + Vehicle = (608.95 ± 45.07) μm, $p = 0.0005$], demonstrating that the MetS group presented more significant bone resorption than the SAL group.

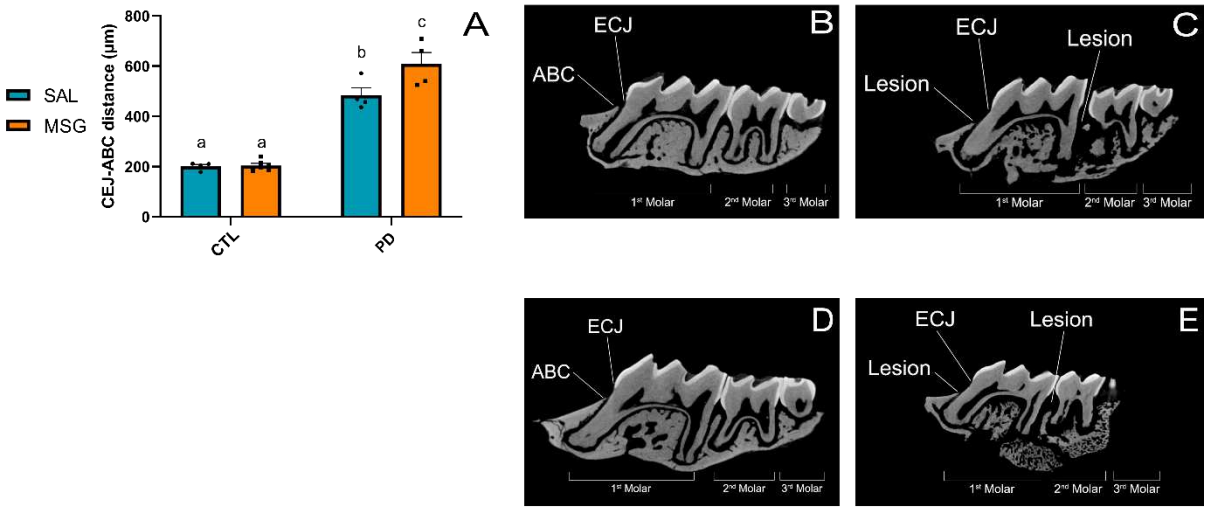

Figure 4. Validation of the PD model. Swiss mice aged 75 days were euthanized. The PD group underwent ligature procedure, while the CTL group served as the control group without ligature. (A) Measurement of the CEJ-ABC distance in micrometers (µm); (B - E) 2D slices of micro-CT analysis from a maxilla of their respective groups. Values followed by the same letter are not significantly different ($p > 0.05$) as determined by two-way ANOVA.

3.4. ASA prevented bone loss only in SAL groups

To observe the effect of ASA on the development of PD, animals were administered ASA or vehicle as a control for 15 days following the ligature procedure. Bone loss was assessed as previously described. Considering the effect of ASA treatment on the development of PD, when comparing the SAL groups, the following outcomes were obtained: the SAL + CTL + Vehicle and SAL + CTL + ASA groups exhibited no statistical difference in CEJ-ABC distances ($p > 0.05$), whereas the SAL + PD + Vehicle and SAL + PD + ASA groups displayed statistical significance, with the group receiving ASA treatment showing a smaller CEJ-ABC distance (Figure 5A) [SAL + PD + Vehicle = (483.51 ± 30.21) µm vs SAL+ PD + ASA = (256.1 ± 19.23) µm, $p <$

0.0001]. Moreover, no statistical difference was observed between the CTL + Vehicle and PD + ASA groups ($p = 0.65$), nor between CTL + ASA and PD + ASA ($p > 0.99$), despite the presence of ligature for PD induction, showing that ASA affected bone resorption.

In the MetS groups, MetS + CTL + Vehicle and MetS + CTL + ASA groups did not display statistical differences ($p = 0.93$). Similarly, the MetS + PD + Vehicle and MetS + PD + ASA groups showed no statistical difference ($p > 0.05$), which differs from the results obtained in the SAL groups (Figure 5A). ASA showed a potential effect in preventing bone loss in SAL groups, but this effect was not replicated in animals with MetS. Figures 5B to 5I show the CEJ-ABC distances in slices of the micro-CT analysis of their respective groups.

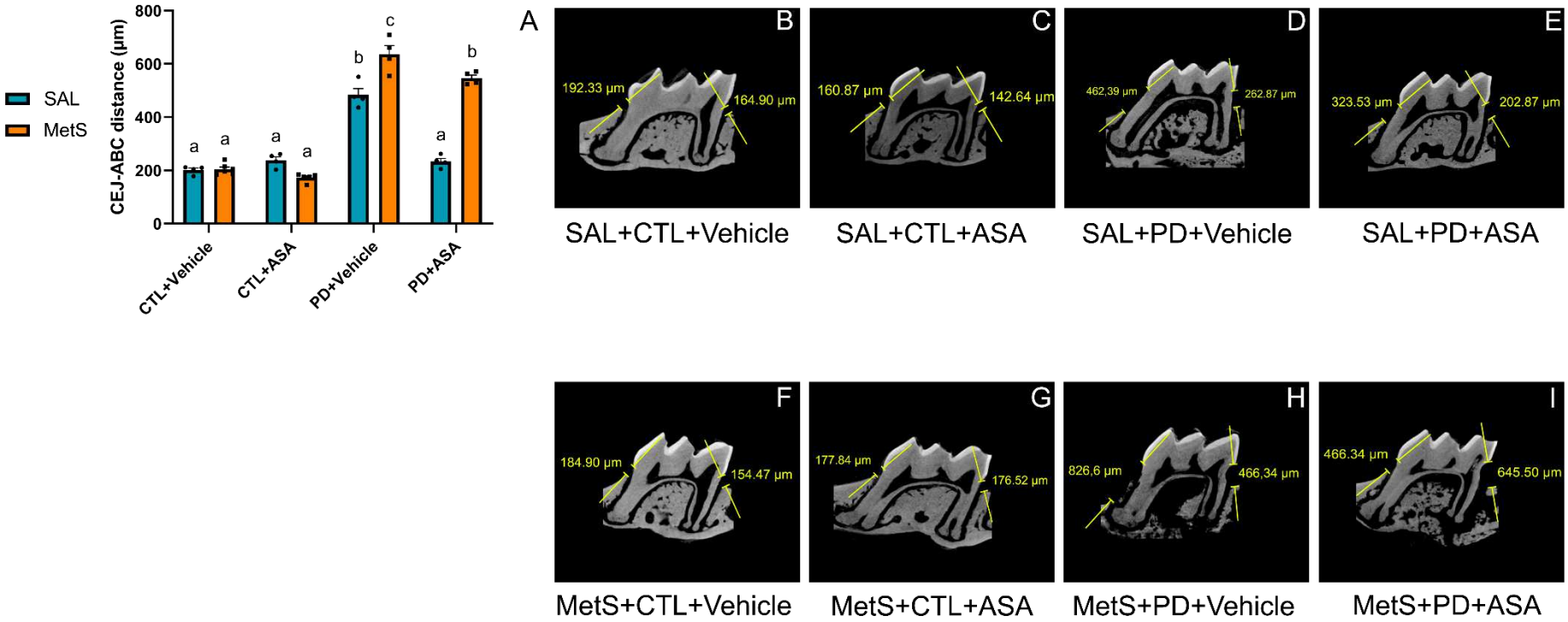

Figure 5. ASA effects on bone loss. Swiss mice, 75 days old, previously subjected to ligature procedure or left untreated were euthanized. Maxillary samples were obtained and scanned by micro-CT, and the 2D images were analyzed using the software DataViewer v1.7 (Bruker; 2016). (A) The measurements show the CEJ-ABC distance in micrometers (µm). CTL + Vehicle: animals that did not undergo the ligature procedure and received water via gavage; CTL + ASA: animals that did not undergo the ligature procedure and received ASA via gavage; PD + Vehicle: animals that underwent the ligature procedure and received water via gavage; PD + ASA: animals that underwent the ligature procedure and received ASA via gavage. (B – I) Representative micro-CT slices of the maxilla from their respective groups. Data are shown as mean ± SEM. Values followed by the same letter are not significantly different ($p > 0.05$), as determined by two-way ANOVA with Tukey's multiple comparisons post-test.

3.5. Hematological analysis of ASA treatment

To evaluate the effect of ASA treatment on blood cell counts in both the control (CTL) and periodontal disease (PD) groups, hematological analysis was performed in 75-day-old mice 15 days after ligature procedure and daily treatment with ASA. In the SAL groups, a decrease in neutrophil count (Figure 6A) was observed in the CTL + ASA group ($p < 0.05$) and the PD + ASA group ($p < 0.05$). Lymphocytes (Figure 6B) did not show any statistical differences between groups, while platelets (Figure 6C) were significantly higher only in the PD + Vehicle group ($p < 0.05$). Conversely, neutrophil count (Figure 6D) in MetS groups showed a higher value in the PD + Vehicle group ($p < 0.05$). Lymphocytes (Figure 6E) exhibited lower counts within the PD + ASA group ($p < 0.05$), and platelets (Figure 6F) were elevated across all groups compared to the CTL + Vehicle group. ASA treatment reduced neutrophil counts in SAL groups and platelet numbers in SAL mice with PD, events that did not occur in the MetS groups. Leukocytes, reticulocytes, and monocytes presented no statistically significant differences, as shown in Figure S4.

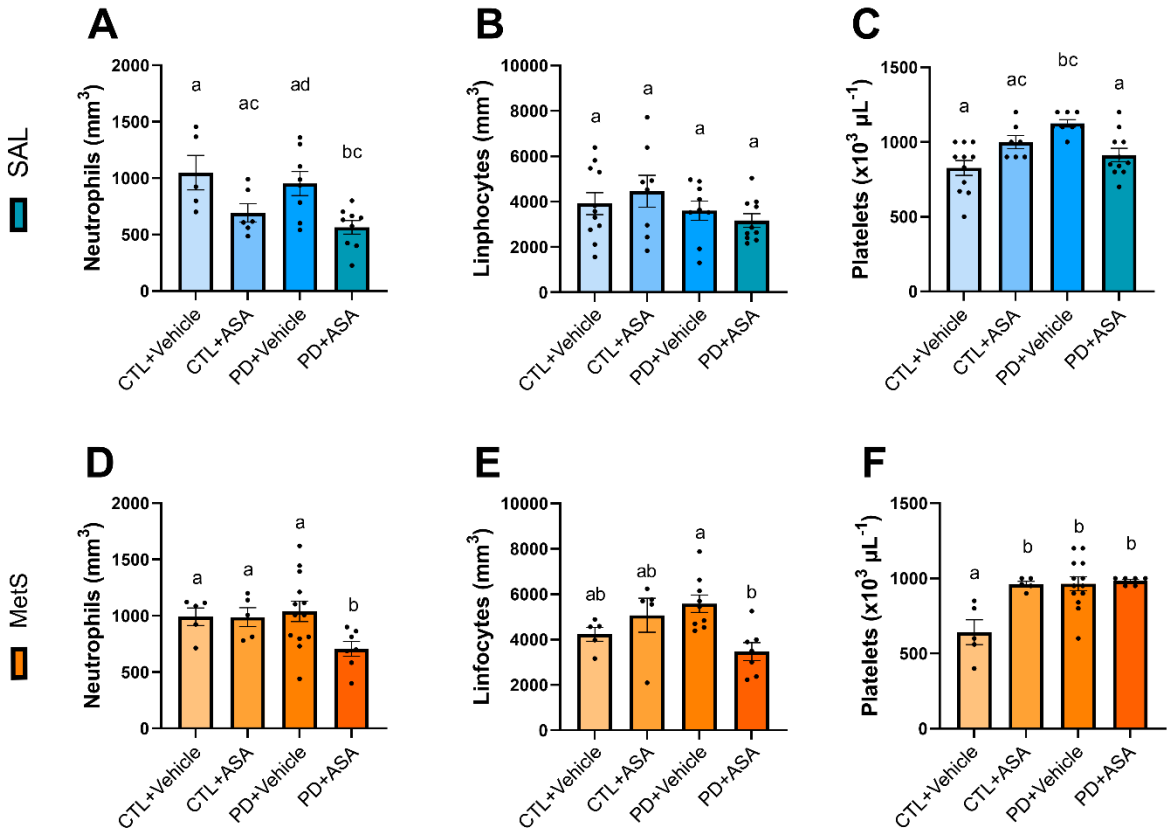

Figure 6. Hematological parameters in mice post ligature procedure and ASA treatment. Peripheral blood analysis was performed at 75 days of age in the CTL and MetS groups, with or without ligature placement. (A) Neutrophils from SAL groups expressed as the number of cells per cubic millimeter (mm^3); (B) Lymphocytes from SAL groups expressed as the number of cells per cubic millimeter (mm^3); (C) Platelets from SAL groups expressed in millions per microliter ($\times 10^3\ \mu L^{-1}$); (D) Neutrophils from MetS groups; (E) Lymphocytes from SAL groups; (F) Platelets from MetS groups. CTL + Vehicle: animals that did not undergo ligature procedure and received water via gavage; CTL + ASA: animals that did not undergo ligature procedure and received ASA via gavage; PD + Vehicle: animals that underwent ligature procedure and received water via gavage; PD + ASA: animals that underwent ligature procedure and received ASA via gavage. Bars represent the mean ± SEM of the separate groups.

Values followed by the same letter are not significantly different ($p > 0.05$, as determined by one-way ANOVA and Tukey's multiple comparisons test).

3.6. Gingival NO levels and correlation with bone loss

To evaluate the production of NO in the gingival tissue surrounding the ligated molar, samples were collected, and NO concentration was determined using the cadmium-Griess technique. The results are shown in Figure 7A. When comparing CTL + Vehicle groups, SAL and MetS animals showed no significant difference in NO concentration ($p \geq 0.9$), as neither group underwent the procedure of periodontal ligature. Similarly, no significant difference was observed in the CTL + ASA groups ($p > 0.99$). Moreover, SAL + PD + Vehicle and MetS + PD + Vehicle groups showed higher NO levels in the tissue compared to their respective CTL groups (SAL + PD + Vehicle = 29.38 μM vs SAL + CTL + Vehicle = 19.36 μM ± 1.514, $p < 0.0001$; MetS + PD + Vehicle = 35.04 μM vs MetS + CTL + Vehicle = 21.07 μM ± 1.514, $p < 0.0001$). When comparing PD + Vehicle groups, MetS animals presented a higher NO concentration than SAL animals (MetS + PD + Vehicle = 35.04 μM vs SAL + PD + Vehicle = 29.38 μM ± 1.514, $p = 0.0178$). Treatment with ASA resulted in a lower NO concentration in the SAL + PD + ASA group compared to SAL+ PD + Vehicle (SAL + PD + ASA = 29.38 μM vs SAL + PD + Vehicle = 16.61 μM ± 1.439, $p < 0.0001$). However, ASA treatment showed no alteration in NO concentration when analyzing MetS + PD groups (MetS + PD + Vehicle = 35.04 μM vs MetS + PD + ASA = 30.29 μM ± 1.636, $p = 0.1608$). These data suggest that ASA treatment can be effective against bone loss but not in MetS conditions.

A correlation can be established between NO levels in gingival tissue and bone resorption, as both are involved in inflammatory conditions. When correlating NO levels

in gingival tissue [μM] and bone loss (μm) from the SAL + PD + Vehicle group, R = 0.9887 and R2 = 0.9775 can be obtained (Figure 7B). Similarly, the correlation of the same parameters in the MetS + PD + Vehicle group resulted in R = 0.9545 and R2 = 0.9111 (Figure 7C). Correlation from the SAL + DP + ASA and MetS + DP + ASA groups resulted in R = 0.9848, R2 = 0.9698 (Figure 7D), and R = 0.9681, R2 = 0.9372 (Figure 7E), respectively.

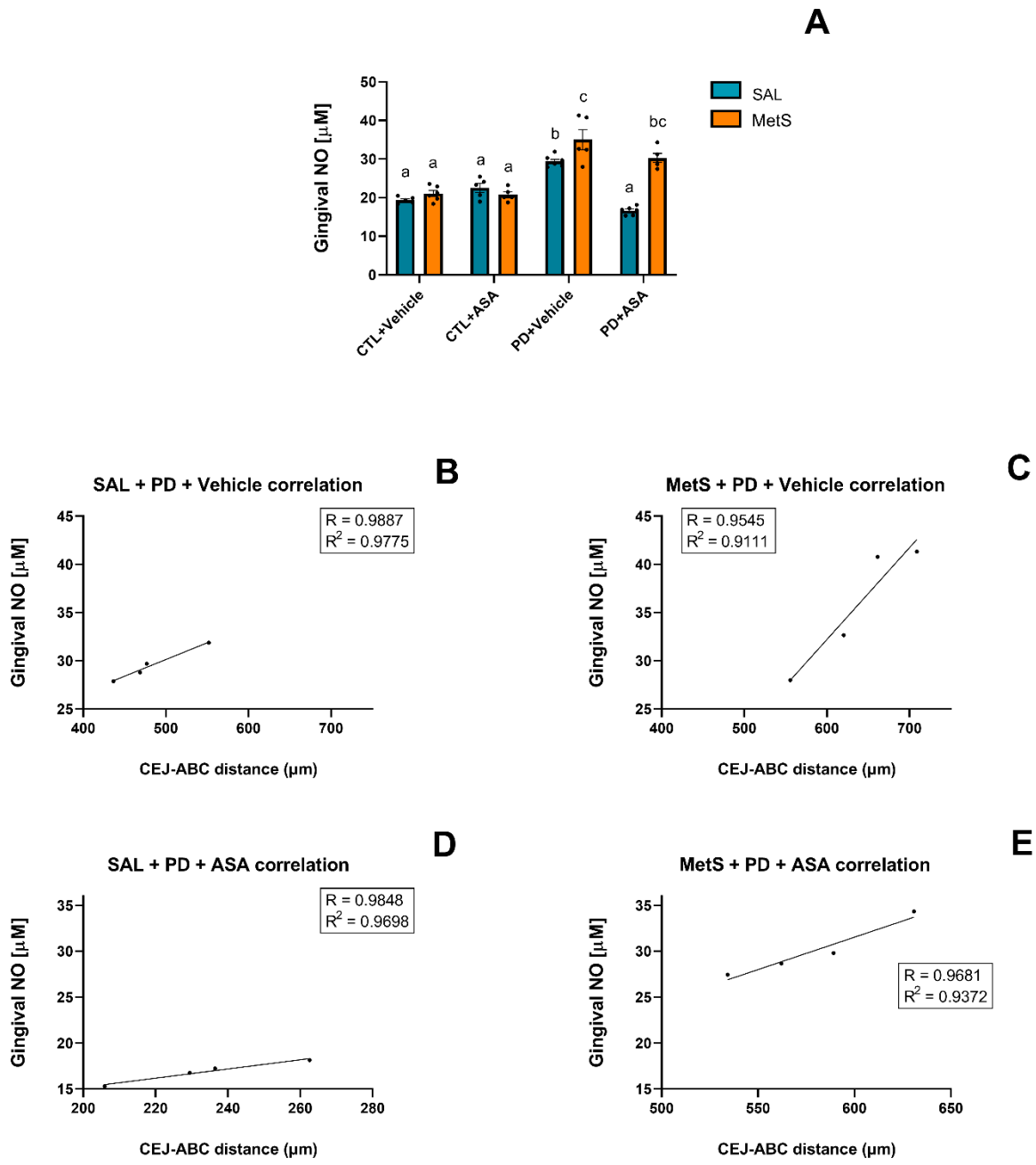

Figure 7. Quantification of NO in gingival tissue and Pearson correlation between gingival NO and bone loss. NO levels were estimated by measuring the production of nitrite through the cadmium-Griess technique and expressed in micromolar (µM). (A) Gingival NO levels in SAL and MSG groups; (B) Pearson correlation in the SAL + PD + Vehicle group; (C) Pearson correlation in the MSG + PD + Vehicle group; (D) Pearson correlation in the SAL + PD + ASA group; (E) Pearson correlation in the MSG

+ PD + ASA group. Bars represent the mean ± SEM of the separate groups. Values followed by the same letter are not significantly different ($p > 0.05$), as determined by one-way ANOVA with Tukey's multiple comparisons test.

4. Discussion

In this study, we demonstrated the protective action of ASA against bone loss in non-obese animals with PD, an effect absent in MetS animals. This finding underscores the importance of ASA utilization in preserving bone density under specific conditions and highlights the complexity of the interaction between MetS and PD in animal study models.

Epidemiological studies corroborate an association between obesity, metabolic syndrome (MetS), and periodontitis [27, 28]. Gingivitis in two or more teeth has been related to obesity, with this association partly mediated by oral hygiene and systemic low-grade inflammation [29]. However, the precise nature of this association is not entirely clear [30], and an understanding the effects of low-grade inflammation in obese individuals on periodontitis and the influence of MetS remains incomplete.

MetS encompasses a spectrum of metabolic dysfunctions, ranging from obesity to the development of cardiometabolic conditions such as type 2 diabetes, coronary artery disease, and stroke [31, 32]. Obesity is associated with high plasma levels of TNF-α and its soluble receptors, which may lead to a hyperinflammatory state, increasing the risk of PD development and insulin resistance [33].

Persistent low-grade inflammation is thought to contribute to the onset of insulin resistance and disrupt the delicate balance of cytokine interactions within periodontal tissues [28]. Additionally, the impact of PD on overall health can heighten susceptibility to systemic illnesses due to the presence of Gram-negative bacteria and inflammatory mediators in the bloodstream, ultimately leading to inflammation. These shared

characteristics indicate the relationship between MetS and PD [34]. Although all this evidence points to an increased risk of severe PD in cases of obesity and MetS, the precise underlying biological mechanism remains to be fully comprehended.

Several studies employing neonatal administration of MSG following the methodology used in this study have observed that the animals developed hypertension, insulin resistance, increased fat deposition, and a reduced nasal-anal length without alterations in body weight [14] and neutropenia [15], which supports our findings. In this study, no significant differences were observed in lymphocytes in the control and PD groups in peripheral blood. ASA decreased neutrophil counts in SAL groups and platelet numbers in SAL animals with PD, events not observed in the MetS groups. Further studies are needed to assess the functional role of neutrophils and platelets, their usefulness as diagnostic or therapeutic markers in periodontitis, and the effects of ASA on these cells in the context we investigated.

Recent research has proposed the use of micro-CT imaging for assessing bone resorption in animal models of periodontitis to assess the extent of alveolar bone loss [35-37]. Indeed, our data show that Micro-CT analysis may also be used to obtain linear measures to assess and compare periodontal bone destruction in MetS.

The biological markers TNF-α and IL-6, shared in MetS and PD, reinforce the association between MetS and periodontitis [38]. In addition, NO has been recognized as an important mediator in periodontitis [33]. Our results demonstrate that NO levels in gingival tissue were higher in animals with PD, in both the saline (SAL) and MetS group. These findings closely align with the observed bone loss results. Comparing the data from both aspects suggests that NO may have a direct influence on the progression of bone resorption in PD [39]. The increased alveolar bone resorption may be due to the stimulatory effect of NO on the activity of osteoclasts [40, 41], and its

capacity to stimulate production of metalloproteinases by osteoblasts [42]. The significantly higher levels of NO in the case group may contribute to the development of the frequently found clinical symptoms of periodontitis. Gingival erythema may be explained by the vasodilatory effect of NO, and the gingival edema by the vascular permeability-increasing effect of NO. Another signal of PD development is bleeding on probing due to the inhibitory effect of NO on platelets [43].

The ASA treatment showed promise in preventing bone loss. This outcome can be attributed to its non-selective inhibition of COX-1 and COX-2 enzymes. As these enzymes reduce prostaglandin production, the levels of pro-inflammatory cytokines decrease [44]. Additionally, ASA exerts direct effects on osteoclast differentiation activated by RANKL via NF-κB inhibition [45, 46]. However, the same cannot be asserted for PD treatment in animals with MetS.

It has been demonstrated that systemic administration of ASA attenuated inflammation associated with periodontitis induced in Holtzman rats without affecting the repair process upon stimulus removal [47]. Ligatures were removed after 15 days of periodontitis induction, and ASA was administered starting the following day for 15 days. Periodontal repair was evaluated by microcomputed tomography [47]. In another study [48], rats received aspirin (30 mg/kg) or Clopidogrel (75 mg/kg) intragastrically once daily for 3 days. On the third day, they were sacrificed, and gingival tissue was used to assess myeloperoxidase activity and the expression of the chemokine CXCL4. Hemi-mandibles were used for microscopic evaluation, revealing that Clopidogrel, but not ASA, prevented bone loss in experimental periodontitis. Different treatment regimens with ASA and early stimulus removal may explain the different results compared to our findings.

As previously mentioned, hyperglycemia is a constituent of the MetS model used, and it has been extensively observed in the literature. It is clear that oxidative stress is a common factor in MetS and PD [49, 50]. Accordingly, we conjecture that ASA treatment was ineffective in animals with this characteristic due to the effects of hyperglycemia on tissues, such as reactive oxygen species (ROS) production, activation of NF-κB-dependent pathways, and inflammasome activation [51, 52]. In addition, ASA gel and mouthwash showed an effect in lowering the levels of salivary biomarkers PGE_2, TNF-α, and NO in patients with periodontal diseases [53].

Therefore, based on the results of the present study, it is appropriate to suggest that ASA alternative therapy may be an efficient approach to preventing bone loss and elevating proinflammatory mediator levels, such as gingival NO, caused by PD. A limitation of this study is that ASA resistance observed in mice with MetS was validated only for the dose of 40 mg/day of ASA, as other doses were not tested. Future studies are needed to clarify these findings.

5. Conclusion

Our findings suggest that ASA has potential in the treatment of damage caused by PD. However, in patients with MetS, ASA alone may not confer therapeutic benefits. These findings complement existing knowledge regarding PD and its treatment approaches in the literature.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this article.

Ethics approval

All executed procedures were conducted in strict accordance with the guidelines for laboratory animals established by the National Institute of Health (Bethesda, MD, USA), as recommended by the Guide for the Care and Use of Laboratory Animals proposed by the Brazilian National Council of Animal Experimentation (COBEA). The protocol was approved by the Internal Scientific Commission and the Ethics in Animal Experimentation Committee of the State University of Londrina (CEUA – process number 063.2021). Every effort was made to minimize pain and suffering in the animals involved in these experiments.

Author contributions

Lucas Sobral de Rossi: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Original Draft, Visualization, Formal Analysis.; **Raquel Pires Nakama:** Formal analysis, Investigation, Validation.; **Lucas Felipe Dos Santos:** Investigation, Validation,; **Leonardo Berto Pereira:** Investigation, Validation,;

Aparecida Donizette Malvezi: Investigation, Validation,; **Maria Isabel Lovo-Martins:** Investigation, Validation,; **Ana Paula Cardoso Canizares:** Investigation, Validation,; **Luiz Claúdio Tozoni Filho:** Investigation, Validation,; **Eduardo Inocente Jussiani:** Methodology, Resources.; **Andressa de Freitas Mendes Dionísio:** Methodology, Resources.; **Marli Cardoso Martins-Pinge:** Methodology, Resources, review & editing; **Phileno Pinge-Filho:** Conceptualization, Methodology, Resources, Writing – review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

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Supplementary Figures

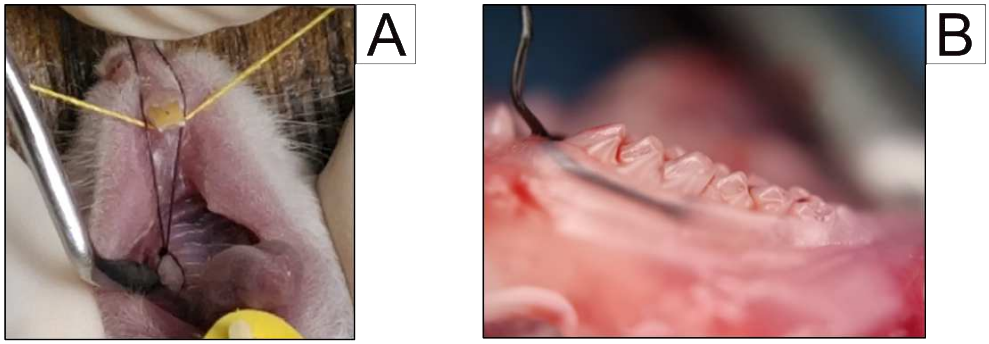

Figure S1. Periodontal ligature procedure. Swiss mice, 60 days old, previously treated with either saline solution (SAL) or monosodium glutamate (MSG), underwent anesthesia. The procedure was performed with a 6-0 nylon suture wrapped around the first upper molar tooth on the right side of the animal. The nylon suture was then inserted into the gingival sulcus, the space between the tooth and the surrounding gum tissue, with the help of a Hollemback spatula, and fixed with a surgical knot. (A) A photograph showing the correct positioning of the suture around the molar tooth. (B) A proximal view reveals the placement of the suture within the gingival sulcus.

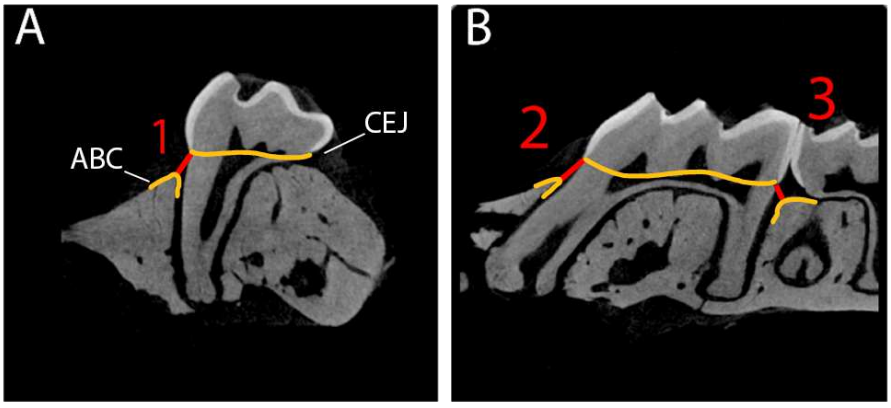

Figure S2. Bone level measurements. (A) Coronal section showing the measurement site (1) at the palatal root of the first upper right molar. (B) Sagittal section shows the measurement sites at the mesiobuccal (2) and distobuccal (mm^3) roots of the first

upper molar. ABC - alveolar bone crest; CEJ - cement-enamel junction. The mean for the sample was obtained from three measurements at each point.

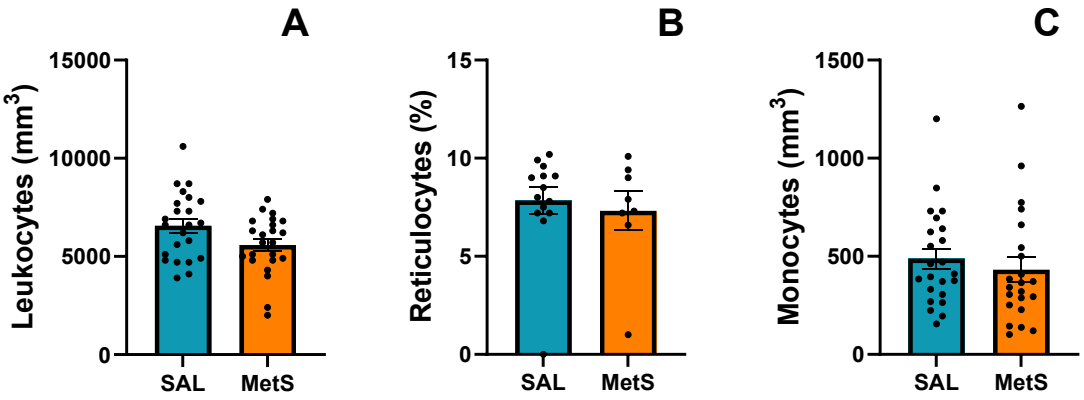

Figure S3. Hematological parameters in SAL and MetS mice. The analysis of peripheral blood was performed at 60 days of age in the control and obese groups. (A) Leukocytes expressed as the number of cells per cubic millimeter (mm^3); (B) Reticulocytes expressed as a percentage (%); (C) Monocytes expressed as the number of cells per cubic millimeter (mm^3). Bars represent the mean ± SEM of the diverse groups (* $p < 0.05$; unpaired Student's t-test).

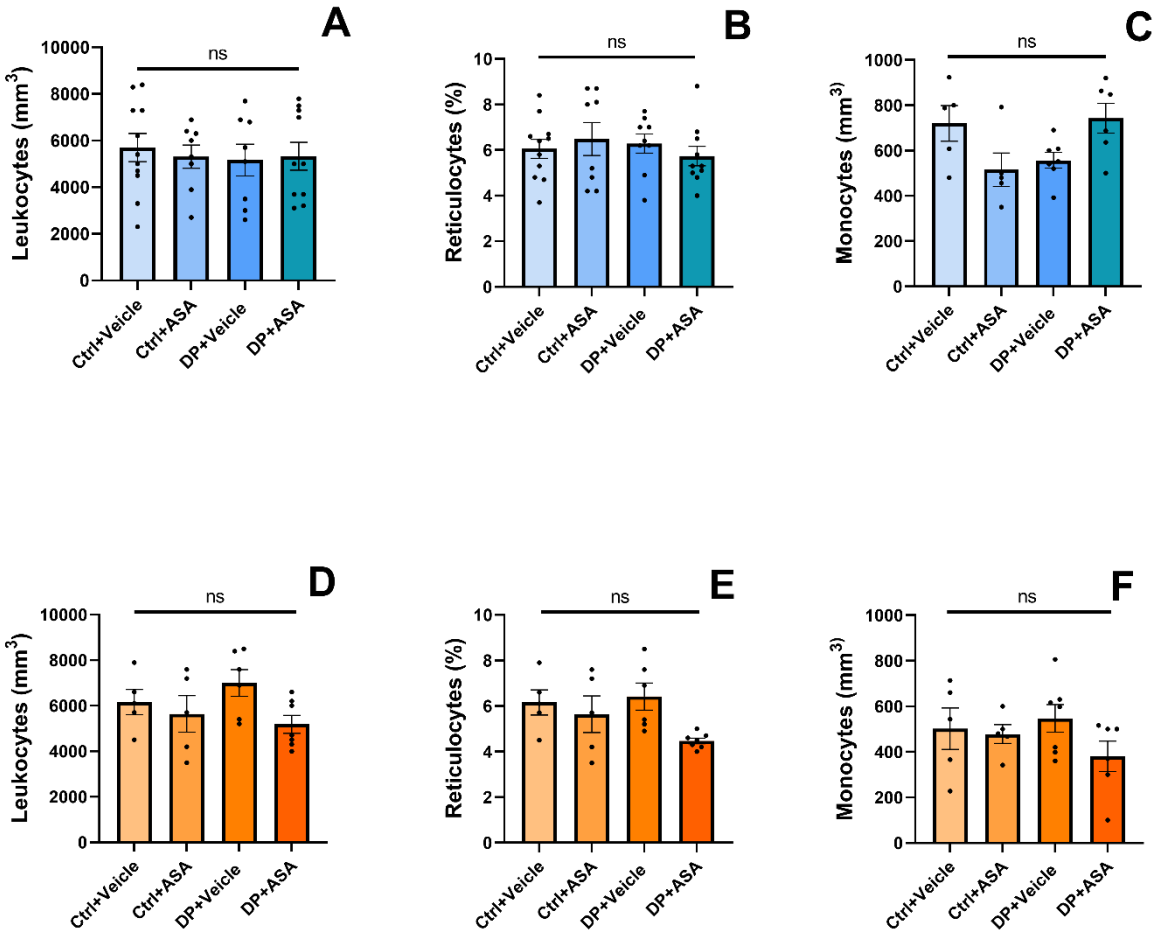

Figure S4. Hematological parameters in mice following ligature procedure and ASA treatment. The analysis of peripheral blood was performed at 75 days of age in the SAL and MetS groups with or without the ligature procedure. (A) Leukocytes in SAL groups expressed as the number of cells per cubic millimeter (mm^3); (B) Reticulocytes in SAL groups expressed as a percentage (%); (C) Monocytes in SAL groups expressed as the number of cells per cubic millimeter (mm^3); (D) Leukocytes in MetS groups expressed as the number of cells per cubic millimeter (mm^3); (E) Reticulocytes in MetS groups expressed as a percentage (%); (F) Monocytes in the MSG groups expressed as the number of cells per cubic millimeter (mm^3). CTL + Vehicle: animals that did not undergo ligature procedure and received water via gavage; CTL + ASA: animals that did not undergo ligature procedure and received ASA via gavage; PD +

Vehicle: animals that underwent ligature procedure and received water via gavage; PD + ASA: animals that underwent ligature procedure and received ASA via gavage. Bars represent the mean ± SEM of the different groups ($p < 0.05$; unpaired t-test). Values followed by the same letter are not significantly different ($p < 0.05$, as determined by one-way ANOVA followed by Tukey's posttest).

Highlights

- Metabolic syndrome worsens bone levels in animals with PD.
- Aspirin prevented bone loss only in non-obese animals with PD.
- Aspirin reduced neutrophil and platelet levels in PD mice, but not in MetS mice.
- High gingival NO levels correlate with higher bone loss levels.