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**ALTERAÇÃO DO ESTRESSE OXIDATIVO DA PERIODONTITE COMO FATOR DE  
RISCO PARA O DESENVOLVIMENTO DA PNEUMONIA ASSOCIADA À  
VENTILAÇÃO MECÂNICA: ESTUDO CLÍNICO, OBSERVACIONAL DO TIPO  
TRANSVERSAL**

CASCAVEL - PR  
NOVEMBRO/2023

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Dissertação apresentada à Universidade Estadual do Oeste do Paraná, campus Cascavel, PR para obtenção do título de Mestre/Doutor, do Programa de Pós-graduação Biociências e Saúde.

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## RESUMO

A saliva carregada de enzimas associada à doença periodontal pode modificar as superfícies da mucosa para promover adesão e colonização por patógenos respiratórios. O objetivo deste estudo foi avaliar a ocorrência de estresse oxidativo, decorrente da inflamação periodontal, no desenvolvimento da pneumonia associada a ventilação mecânica (PAV) em pacientes internados em Unidade de Terapia Intensiva (UTI). Metodologia: Estudo clínico, transversal e observacional, com análise quantitativa dos dados, com base clínica e laboratorial. As coletas foram realizadas no período de abril de 2022 a janeiro de 2023. Os pacientes selecionados foram avaliados quanto à presença ou ausência de periodontite e PAV. Foram avaliados 117 pacientes e, após a aplicação dos critérios de inclusão e exclusão, 53 pacientes foram incluídos no estudo e foram divididos em 3 grupos: 1- Controle – sem periodontite e sem PAV (n=17), 2- Periodontite e sem PAV (n=30), 3- Periodontite e com PAV (n=6). Foi realizado exame clínico periodontal de: profundidade clínica de sondagem (PCS), sangramento à sondagem (SS) e profundidade clínica de inserção (PCI), além da coleta e quantificação do fluido crevicular gengival, coleta de saliva e secreção traqueal para análise do sistema antioxidante e estresse oxidativo. Os exames hematológicos e os dados dos pacientes foram coletados de prontuário eletrônico (Tasy®) e os dados para diagnóstico da PAV foram fornecidos pela Comissão de Controle de Infecção Hospitalar. As variáveis referentes às avaliações clínicas e as análises bioquímicas foram avaliadas quanto a normalidade (Teste de Shapiro-Wilk) e homocedasticidade (Teste de Bartlett) e em seguida, foram realizadas as análises entre os grupos por meio do teste não paramétrico de Kruskal-Wallis, seguido pelo teste de Dunn. Resultados: A média de idade dos pacientes foi de 49,8 anos, sendo a maioria do sexo masculino. As variáveis clínicas de PCS, PCI e SS, no grupo de pacientes com periodontite, mas sem PAV, apresentaram valores significativamente mais elevados do que o grupo controle. Os exames hematológicos ureia e creatinina apresentaram diferenças estatísticas significativas no grupo controle. Ao avaliar as variáveis relativas ao sistema antioxidante e estresse oxidativo na saliva, a enzima SOD mostrou-se inibida e a LPO aumentada, no grupo periodontite, quando comparado com o grupo controle. Houve aumento de SOD, CAT e GR e diminuição de LPO, no grupo Periodontite+PAV em relação ao grupo Periodontite. A análise integrativa mostrou maior contribuição da enzima CAT e GST com escores positivos no grupo Periodontite+PAV. A análise das mesmas variáveis, mas na secreção traqueal, mostrou que GST e LPO apresentaram diminuição no grupo de pacientes com periodontite comparado ao controle. As enzimas GR e GST mostraram-se em indução e inibição, respectivamente, no grupo Periodontite + PAV, tendo os mesmos resultados sido observados na análise integrativa da secreção traqueal. Conclusão: a alteração do estresse oxidativo, causado pela inflamação da periodontite com um desequilíbrio do estado antioxidante, pode ser um fator de risco para o desenvolvimento da PAV. Estudos clínicos com maior amostra e maior tempo de coleta são necessários para confirmar esses achados.

Palavras-chave: periodontite; pneumonia associada a ventilação mecânica; estresse oxidativo; unidade de terapia intensiva.

**Change in oxidative stress in periodontitis as a risk factor for the development of pneumonia associated with mechanical ventilation: a clinical, observational, cross-sectional study.**

**ABSTRACT**

Enzyme-laden saliva associated with periodontal disease can modify mucosal surfaces to promote adhesion and colonization by respiratory pathogens. The objective of this study was to evaluate the occurrence of oxidative stress resulting from periodontal inflammation in the development of VAP in patients admitted to the Intensive Care Unit (ICU). Methodology: Clinical, cross-sectional and observational study, with quantitative analysis of data, with clinical and laboratory basis. Collections were carried out from April 2022 to January 2023. The selected patients were evaluated for the presence or absence of periodontitis and VAP. 117 patients were evaluated, and after applying the inclusion and exclusion criteria, 53 patients were included in the study and were divided into 3 groups: 1- Control – without periodontitis and without VAP (n=17), 2- Periodontitis and without VAP (n=30), 3- Periodontitis and with VAP (n=6). A periodontal clinical examination was carried out: Clinical Probing Depth (PCS), Bleeding on Probing (SS) and Clinical Insertion Depth (PCI), in addition to collecting and quantifying gingival crevicular fluid, collecting saliva and tracheal secretion for system analysis antioxidant and oxidative stress. Hematological exams and patient data were collected from electronic medical records (Tasy®) and data for diagnosing VAP were provided by the Hospital Infection Control Committee. The variables referring to clinical evaluations and biochemical analyzes were evaluated for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test) and then analyzes were carried out between groups using the Kruskal-Wallis non-parametric test., followed by the Dunn test. Results: The average age of the patients was 49.8 years, with the majority being male. The clinical variables of PCS, PCI and SS, in the group of patients with periodontitis but without VAP, presented significantly higher values than the control group. Regarding hematological tests, urea and creatinine showed statistically significant differences. When evaluating variables related to the antioxidant system and oxidative stress in saliva, the SOD enzyme was inhibited and LPO was increased in the periodontitis group when compared to the control group. There was an increase in SOD, CAT and GR and a decrease in LPO in the Periodontitis+Pav group in relation to the Periodontitis group. The integrative analysis showed a greater contribution of the CAT and GST enzymes with positive scores in the Periodontitis+VAP group. Analysis of the same variables, but in tracheal secretion, showed that GST and LPO presented a decrease in the group of patients with periodontitis compared to the control group. The GR and GST enzymes showed induction and inhibition, respectively, in the Periodontitis + VAP group and the same results were observed in the integrative analysis of tracheal secretion. Conclusion: the change in oxidative stress caused by the inflammation of periodontitis with an imbalance in the antioxidant status may be a risk factor for the development of VAP. Clinical studies with larger samples and longer collection times are needed to confirm these findings.

Keywords: periodontitis; pneumonia associated with mechanical ventilation; oxidative stress; intensive care unit.

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## 1. INTRODUÇÃO

A pneumonia é responsável pelo acometimento do parênquima pulmonar e pode ser causada por bactérias, micoplasmas, fungos, parasitas e vírus, sendo a causa bacteriana mais comum (OLIVEIRA *et al.*, 2007). A pneumonia associada a ventilação mecânica (PAV) é responsável pela inflamação do parênquima pulmonar, instalada de 48 a 72 horas após a intubação orotraqueal e o início da ventilação mecânica (SOUZA; SANTANA, 2012; NOBAHAR *et al.*, 2016). Esses pacientes estão sob ventilação mecânica através de um tubo traqueal, traqueostomia ou podem estar em processo de desconexão do ventilador nas 48 horas anteriores ao início dos sintomas (NOBAHAR *et al.*, 2016).

O alto risco de desenvolvimento da PAV decorre da presença do tubo orotraqueal, nível de consciência alterada, boca seca e aberta, alteração na flora da orofaringe, inflamação oral, mecanismo de autolimpeza das vias aéreas prejudicado, inibição do reflexo da tosse e microaspiração de secreções, permitindo que a cavidade oral seja colonizada por bactérias patogênicas (CHACKO *et al.*, 2017).

Além disso, fatores do indivíduo, como: idade acima de 70 anos, desnutrição, doenças de base, depressão do nível de consciência, intubação e reintubação traqueal, situação imunológica, uso de drogas imunodepressoras, gravidade da doença, aspiração de secreções contaminadas, posição do paciente no leito e a elevação insuficiente da cabeceira, tempo prolongado de ventilação mecânica também colaboram no desenvolvimento da PAV (RODRIGUES *et al.*, 2016; SOUZA *et al.*, 2013).

O tubo orotraqueal fornece uma superfície inerte e sem descamação, à qual as bactérias aderem e crescem para formar biofilmes, de onde as bactérias são eliminadas e aspiradas para as vias aéreas inferiores. Além disso, o tubo orotraqueal induz à abrasão mecânica, irritação das vias da mucosa respiratória, comprometimento da função normal da laringe e aumento da secreção, o que leva ao aumento do risco de aspiração de secreções do trato respiratório superior (RAGHAVENDRAN; MYLOTTE; SCANNAPIECO, 2007).

Raghavendram e colaboradores (2007) citam ainda que a microbiota bacteriana e/ou conteúdo gástrico também podem ser importantes na patogênese da pneumonia pós-operatória. Embora os ventiladores, por si só, não sejam

considerados uma importante fonte de disseminação bacteriana, os circuitos respiratórios podem ficar fortemente contaminados com microrganismos da orofaringe e da traqueia (RAGHAVENDRAN; MYLOTTE; SCANNAPIECO, 2007).

A ligação entre placa dentária/biofilme e a PAV também tem sido estudada. A incidência de infecções do trato respiratório em pacientes que necessitam de um tubo orotraqueal é substancial, e o risco de adquirir PAV aumenta de 1 a 5% por dia de internação hospitalar (RAGHAVENDRAN; MYLOTTE; SCANNAPIECO, 2007). Estudos demonstram a associação entre a periodontite e a PAV, o que é biologicamente realístico, pois a proliferação bacteriana nos indivíduos com periodontite pode resultar na colonização da orofaringe, o que facilita a aspiração direta de patógenos e sustenta a infecção, mediada por fatores inflamatórios e imunológicos (JERÔNIMO *et al.*, 2020; CAMARGO *et al.*, 2019); (RAGHAVENDRAN *et al.*, 2011; PADILHA *et al.*, 2010).

A saliva é um importante componente fisiológico, útil para o diagnóstico e monitoramento de muitas condições patológicas orais e sistêmicas. Marcadores de estresse oxidativo (OS) na saliva são indicadores do local do processo de inflamação, da progressão da periodontite e da quantidade de bactérias periodontopáticas nas bolsas periodontais (SÁNCHEZ-VILLAMIL *et al.*, 2020). Além disso, a saliva, carregada de enzimas, associada à doença periodontal tem potencial de modificar as superfícies da mucosa para promover a adesão e colonização por patógenos respiratórios. As citocinas originárias dos tecidos periodontais pode promover a inflamação das vias aéreas inferiores após a aspiração e, assim, alterar o epitélio para promover a infecção por patógenos respiratórios (RAGHAVENDRAN; MYLOTTE; SCANNAPIECO, 2007).

O estresse oxidativo é causado pelo desequilíbrio entre a produção de espécies reativas de oxigênio e a atividade de antioxidantes endógenos locais (SÁNCHEZ-VILLAMIL *et al.*, 2020). O estudo de Brock *et al.* (2004) avaliou a capacidade antioxidante total como um indicador de resposta tecidual na periodontite e relatou sua diminuição na saliva de indivíduos com periodontite, enquanto SÁNCHEZ-VILLAMIL *et al.* (2020), observaram uma maior capacidade antioxidante total salivar.

Diante do exposto, acredita-se que o desequilíbrio da atividade do sistema antioxidante em pacientes com periodontite eleva o risco de ocorrência do estresse oxidativo, facilitando, assim, o desenvolvimento da PAV.

## **2. OBJETIVOS**

### **2.1 Objetivo Geral**

Avaliar a ocorrência de estresse oxidativo decorrente da inflamação periodontal no desenvolvimento da PAV em pacientes internados em uma UTI.

### **2.2 Objetivos Específicos**

Comparar o sistema antioxidante (SOD, CAT, GR, GST), bem como a ocorrência de estresse oxidativo na saliva e aspirado traqueal de pacientes em ventilação mecânica invasiva, com e sem periodontite, que desenvolveram ou não a PAV;

Comparar a quantidade de fluido crevicular gengival em todos os grupos de pacientes dessa investigação;

Analisar a temperatura corporal e os parâmetros bioquímicos sanguíneos que podem estar associados as alterações periodontais e sistêmicas dos grupos estudados;

Investigar os parâmetros periodontais, bem como os de fluido crevicular gengival, saliva e aspirado traqueal com a ocorrência de PAV entre os grupos estudados.

### 3. REVISÃO DE LITERATURA

#### 3.1 DOENÇAS RESPIRATÓRIAS, PNEUMONIA COMUNITÁRIA E NOSOCOMIAL

##### 3.1.1 DOENÇAS RESPIRATÓRIAS

O sistema respiratório é composto pelos tratos superior e inferior, sendo ambos responsáveis pela ventilação. O trato respiratório superior tem a função de aquecer e filtrar o ar inspirado, já o trato respiratório inferior realiza as trocas gasosas. As estruturas que compõem as vias aéreas superiores são: nariz, seios paranasais, faringes, tonsilas (adenoide), laringe e traqueia, enquanto o trato inferior é constituído pelos pulmões e estruturas brônquicas e alveolares (SMELTZER *et al.*, 2014).

Os pulmões são revestidos por uma membrana serosa, chamada pleura. Na pleura existe uma pequena quantidade de líquido pleural que possibilita o movimento do pulmão na cavidade torácica a cada inspiração e expiração (SMELTZER *et al.*, 2014).

Montanari (2016) descreve os epitélios que formam a parte respiratória inferior:

1. Traqueia e brônquios: epitélio pseudoestratificado colunar ciliado e com células caliciformes;
2. Bronquíolos: epitélio simples colunar ou cúbico ciliado e com células caliciformes ocasionais;
3. Alvéolo: epitélio simples pavimentoso.

A infecção respiratória inferior começa pela contaminação do epitélio pulmonar por meio de microrganismos contidos em gotículas ou pela aspiração de secreções orais que contém microrganismos. Em adultos saudáveis, os mecanismos de defesa pulmonar mantêm as vias aéreas inferiores estéreis e quando ocorre uma falha nos mecanismos de defesa do hospedeiro, inicia-se um processo infeccioso. A falha do mecanismo de defesa em eliminar os patógenos da superfície respiratória resulta em sua multiplicação e conseqüente destruição de tecidos (SCANNAPIECO, 1999).

Doenças pulmonares são frequentes e, dependendo da sua gravidade, podem desencadear incapacidades significantes e aumentar o risco de morte. As maiores representantes dessas condições crônicas são a asma e a doença pulmonar obstrutiva crônica (DPOC) (KAHN *et al.*, 2019).

A DPOC caracteriza-se pela obstrução crônica do fluxo aéreo, sendo essa obstrução geralmente progressiva e associada a uma resposta inflamatória incomum dos pulmões frente à exposição de partículas e gases tóxicos, causada, primariamente, pelo hábito de tabagismo. O processo inflamatório resulta em alterações nos brônquios (bronquite crônica), bronquíolos (bronquiolite obstrutiva) e parênquima pulmonar (enfisema) (SBPT, 2004).

A DPOC é a 4ª principal causa de morte no mundo, sendo que o envelhecimento da população mundial favorece a carga dessa comorbidade. Essa doença envolve a limitação do fluxo aéreo, causada pela inflamação crônica das vias aéreas e parênquima pulmonar ou destruição do tecido pulmonar. Devido a esses eventos, ocorre o comprometimento da elasticidade pulmonar e a dificuldade de abertura das vias aéreas durante a exalação (KAHN *et al.*, 2019).

Em relação à periodontite, um estudo retrospectivo mostrou evidências de que a periodontite está fortemente associada à mortalidade devido a DPOC, em uma população acima de 75 anos. Os resultados evidenciaram que o IMC e o histórico de tabagismo podem alterar o efeito da doença periodontal, aumentando as chances de mortalidade por doenças respiratórias (QUIAN *et al.*, 2020).

Outro estudo de caso controle trouxe que participantes, com quadro mais avançado de DPOC, eram mais suscetíveis ao desenvolvimento de doença periodontal severa. A perda óssea e a quantidade de dentes foram associadas a todos os estágios da DPOC (QUIAN *et al.*, 2020).

A DPOC e a periodontite possuem fatores de risco em comum, como: fumaça de cigarro, condições socioeconômicas e idade. As duas doenças se beneficiam dos mesmos mediadores pró-inflamatórios, o que facilita a progressão das duas condições (CARDOSO; REIS; CÉSPEDES, 2018). Portanto, existe a correlação entre a periodontite e a DPOC, devido aos mecanismos em comum, como inflamação rica em neutrófilos com subsequente destruição proteolítica de tecido conjuntivo (GOMES-FILHO *et al.*, 2020).

Outra doença respiratória de caráter crônico e inflamatório é a asma, a qual é definida pelo histórico de sintomas respiratórios (sibilos, dispneia, opressão torácica retroesternal e tosse) que podem variar em relação ao tempo e à intensidade e que estão associados a restrição variável do fluxo aéreo (SBPT, 2020). Todas as faixas etárias podem ser acometidas por essa doença, caracterizada por uma desordem genética complexa e variada, sofrendo influência ambiental significativa. Os principais fatores de risco para o início da doença são: tabagismo, obesidade, dieta e exposição a alérgenos (KAHN *et al.*, 2019).

### 3.1.2 PNEUMONIA COMUNITÁRIA E NOSOCOMIAL

A pneumonia é uma doença infecciosa do parênquima pulmonar e a microbiota oral exerce um papel importante na sua história natural (GOMES-FILHO *et al.*, 2020). Pode ser causada por bactérias, vírus, fungos e parasitas (BUI *et al.*, 2019), apresenta-se com sinais e sintomas respiratórios como: tosse, taquipneia, produção de secreção, dores no peito, febre, fadiga, dores musculares e inapetência (SANTI; SANTOS, 2016). Alguns dos fatores de risco associados à pneumonia são: indivíduos com DPOC, condição bucal deficiente, idosos, uso prévio de antibióticos, intubação orotraqueal, rebaixamento do nível de consciência, indivíduos que aspiraram grande volume de secreção, presença de sonda gástrica, traumatismo grave e broncoscopia recente (KAHN *et al.*, 2019).

A pneumonia possui duas classificações:

Pneumonia comunitária: É a infecção que se desencadeia no paciente nas primeiras 48 horas, ou seja, ele adquiriu a infecção fora do ambiente hospitalar (KAHN *et al.*, 2019). Segundo Corrêa *et al.*, (2018) constitui a principal causa de morte mundial com impacto importante nas taxas de morbidade. A disseminação microbiana respiratória é muito ampla e possui agentes potencialmente patogênicos. Dentre esses microrganismos, o *Streptococcus pneumoniae* perdura-se como a bactéria de maior prevalência (CORRÊA *et al.*, 2018), mas também pode ser causada por outros patógenos que residem na mucosa oral, como: *Haemophilus influenza*, *Mycoplasma pneumonia*, *Chlamydia pneumonia*, *Legionella pneumophila*, *Candida albicans* e espécies anaeróbicas (BANSAL; KHATRI; TANEJA, 2013).

O outro tipo de pneumonia é a nosocomial ou hospitalar (PAH), é aquela que ocorre geralmente em ambiente hospitalar, quando o paciente está em unidade de internamento, desenvolvendo-se após as primeiras 48 horas de internação, não possuindo relação com intubação endotraqueal ou ventilação mecânica (SBPT 2007).

A PAH pode ser classificada quanto ao tempo decorrido desde a admissão hospitalar até o início dos sinais e sintomas. A PAH precoce inicia-se até o quarto dia de internação e PAH tardia ocorre após o quinto dia de hospitalização (SBPT 2007), geralmente causada por patógenos presentes no meio ambiente, que não residem na orofaringe, entre esses, destacam-se os bacilos Gram-negativos (entéricos como *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia sps*, *Enterobacter sps.*), *Pseudomonas aeruginosa* e *Staphylococcus aureus* (BANSAL; KHATRI; TANEJA, 2013).

### **3.1.3 PNEUMONIA ASSOCIADA A VENTILAÇÃO MECÂNICA (PAV)**

A PAV é definida como a pneumonia que se desenvolve no paciente em um período de 48 a 72 horas após início da ventilação mecânica. O tubo orotraqueal fornece o oxigênio adequado ao que o paciente precisa, porém, também atua como um canal para bactérias patogênicas que se proliferam na cavidade oral e alcançam os pulmões pelo tubo (HUA et al., 2016). Esse tipo de pneumonia é classificada em precoce e tardia, sendo que a precoce ocorre até o quarto dia de intubação, enquanto a tardia ocorre após o quinto dia (SBPT, 2007).

A ventilação mecânica é utilizada para substituir parcial ou totalmente a ventilação espontânea, sendo indicada nas condições de insuficiência respiratória aguda ou crônica agudizada. A ventilação mecânica (VM) melhora a troca gasosa e diminui o trabalho respiratório, podendo ser utilizada de maneira não invasiva, com o uso de uma máscara facial ou de forma invasiva, por meio de um tubo endotraqueal ou cânula de traqueostomia (SBPT, 2013).

Sendo comum e associada à altas taxas de morbidade e mortalidade, a PAV acomete entre 10% a 25% dos pacientes admitidos em UTI, possuindo taxa de mortalidade de 24% a 76%.

Geralmente ocorre, como citado anteriormente, em um período de 48 a 72 horas após admissão e instalação da VM, estando associada ao aumento de infecções multirresistentes, ventilação mecânica prolongada, uso de antibióticos e tempo de hospitalização (XIE *et al.*, 2019).

A PAV é uma complicação grave, representando cerca de 80% dos episódios de pneumonia hospitalar, sendo a segunda causa mais comum de infecção hospitalar e mortalidade dentre as infecções hospitalares (AMARAL *et al.*, 2009).

Entre as estratégias de prevenção para o desenvolvimento da PAV, estão os cuidados bucais intensivos, pois em pacientes mecanicamente ventilados, a saúde bucal deteriora-se rapidamente (XIE *et al.*, 2019).

Alguns fatores de risco para o desenvolvimento da PAV incluem agentes relacionados ao próprio paciente (sexo, idade, histórico médico, distúrbio neurológico, DPOC, comorbidades, entre outros) e outros relacionados à ventilação (tempo de intubação, reintubação, ausência de drenagem de secreção subglótica e traqueostomia) (XIE *et al.*, 2019).

Algumas medidas preventivas podem auxiliar na diminuição da pneumonia nosocomial, são elas: elevação da cabeceira entre 30 e 45 graus, utilização de pressão do balonete da cânula entre 20 e 30 mmHg a fim de reduzir aspiração da secreção orofaríngea, limpeza rotineira na tubulação do ventilador mecânico, aspiração se necessária e não em horários padronizados, descontaminação oral, entre outros (SPEZZIA, 2019).

Estudo publicado em 2017, com o objetivo de avaliar a associação de patógenos respiratórios presentes na secreção traqueal e o biofilme oral em pacientes intubados, mostrou que a taxa de mortalidade de pacientes que evoluem para PAV é significativa. Dos 32 pacientes que compuseram o estudo, 40,6% evoluíram para PAV e 9,4% para pneumonia por aspiração. Dos treze pacientes que desenvolveram PAV, sete foram a óbito (SOUZA *et al.*, 2017).

O tubo endotraqueal é considerado o principal facilitador na entrada de patógenos das vias aéreas inferiores e serve como reservatório para esses patógenos que aderem à superfície do tubo, produzindo um biofilme. O tubo consiste em um foco resistente aos efeitos de antibióticos, representando um local de superfície inerte e cumulativo de microrganismos multirresistentes. Sua presença



mantém as cordas vocais permanentemente abertas, facilitando a aspiração de secreções que se acumulam acima do *cuff* (PADILHA *et al.*, 2010).

Os tubos são introduzidos na orofaringe e laringe, normalmente colonizadas, e passam para o meio estéril que é a árvore traqueobrônquica, criando, dessa maneira, uma passagem direta do meio externo para os pulmões. Desse modo, após algumas horas, o tubo endotraqueal é possivelmente colonizado por bactérias. Pacientes com periodontite possuem quantidades elevadas de periodontopatógenos no ambiente oral, portanto, infere-se que os patógenos podem migrar por meio do tubo endotraqueal para a via aérea inferior (PORTO *et al.*, 2016).

Outro fator de relevância é a duração da hospitalização, que, em questão de poucas semanas, interfere diretamente na diminuição da secreção salivar e mudança da microbiota oral. Condições que cooperam para a prevalência de bactérias gram-negativas e, desse modo, causam infecções pulmonares devido à aspiração desses patógenos (JERÔNIMO *et al.*, 2020).

### 3.2 DOENÇA PERIODONTAL

Dentre as doenças crônicas não transmissíveis, como diabetes, doenças cardiovasculares, câncer e doenças respiratórias, a doença periodontal é uma das comorbidades bucais mais importantes, representando um relevante problema de saúde pública (CARDOSO; REIS; CESPEDES, 2018), tanto é verdade que a Organização Mundial da Saúde (OMS) ressalta a necessidade de fortalecer a supervisão da doença periodontal no mundo todo (PETERSEN; OGAWA, 2005; PETERSEN; OGAWA, 2012).

Segundo o Centro de Controle e Prevenção de Doenças (CDC) dos Estados Unidos da América, a doença periodontal é considerada uma pandemia que traz consequências como o comprometimento da fala, a baixa autoestima e a redução da qualidade de vida. É uma das doenças inflamatórias mais comuns em adultos e, conforme o envelhecimento da população, passa a ser uma preocupação importante para o sistema de saúde público (BUI *et al.*, 2019).

A doença periodontal compreende um conjunto de doenças inflamatórias que afetam o periodonto, o qual é composto pelas estruturas que concedem suporte aos

dentes: gengiva, cemento, ligamento periodontal e osso alveolar (CARDOSO; REIS; CESPEDES, 2018).

Os primeiros agentes causadores dessa doença foram identificados como: *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* e *Tannerella forsythia*, patógenos esses que continuam sendo objeto de pesquisas até os dias atuais. A microbiota oral está presente na saliva, epitélio gengival e outras partes da cavidade oral, bem como aglomerada na placa dentária (essa última, de maneira sucinta, é um biofilme constituído destes microrganismos que ficam aderidos à superfície do dente (BUI *et al.*, 2019).

Conforme a nova classificação das doenças e condições periodontais e peri-implantares 2018: guia prático e pontos-chave, as condições periodontais podem ser divididas em três grupos principais, com subcategorias: (1) Saúde Periodontal e Condições Gengivais; (2) Periodontite e (3) Demais Condições que afetam o Periodonto.

A periodontite é definida como “doença inflamatória crônica multifatorial associada com biofilme disbiótico e caracterizada pela destruição progressiva do aparato de inserção dental” (STEFFENS; MARCANTONIO, 2018), sendo classificada de acordo com o estágio (relacionado à severidade da doença – ex. estágio I ao IX), progressão da doença em graus (grau A, B ou C) e impacto na saúde sistêmica.

Logo, a gengivite possui quadro reversível, de caráter inflamatório, nos tecidos gengivais (SANZ *et al.*, 2010). É definida como a inflamação local da gengiva, sem sinais de destruição óssea (DURSUN *et al.*, 2016).

Entre algumas espécies de microrganismos na etiologia das doenças periodontais, destaca-se *Prevotella intermedia* (Pi), *Porphyromonas gingivalis* (Pg) e *Actinomyces viscosus* como associados à gengivite e *Tannerella forsythus*, *Treponema denticola*, Pi, Pg, *Campylobacter*; e *Aggregatibacter actinomycetemcomitans* (Aa) como associados à periodontite, estando esse último envolvido, principalmente, nas formas mais agressivas das doenças periodontais e nas infecções extraorais como endocardite, pneumonias e abscessos (SOUTO *et al.*, 2014).

A periodontite caracteriza-se por ser uma doença de natureza inflamatória que afeta as estruturas da gengiva e osso ao redor dos dentes, a sua principal causa é a

microbiota da placa dentária (HEGDE; AWAN, 2018), local em que ocorre a mudança do ambiente de uma comunidade microbiana simbiótica para uma comunidade microbiana disbiótica, na região subgengival (WANG *et al.*, 2019). Embora seja iniciada pela presença de placa subgengival, a maior parte da destruição do tecido é desencadeada pela resposta anormal do hospedeiro às bactérias e seus produtos. Essa inflamação exacerbada envolve liberação de enzimas proteolíticas e espécies reativas de oxigênio (DURSUN *et al.*, 2016).

A periodontite pode se agravar de acordo com a resposta inflamatória desregulada do hospedeiro contra os patógenos orais. Enquanto a saúde do paciente se desregula, a doença tende a progredir (WANG *et al.*, 2019).

A disbiose da microbiota oral e os eventos pró-inflamatórios envolvem células e mediadores da resposta imune inata e adaptativa. A inflamação crônica é intermediada por diversos mediadores, como as citocinas pró-inflamatórias, incluindo IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IL-6 e IL-17 que exercem papel importante na inflamação aguda e crônica, bem como na lesão tecidual. Essas respostas imunes desreguladas, resultam na inflamação crônica dos tecidos periodontais (CARDOSO; REIS; CESPEDES, 2018).

Citocinas como TNF- $\alpha$ , IL-1 $\beta$  e IL-6 são os primeiros componentes da resposta imunidade inata a aparecer na patogênese da doença periodontal. Uma das funções do TNF- $\alpha$  inclui a regulação positiva da adesão de moléculas e estimulação da secreção das metaloproteinases de matriz (MMPs) para desencadear migração celular e destruição de tecidos. A dificuldade em eliminar a infecção, desencadeia o processo de transição da imunidade inata para a imunidade adaptativa (WANG *et al.*, 2019).

A principal alteração clínica, causada pela periodontite, é a perda de inserção dos tecidos periodontais com formação de bolsa periodontal (ALVES *et al.*, 2007). A concentração elevada de bactérias patogênicas dentro da placa e a ativação exacerbada do sistema imune, causam aumento nas moléculas de superfície bacteriana, estimulando a produção de citocinas e mediadores inflamatórios, possibilitando a liberação de MMPs. Tais enzimas contribuem para a remodelação da matriz extracelular e destruição óssea. Os periodontopatógenos podem romper a bolsa periodontal, resultando na entrada de endotoxinas e exotoxinas na corrente

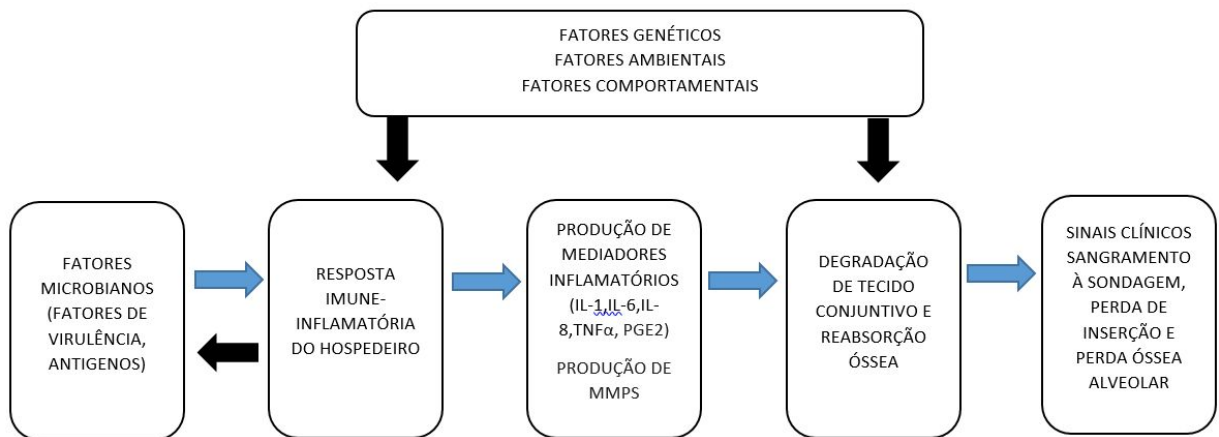
sanguínea, o que possui potencial para levar a disseminação bacteriana e infecção sistêmica, causando aumento da resposta inflamatória (LICCARDO *et al.*, 2019).

As bactérias, nesses casos, conseguem adentrar no sulco gengival por mecanismo direto ou indireto. O direto é caracterizado pela secreção de enzimas histolíticas (por exemplo: proteases, colagenases, hialuronidases), produtos tóxicos (endotoxinas e leucotoxinas) e produtos finais metabólicos (amônia, sulfeto de hidrogênio e ácidos orgânicos). O mecanismo indireto inclui a estimulação da resposta imuno-inflamatória do hospedeiro por seus componentes estruturais, como, por exemplo, lipopolissacarídeos (LPS), o que acarreta a liberação de citocinas e enzimas, resultando na destruição dos tecidos periodontais (colapso das fibras de tecido conjuntivo no ligamento periodontal e a reabsorção do osso alveolar) (WANG *et al.*, 2019).

Os LPS presentes nas bactérias, por meio de receptores *Toll-like* (TLRs) na superfície da célula hospedeira, levam a secreção de citocinas pró-inflamatórias, como: IL-1 $\beta$ , IL-8 e TNF- $\alpha$ , neutrófilos e fibroblastos. Os neutrófilos ativados levam a um estado de inflamação prolongada, causando danos ao tecido circundante. Os TLRs reconhecem as bactérias do biofilme oral, na lesão inicial, e liberam os mediadores pró-inflamatórios. Essa liberação acarreta vasodilatação local e aumento da permeabilidade vascular, fazendo com que as células imunes sejam recrutadas e ativadas, sendo os neutrófilos predominantes nessa fase da inflamação (WANG *et al.*, 2019).

Em resumo, a peça fundamental para a criação de um ambiente disbiótico e a patogênese da periodontite é uma complexa interação do biofilme ou placa subgengival com a ampla resposta imune do hospedeiro a presença deste biofilme (SCZEPANIK, 2020).

Figura 1 - Patogênese da periodontite



Fonte: Traduzido de WANG *et al.*, 2019.

A deficiência no controle clínico da doença provoca aumento de marcadores inflamatórios e sensibiliza todo o organismo (MACHADO *et al.*, 2020). Assim, a periodontite pode provocar uma resposta inflamatória sistêmica por ativação da resposta de fase aguda hepática, o que tem potencial para representar uma fonte distante de inflamação sistêmica de baixo grau (BRITO *et al.*, 2013).

Portanto, é necessário estudar o impacto da doença (MACHADO *et al.*, 2020) bem como os distúrbios sistêmicos associados à doença periodontal, isto é, as doenças cardiovasculares, diabetes, obesidade, desfechos adversos na gravidez, câncer oral e colorretal, doença de Alzheimer e infecções respiratórias (BUI *et al.*, 2019).

### 3.3 INFLUÊNCIA DA PERIODONTITE NO DESENVOLVIMENTO DA PAV

A relação entre a periodontite e a PAV é biologicamente aceitável, visto que a colonização de bactérias, no ambiente oral, de pacientes com periodontite, facilita a aspiração direta desses patógenos, sendo a infecção continuada por meio de fatores inflamatórios e imunológicos. Nesse sentido, a periodontite contribui para o acúmulo de bactérias e alteração do epitélio pulmonar, favorecendo o desenvolvimento da pneumonia nosocomial (JERÔNIMO *et al.*, 2020).

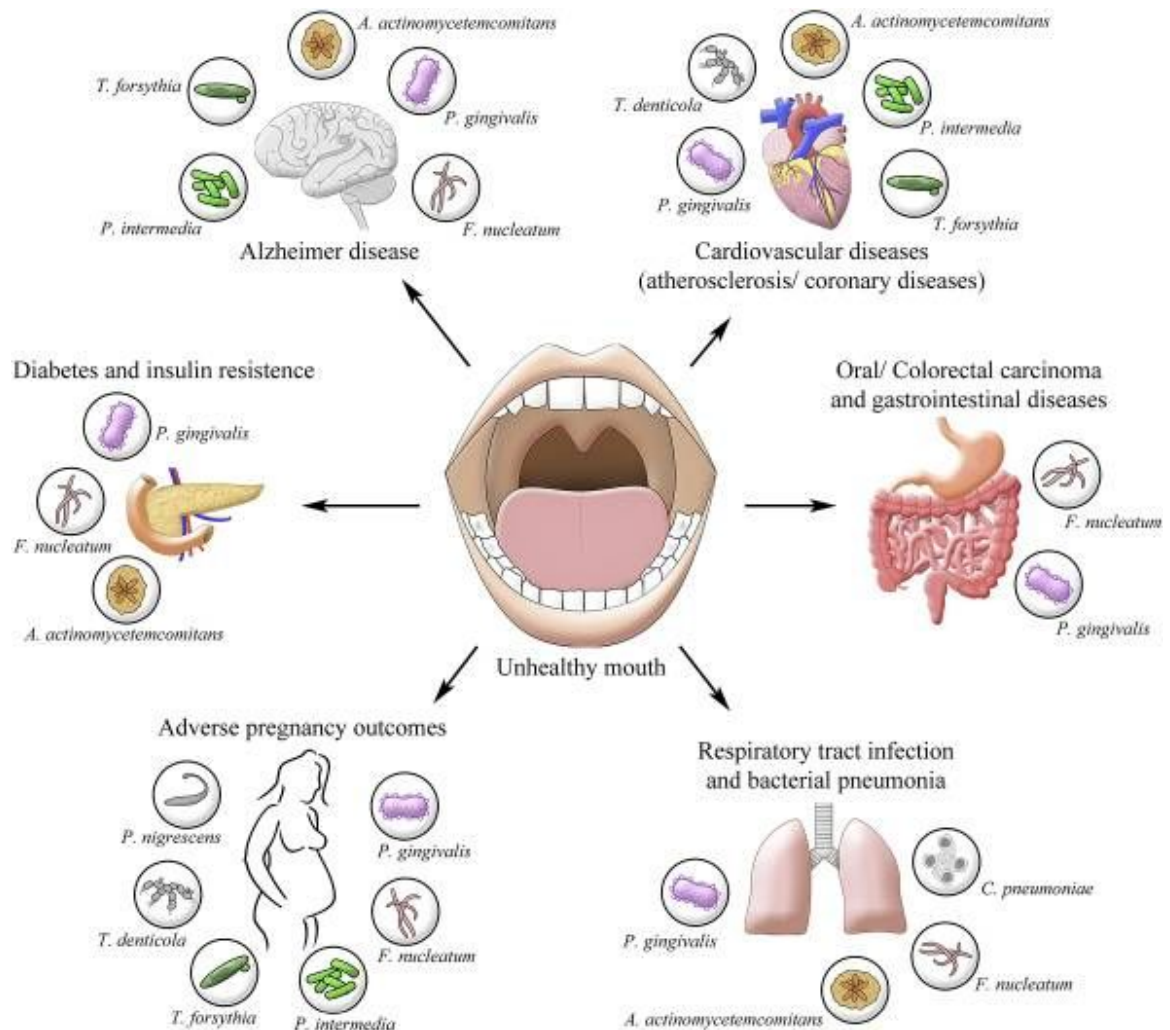
Como citado anteriormente, estudos relataram a inter-relação da doença oral, condição inflamatória e doença sistêmica (Figura 2). A microbiota, presente na cavidade oral, pode induzir um processo inflamatório e contribuir diretamente para a inflamação sistêmica devido à liberação de toxinas ou produtos microbianos na

corrente sanguínea. Considerando as evidências, é fundamental compreender os efeitos nocivos da inflamação oral e a capacidade desses efeitos em aumentar o desenvolvimento de doenças não orais (BUI *et al.*, 2019; WHITMORE; LAMONT, 2014; KIM; AMAR, 2006).

O ambiente oral é alterado devido ao acúmulo de patógenos, acarretando a infecção das vias aéreas por novos patógenos. É comum que pequenas quantidades de secreções da via oral sejam aspiradas continuamente por indivíduos saudáveis, porém, em pacientes com rebaixamento de nível de consciência, a quantidade da secreção aspirada pode aumentar (JERÔNIMO *et al.*, 2020).

Além disso, enzimas do aspirado contribuem para destruir macromoléculas na superfície da mucosa, expondo os receptores e permitindo a adesão e colonização de patógenos respiratórios, ademais, podem destruir a mucina, responsável por limpar as bactérias da superfície da mucosa. Logo, produtos bacterianos e citocinas, como interleucina (IL-1, IL-6, IL-8 e TNF- $\alpha$ ), podem induzir a produção de mais citocinas, resultando no recrutamento de células inflamatórias. O epitélio da mucosa bucal inflamada, torna-se mais suscetível à adesão por patógenos respiratórios (SCANNAPIECO; HO, 2001).

Figura 2 - Representação esquemática da relação de patógenos orais com outras doenças sistêmicas



Fonte: Bui *et al.*, 2019.

A cavidade bucal é uma fonte de reservatório microbiológico para a infecção pulmonar. Alguns estudos mostram evidências que a periodontite está possivelmente associada a um maior risco de desenvolver pneumonia. As citocinas e enzimas produzidas pela inflamação do tecido periodontal podem ser transferidas para os pulmões, onde possuem potencial para iniciar processos inflamatórios locais que irão preceder a colonização de patógenos (CARDOSO; REIS; CESPEDES, 2018).

A disseminação sistêmica de bactérias orais e mediadores pró-inflamatórios (TNF- $\alpha$ , IL-1, IL-6) são capazes de iniciar ou manter mecanismos associados ao desenvolvimento de doenças sistêmicas crônicas (CARDOSO; REIS; CESPEDES, 2018), portanto, são fatores de risco para o desenvolvimento de outras condições sistêmicas.

O estudo de Reis *et al.*, (2014) mostrou que esses mediadores estavam elevados no fluido crevicular das fendas gengivais em pacientes com periodontite,

transformando, assim, a boca em um reservatório para a propagação da infecção respiratória.

A aspiração de conteúdo estranho para as vias aéreas, além das cordas vocais, pode variar entre: secreções, sangue, bactérias, líquidos e partículas de alimentos. Em pacientes gravemente enfermos, esse processo é comum no cenário clínico, sendo que é a composição do aspirado que determina a gravidade e progressão da infecção no parênquima pulmonar (RAGHAVENDRAN *et al.*, 2011).

Pesquisas mostraram evidências da periodontite estar associada ao risco de pneumonia. Possivelmente as bactérias orais presentes na placa dentária são difundidas com a saliva e, logo após, aspiradas para o trato respiratório inferior, causando infecção. Além disso, citocinas e enzimas, produzidas em razão do tecido periodontalmente inflamado, podem ser transportados para os pulmões, estimulando processos inflamatórios, facilitando a colonização de patógenos (PAJU; SCANNAPIECO, 2007; CARDOSO; REIS; CÉSPEDES, 2018).

Os periodontopatógenos são bactérias gram-negativas que predominam no ambiente oral do paciente nas primeiras 48 horas após a admissão hospitalar. A colonização oral por patógenos respiratórios, desencadeada pela higiene oral insuficiente, está fortemente associada ao desenvolvimento de pneumonia nosocomial (PORTO *et al.*, 2016).

Os pacientes internados em UTI apresentam uma quantidade importante de patógenos respiratórios em sua microbiota. Essa relação foi evidenciada por meio de estudo realizado por Souza e colaboradores (2017), através do qual, detectaram a presença de *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* e *Enterobacter cloacae* no biofilme oral de pacientes, bem como identificado e observado que após 48 horas, 25% desses pacientes desenvolveram PAV, tendo a presença desses patógenos sido confirmada por análise do aspirado da secreção traqueal.

Em uma revisão sistemática realizada por Cagnani *et al.* (2016), apurou-se que intervenções relacionadas a higiene bucal, com o objetivo de diminuir a carga microbiana, reduziram o risco de desenvolvimento da PAV. Os indivíduos que não receberam a higienização bucal, tiveram mais chances de desenvolver pneumonia, concluindo-se, portanto, que a higiene oral precária, somada à colonização da



mucosa oral por patógenos respiratórios e periodontite, são fatores que predis põe o desenvolvimento da pneumonia nosocomial.

Hua *et al.* (2016) analisaram dezessete estudos clínicos controlados e randomizados, tendo, em um dos estudos, observado que o uso de clorexidina, como forma de descontaminação, quando comparado ao uso de placebo, associado ou não a remoção mecânica da placa, mostrou efeito positivo na redução da PAV. Os autores encontraram evidências de que o uso de clorexidina, como colutório bucal ou gel, pode reduzir o risco de PAV de 24% para 18%.

Gomes-Filho *et al.* (2014) produziram um estudo tipo caso-controle, por meio do qual realizaram exame periodontal e o diagnóstico de pneumonia nosocomial de acordo com critérios médicos em 315 indivíduos. Os resultados do estudo apontaram que indivíduos com periodontite apresentaram três vezes mais chances de desenvolver pneumonia nosocomial. Também foi observado que em um período de 48 horas ocorrem mudanças importantes na microbiota oral, sendo essa predominantemente Gram-negativa.

Jerônimo *et al.* (2020) mostraram evidências positivas na relação entre periodontite e pneumonia nosocomial, resultados esses que foram obtidos por meio de meta-análise. Sujeitos com periodontite admitidos em UTI foram mais suscetíveis a desenvolver pneumonia nosocomial quando comparados com os sujeitos sem periodontite.

O número de bactérias presentes na boca está relacionado com a condição da higiene oral do paciente. Nos pacientes internados em UTI, a higienização oral é considerada precária, estando esses expostos a outros fatores agravantes como: limitação da movimentação da língua e das bochechas (AMARAL *et al.*, 2009) e redução do fluxo salivar e do pH, devido ao uso de alguns medicamentos, o que contribui para o aumento da colonização oral de patógenos respiratórios (BANSAL; KHATRI; TANEJA, 2013).

Alguns mecanismos foram propostos para explicar o papel das bactérias orais na patogênese da infecção respiratória:

- 1) Os periodontopatógenos podem ser aspirados para o pulmão;
- 2) Enzimas associadas a doença periodontal, presentes na saliva, podem modificar a superfície da mucosa, ajudando a facilitar a adesão e colonização por patógenos respiratórios, para então serem microaspirados;
- 3) As películas salivares nas bactérias podem ser

destruídas, dificultando sua eliminação; 4) Citocinas originadas das superfícies periodontais podem alterar o epitélio respiratório para promover a infecção por patógenos respiratórios (SCANNAPIECO; MYLOTTE, 1996; BANSAL; KHATRI; TANEJA, 2013).

No ambiente hospitalar, a higiene bucal é primordial para evitar colonização de microrganismos no biofilme dentário, eis que a boa higiene bucal reduz a adesão dos periodontopatógenos (BANSAL; KHATRI; TANEJA, 2013). A aplicação de meios para redução da carga microbiana oral está associada a uma menor chance de desenvolver pneumonia (CARDOSO; REIS; CESPEDES, 2018).

A higiene oral torna-se relevante aos pacientes internados em UTI, mesmo que a sua qualidade tende a reduzir durante a hospitalização. Os cuidados orais eficazes estão associados a uma redução de 40% das chances de desenvolver a PAV em pacientes criticamente enfermos (PORTO *et al.*, 2016).

A periodontite e a pneumonia nosocomial exercem relação entre si, pois a primeira pode predispor o aparecimento da segunda em pacientes internados em UTI (GOMES-FILHO *et al.*, 2014). Sendo assim, é possível inferir que o acúmulo de patógenos orais relacionados a doença periodontal é capaz de aumentar o risco de infecção da árvore brônquica, incluindo a pneumonia em indivíduos hospitalizados (SCANNAPIECO; HO, 2001).

A persistência de patógenos microbianos (resposta inflamatória prolongada e condições sistêmicas) resulta no chamado estresse oxidativo, caracterizado pelo aumento de EROs e comprometimento do mecanismo de defesa antioxidante (SÁNCHEZ-VILLAMIL *et al.*, 2020). Dessa forma, o sistema de defesa antioxidante é definido como a presença de substâncias que podem atrasar, prevenir ou remover o dano oxidativo de uma molécula alvo (HALLIWELL; GUTTERIDGE, 2007). As principais enzimas antioxidantes são superóxido dismutase (SOD) e catalase (CAT), sendo a SOD a mais importante do sistema de defesa enzimático (PERRY *et al.*, 2010).

O aumento danoso de EROs ocorre quando há um desequilíbrio entre os níveis de produção de EROs e a diminuição das defesas antioxidantes do hospedeiro, o que resulta em danos no DNA, lipídeos e proteínas. Os neutrófilos, em resposta contra as bactérias, agem liberando as EROs, gerando danos significativos nos tecidos do corpo humano (DURSUN *et al.*, 2016).

As EROs, em concentrações baixas/moderadas, apresentam efeitos benéficos em relação às funções fisiológicas em respostas celulares, como, por exemplo: a defesa contra os agentes infecciosos, sinalização intracelular e indução de resposta mitogênica. Em elevadas concentrações, causam danos biológicos que afetam as estruturas celulares (KOVACIC; JACINTHO, 2001; VALKO *et al.*, 2001; VALKO *et al.*, 2007; RIDNOUR *et al.*, 2005).

Um estudo realizado na Colômbia, com o objetivo de determinar o nível de malondialdeído (MDA) como marcador do estresse oxidativo e atividade antioxidante total na saliva dos participantes com periodontite, mostrou que pacientes com periodontite crônica tinham níveis mais elevados de MDA e capacidade antioxidante total em comparação ao grupo controle saudável. As bactérias mais prevalentes apresentadas no estudo foram *A. actinomycetemcomitans* e *P. gingivalis*, todavia, não foi observado correlação positiva entre a capacidade antioxidante total e os níveis de MDA na presença dessas bactérias (SÁNCHEZ-VILLAMIL *et al.*, 2020).

Evidências crescentes sugerem que as espécies reativas de oxigênio (EROs) estão envolvidas na patogênese e progressão da periodontite. EROS é um termo coletivo para designar radicais de oxigênio e alguns não radicais que são agentes oxidantes ou que podem ser facilmente convertidos em radicais. Baixos níveis de EROS são indispensáveis em vários processos bioquímicos; no entanto, eles podem causar dano tecidual por meio de múltiplos mecanismos, incluindo dano ao DNA, peroxidação lipídica (LPO), dano às proteínas e oxidação enzimática. O estresse oxidativo ocorre quando a defesa antioxidante celular é inadequada para inativar completamente as ROS geradas devido à sua produção excessiva, perda de defesa antioxidante ou ambos (NGUYEN *et al.*, 2016; TRIVEDI *et al.*, 2014).

A LPO é uma das principais consequências do estresse oxidativo e pode ser avaliada através de monitoramento os níveis de MDA, um dos produtos da quebra de longas cadeias de carbono de ácidos graxos. Anteriormente, os níveis de MDA foram avaliados no plasma, soro, fluido crevicular gengival e saliva de pacientes com periodontite. A saliva é uma ferramenta de pesquisa translacional ideal e meio de diagnóstico, sendo usada como um novo meio para estudar biomarcadores para várias doenças e condições orais e sistêmicas. Além disso, a saliva como amostra é um meio de diagnóstico barato, não invasivo, sendo ferramenta acessível na prática clínica (NGUYEN *et al.*, 2016).

### 3.4 ESTRESSE OXIDATIVO

Muito estudado e comentado na literatura, o estresse oxidativo consiste, resumidamente, no desequilíbrio entre os antioxidantes e a produção dos pró-oxidantes. O organismo humano possui um sistema de defesa antioxidante que age para neutralizar os radicais livres, os quais são formados ininterruptamente no metabolismo celular fisiológico, inclusive em condições patológicas e, quando em demasia, podem resultar na oxidação de moléculas biológicas, alterando suas funções (MACHADO *et al.*, 2009).

A simples respiração humana resulta na produção de oxigênio necessário para fornecer energia para todas as células do corpo, processo conhecido como metabolismo oxidativo. A principal organela envolvida nesse processo é a mitocôndria, onde atuam outras enzimas responsáveis por catalisar as demais etapas desse processo. Em cada etapa originam-se os subprodutos, alguns deles são benéficos, porém, em média 5% desses subprodutos podem ser tóxicos para as células quando altamente concentrados (SILVA; JASIULIONIS, 2014).

As mitocôndrias são organelas essenciais, pois atuam na produção de trifosfato de adenosina (ATP) e na produção de EROs. Essas organelas, quando não estão exercendo sua devida função, influenciam na: contratilidade das vias aéreas, expressão gênica, estresse oxidativo, proliferação celular, apoptose e respostas imunes e inflamatórias (WIEGMAN *et al.*, 2020).

O oxigênio é um exemplo dos subprodutos gerados pela mitocôndria, pois o transporte de elétrons que ocorre na mitocôndria pode ser diminuído moderadamente, originando espécies reativas de oxigênio (EROs), como: ânion superóxido ( $O_2^-$ ), peróxido de hidrogênio ( $H_2O_2$ ) e radical hidroxila ( $OH^-$ ) (SILVA; JASIULIONIS, 2014).

As EROs são fundamentais no metabolismo natural, porém sua produção excessiva pode resultar em dano (CHAPPLE, 1997). O resultado do excesso das EROs, pode ser prejudicial para as células, modificando suas funções e estruturas, como: danos nas membranas ou outra estrutura lipídica; modificação das proteínas (alterando sua estrutura e resultando em perda da função); fragmentação e ligação

cruzada e modificações no DNA (que podem ser reparadas ou levar a mutações) (HALLIWELL; GUTTERIDGE, 1999).

Com um papel muito importante no organismo, as EROs auxiliam na eliminação dos agentes patogênicos invasores, sinalização celular, regulação gênica, todavia, se presentes em abundância, também podem se tornar citotóxicas (SCZEPANIK, 2020).

A literatura elenca como espécies reativas: átomos, moléculas ou íons que são derivados do oxigênio, sendo que esses últimos são altamente reativos e são classificados em três classes: espécies reativas de oxigênio (EROs), espécies reativas de enxofre (EREs) e espécies reativas de nitrogênio (ERNs) (MARTELLI; NUNES, 2014).

As espécies reativas podem ainda ser divididas em dois grupos: radicais livres e compostos não radicalares. Os radicais livres são moléculas que possuem um elétron ímpar na sua última camada, para fins de exemplificação, traz-se:  $\text{OH}\cdot$  (íon hidroxila),  $\text{HOH}\cdot$  (íon peroxil),  $\text{O}_2^{-\cdot}$  (ânion superóxido),  $\text{NO}$  (óxido nítrico) e  $\text{O}_2$  (oxigênio molecular) (MARTELLI; NUNES, 2014). Os radicais livres tendem a ser muito reativos e causam reações em cadeia que afetam diversas outras células, danificando várias moléculas (JONES, 2008).

O outro grupo que compõe os radicais livres é denominado compostos não radicalares que, diferentemente do grupo anterior, os radicais não possuem um elétron a mais, sendo, portanto, mais estáveis. Apesar da estabilidade, também podem interagir com outras moléculas, entre elas:  $\text{H}_2\text{O}_2$  (peróxido de hidrogênio) e  $\text{HOCl}$  (ácido hipocloroso) (MARTELLI; NUNES, 2014).

A maior geradora endógena de radicais livres, como já mencionado, é a mitocôndria. Além dela, as células imunes também são fontes geradoras de radicais livres, eis que são produtoras de enzimas, como NADPH Oxidase, produzindo, assim, uma grande quantidade de  $\text{O}_2$ , o qual, por sua vez, age na destruição dos agentes agressores. Já as responsáveis pela produção de óxido nítrico são as: células nervosas, células endoteliais e macrófagos. Para além dessas, esclarece-se que existem produtores exógenos de radicais livres, como: radiação UV, tabagismo, poluentes, dieta calórica, exercício físico intenso, uso de drogas, agrotóxicos (HERRLING; JUNG; FUCHS, 2006; MARTELLI; NUNES, 2014).

Entre os marcadores do estresse oxidativo, tem-se a lipoperoxidação (LPO) que, em síntese, é o processo que ocorre nas membranas das células. As EROs causam danos aos ácidos graxos polinsaturados dos fosfolipídios presentes na membrana celular, desintegrando-as e, com isso, facilitando a entrada das espécies no interior da célula (HALLIWELL; GUTTERIDGE, 1999). O produto final desse processo de degradação dos ácidos graxos é o malondialdeído (MDA), sendo que a mensuração de altos níveis de MDA indica aumento da lipoperoxidação (CHERUBINI *et al.*, 2005; KASHYAP *et al.*, 2005).

As EROs podem causar danos nos tecidos por meio de vários mecanismos como: danos às moléculas de DNA, peroxidação lipídica, por meio da ativação das ciclooxigenases e lipoxigenases, danos proteicos, estimulação de citocinas pró-inflamatórias liberadas por monócitos e macrófagos (CHAPPLE, 1997; WADDINGTON; MOSELEY; EMBERY, 2000).

A membrana celular é a estrutura mais atingida do processo de LPO, embora todos os componentes sejam suscetíveis a ação das EROs, a LPO provoca alterações na estrutura e na permeabilidade das membranas celulares, culminando em perda na troca iônica e liberação do conteúdo das organelas, como enzimas hidrolíticas dos lisossomas, resultando na formação de produtos citotóxicos e, eventualmente, na morte da célula. O radical hidroxila é o mais conhecido da LPO (FERREIRA; MATSUBARA, 1997).

Em contrapartida, a fim de minimizar os danos causados pelos oxidantes e manter a homeostasia das células, tem-se o sistema antioxidante, formado por compostos não enzimáticos adquiridos por meio de alimentação adequada. Entre esses compostos, destaca-se: vitaminas lipossolúveis (vitamina A, vitamina E), vitaminas hidrossolúveis (vitamina C, complexo B) e minerais como zinco, cobre, magnésio. Adicionalmente, o sistema antioxidante também engloba os antioxidantes enzimáticos, os quais agem na destruição ou produção das EROs, sendo exemplos desses antioxidantes: superóxido dismutase ou SOD, catalase ou CAT, glutathiona peroxidase (GPx) (SILVA; JASIULIONIS, 2014).

Abaixo, tabela com resumo da função de cada antioxidante:

Tabela 1 - Antioxidantes enzimáticos e não enzimáticos e suas respectivas funções

Sistema	Função
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<b>Não enzimáticos</b>	
α Tocoferol	Intercepta reação de LPO
Beta-caroteno	Neutraliza $^1O_2$
Licopeno	Neutraliza $^1O_2$ e outros radicais
Ubiquinol 10	Sequestrador de radicais
Ácido ascórbico	Inúmeras funções antioxidantes
Ácido úrico	Sequestrador de radicais
Glutationa	Inúmeras funções antioxidantes
Flavonoides	Sequestrador de radicais
<b>Enzimáticos</b>	
Superóxido dismutase	Reações de dismutação de $O_2^-$
Catalase	Catalisa reação sobre $H_2O_2$
Glutationa peroxidase	Catalisa reação sobre hidroperóxidos
<b>Enzimáticos auxiliares</b>	
Glutationa-S-Transferase	Reação de conjugação e excreção de xenobióticos
Glutationa redutase	Colabora no ciclo de GSH

Fonte: Adaptada de SIES, 1993.

Essas substâncias antioxidantes têm a função de captar e neutralizar os radicais livres e diversas espécies reativas de oxigênio, contribuindo, assim, para a formação de um ambiente benéfico para o organismo, impedindo as lesões teciduais (HALLIWELL; GUTTERIDGE, 1999c).

A periodontite é uma condição que atinge cerca de 10 - 15% da população. É altamente inflamatória devido à presença crônica da placa na superfície do dente, sendo que a persistência dessa placa leva ao recrutamento de neutrófilos polimorfonucleares ao local da inflamação. Tais neutrófilos são elementos-chave nesse evento e atuam de maneira fundamental, pois são a primeira resposta do corpo humano frente a uma ameaça, atuando por meio de mecanismos de defesa como degranulação, quimiotaxia, fagocitose e uma acentuada liberação de espécies reativas de oxigênio (SCZEPANIK, 2020).

A excessiva carga oxidante, juntamente com o sistema antioxidante alterado ou reduzido, resulta no estresse oxidativo nos tecidos afetados. Esse desequilíbrio pode levar a alterações patológicas e, conseqüentemente, à destruição das estruturas de suporte dos dentes e perda dentária. Além disso, os fibroblastos são afetados pelas EROs, o que diminui a produção de colágeno. A liberação em excesso no local da infecção das metaloproteinases resulta na degradação do tecido conjuntivo e da matriz óssea (SCZEPANIK, 2020).

Da mesma maneira, nas infecções do trato respiratório inferior, os neutrófilos são recrutados para agir na destruição de microrganismos por meio da fagocitose. No entanto, essa atividade dos neutrófilos pode se tornar aumentada e resultar em dano do tecido pulmonar devido à liberação de EROs (SILVA, GONÇALVES, 2010) ou seja, a injúria oxidativa pode levar a efeitos danosos direto na integridade das membranas e organelas das células epiteliais que estão expostas nas vias aéreas, levando a uma resposta desse estresse com a liberação de EROs (WIEGMAN, *et al.*, 2020).

As células do epitélio pulmonar também estão vulneráveis aos efeitos oxidativos das EROs, pois essas células são as primeiras a entrar em contato com agentes oxidantes inalados durante a respiração, levando o organismo a desenvolver mecanismos de defesa antioxidante (SILVA, GONÇALVES, 2010).

No aparelho respiratório, os efeitos das EROs podem resultar em destruição direta de células, incluindo pneumócitos tipo I e células endoteliais. As EROs causam destruição da elasticidade do tecido pulmonar, diminuindo sua capacidade de expansão durante a respiração. Além disso, elas podem alterar o tônus da musculatura lisa, resultando no desbalanço entre receptores muscarínicos (responsáveis pela contração) e receptores beta-adrenérgicos (responsáveis pelo relaxamento).

As EROs têm capacidade de alterar ou interferir em vias metabólicas como a cascata de inflamação, onde estão os mediadores químicos, exacerbando a resposta inflamatória. O aumento de EROs pode ocasionar danos na microcirculação regional, resultando no derrame de fluido rico em proteínas no interior dos alvéolos. Esse derrame, aliado à ação das EROs, degrada o surfactante, ocasionando um colapso dos alvéolos e o aumento da resistência pulmonar (SILVA, GONÇALVES, 2010).



Apesar dos danos causados, as EROs são fundamentais para o funcionamento de funções fisiológicas, pois atuam como mecanismo de defesa celular. A ausência das EROs torna os processos que mantêm a vida inviáveis, por isso, é substancial haver equilíbrio entre a produção EROs e o funcionamento do sistema antioxidante (SILVA, GONÇALVES, 2010).

## **4. METODOLOGIA**

### **4.1 COMITÊ DE ÉTICA**

Todas as normativas para a realização de estudos com seres humanos foram seguidas, a pesquisa foi aprovada pelo Comitê de Ética de Pesquisa em Humanos da UNIOESTE, sob parecer 5.340.334.

Foi explicado a todos os acompanhantes/responsáveis pelos pacientes o objetivo e a natureza do estudo, sendo incluídos como participantes do estudo apenas após a concordância e assinatura do termo de consentimento livre e esclarecido (TCLE).

### **4.2 TIPO DE ESTUDO**

Trata-se de um estudo clínico do tipo transversal e observacional com análise quantitativa dos dados, com base clínica e laboratorial, visando a análise dos mecanismos pelos quais a inflamação causada pela periodontite pode levar ao desenvolvimento da PAV em pacientes internados em uma UTI de um hospital da macrorregião de Cascavel.

### **4.3 DELINEAMENTO EXPERIMENTAL**

#### **4.3.1 População amostral**

Os pacientes selecionados foram avaliados com exame clínico extrabucal e intrabucal completos, considerando a condição de internamento, com enfoque no diagnóstico das alterações periodontais. Além do exame clínico, foi realizada a análise dos exames hematológicos, bem como o histórico de saúde, considerando a possível associação com as alterações periodontais avaliadas e correlacionando os dados sistêmicos, sanguíneos e periodontais. O levantamento dos dados de caracterização do paciente (idade, sexo, temperatura corporal, exames e histórico de saúde) foi realizado pelo sistema de informação do hospital e prontuários eletrônicos - Tasy®, no mesmo período da coleta.

O estudo foi realizado no período de abril de 2022 a janeiro de 2023 com pacientes adultos em uso de VM por tubo orotraqueal. Todos os pacientes selecionados deveriam estar dentro do período de até 72 horas de intubação orotraqueal (IOT). Foram avaliados os efeitos dos fatores presença e ausência de periodontite e presença e ausência de PAV. A interação desses fatores estabeleceu 4 grupos para comparação (Sem Perio e sem PAV, Sem Perio e com PAV, Perio sem PAV e Perio com PAV), no entanto, o grupo sem periodontite e com PAV não teve pacientes, formando-se assim, 3 grupos para o estudo:

1. Grupo controle (sem periodontite e sem PAV): grupo de pacientes sem periodontite e que não desenvolveu PAV;
2. Periodontite sem PAV: grupo de pacientes com periodontite e que não desenvolveu PAV;
3. Periodontite com PAV: grupo de pacientes com periodontite e que desenvolveu a PAV.

#### 4.3.2 Critérios de inclusão

Indivíduos acima de 18 anos, internados na UTI de um hospital de referência da macrorregião do oeste do Paraná, estando esses pacientes em ventilação mecânica há 24 a 72 horas da coleta e com, no mínimo, seis dentes. (ALMONDES *et al.*, 2017).

#### 4.3.3 Critérios de exclusão

Pacientes com trauma maxilofacial grave que dificultasse a possibilidade de exame, edentados totais superior e inferior, gestantes, imunossuprimidos, pacientes com complicações clínicas ou cirúrgicas graves, segundo registros em prontuário médico.

#### 4.3.4 Cálculo amostral

O presente cálculo amostral foi realizado *a posteriori*, visto que a amostra foi composta por 53 sujeitos. Assumiu-se para o cálculo uma família t-Student, com um

tamanho de efeito de 0,57 (médio) e nível de significância de 0,05, atingindo um poder de análise de 0,80. O cálculo amostral foi realizado pelo software *G-power*.

#### 4.4 MÉTODO DE COLETA DOS DADOS

##### 4.4.1 Exame clínico periodontal

O exame clínico foi realizado por um examinador previamente treinado em condições ergonômicas e de iluminação, adequadas em relação ao posicionamento e inclinação da sonda milimetrada, bem como em relação à pressão da sondagem (de aproximadamente 25 gramas). O treinamento foi mantido durante a coleta dos dados e, para sua realização, o examinador utilizou um manequim periodontal, posicionado sobre a bandeja da balança digital de precisão AX200 (Shimadzu®, Japão), simulando a sondagem periodontal, aplicando força até atingir o valor de 25 gramas, utilizando uma sonda periodontal milimetrada, modelo Carolina do Norte (Millennium®, São Paulo), com empunhadura em forma de caneta modificada e mantendo a ponta ativa perpendicular ao dente do manequim até atingir 10 repetições (SILVA *et al.*, 2022).

##### 4.4.2 Parâmetros avaliados

Em ordem sequencial e por conveniência foram avaliados:

1. Profundidade clínica de sondagem (PCS): distância da margem gengival (MG) até o fundo do sulco/bolsa com registro de presença ou ausência e mensuração em mm.
2. Sangramento a sondagem (SS): a cada três dentes, com intervalo de, aproximadamente, 30 segundos para registro do parâmetro que corresponde ao tempo de sondagem, com registro de presença ou ausência.
3. Perda clínica de inserção (PCI): distância da junção cimento-esmalte (JCE) até o fundo do sulco/bolsa, com registro de presença ou ausência e mensuração em milímetros. Obtenção a partir da soma entre a recessão gengival e profundidade clínica de sondagem ( $PCI = RG + PCS$ ) – método utilizado em estudo clínico de TRAN *et al.*, 2013.

#### 4.4.3 Grupos dentários e sítios avaliados

Considerando que a população do estudo foi composta por pacientes com possíveis alterações hematológicas, bucais e sistêmicas, que precisavam de exames práticos e minimamente invasivos, foi realizado o exame de boca toda, excluindo terceiros molares. Foram avaliados no mínimo 6 dentes e, em cada dente, três sítios, sendo eles: méso-vestibular (MV), vestibular (V) e disto-vestibular (DV). As faces palatinas e linguais não foram avaliadas, considerando que os pacientes estavam com ventilação mecânica invasiva por tubo orotraqueal, tornando, desta maneira, a avaliação dificultosa e demorada (ALMONDES *et al.*, 2017).

#### 4.4.4 Quantificação do Fluido Crevicular Gengival (FCG)

Foram selecionados três sítios mais profundos, em milímetros (mm), de dentes diferentes, de acordo com exame clínico periodontal, nas faces vestibulares e com inflamação gengival detectada previamente, empregando-se cone de papel absorvente número 40. A placa supragengival foi cuidadosamente removida de acordo com o procedimento operacional padrão (POP) estabelecido na UTI em questão. Após os sítios terem sido isolados com rolos de algodão e secos, os cones de papel foram inseridos abaixo da margem gengival por 30 segundos e, imediatamente, colocados em solução alcoólica de ninidrina a 0,2% (2,2-diidroxihidríndeno-1,3-diona) durante 1 minuto. Em seguida, foram fotografados em uma distância de 10 cm e analisados com software (Image Pro Plus® 4.5.0.29, Média Cybernetics, Silver Spring, MD, USA) para determinação da quantidade de fluido absorvido em milímetros quadrados (mm<sup>2</sup>) (LAGOS *et al.*, 2011).

#### 4.4.5 Exames hematológicos para análise

O resultado dos exames laboratoriais foi coletado do Tasy, no mesmo dia das demais coletas. As alterações dos exames laboratoriais também podem estar associadas ao surgimento de alterações em cavidade bucal, portanto, neste estudo foi realizada a análise dos seguintes parâmetros:

1. Leucócitos
2. Ureia
3. Creatinina
4. Proteína C-Reativa (PCR)

#### 4.4.6 Coleta de saliva

A coleta da saliva foi realizada imediatamente antes do exame clínico periodontal e foi coletada com auxílio do sistema a vácuo por meio de sucção em um tubo de vácuo limpo. Em seguida, o conteúdo foi aspirado com uma seringa e acondicionado em um único *ependorf* estéril. Essa amostra foi utilizada para avaliação do sistema antioxidante (SOD, CAT, GR, GST), bem como de ocorrência de estresse oxidativo. As amostras foram armazenadas e congeladas em freezer a -70°C.

#### 4.4.7 Coleta endotraqueal

A coleta da secreção traqueal foi realizada após o exame clínico periodontal e a aspiração do paciente, por um profissional capacitado para tal procedimento e utilizando dos equipamentos de proteção individual (EPI) recomendados. Foi feita a introdução de uma sonda de calibre adequado conectada ao sistema a vácuo pelo tubo endotraqueal até encontrar resistência. Após a sonda estar com a quantidade ideal de secreção, ela foi clampeada e desconectada do extensor de aspiração. Em seguida, o material coletado foi acondicionado em um único tubo *ependorf* estéril, após, foi congelado em freezer, a -70°C, para posterior análise do sistema antioxidante (SOD, CAT, GR, GST), bem como verificação de estresse oxidativo.

#### 4.4.8 Critérios de diagnóstico para PAV

As notificações para diagnóstico da PAV foram fornecidas por meio de relatório da Comissão de Controle de Infecção Hospitalar (CCIH), o qual foi baseado no caderno “Critérios Diagnósticos de Infecção Relacionada à Assistência à Saúde” da Agência Nacional de Vigilância Sanitária (Anvisa), referente aos anos de 2017

(Agência Nacional de Vigilância Sanitária, 2017) e de 2021 (Agência Nacional de Vigilância Sanitária, 2021).

#### 4.4.9 Preparo das amostras

As amostras de saliva e secreção traqueal foram homogeneizadas em tampão tris-HCl pH 7,4, centrifugadas a 13680 g e 4°C, durante 10 minutos e, posteriormente, foram acondicionadas em ultrafreezer -80°C. No momento das análises, as amostras foram descongeladas, mas mantidas resfriadas durante todos os procedimentos, conforme será descrito mais adiante. A determinação de proteínas seguiu o método de Bradford (1976), utilizando como padrão soro albumina bovina e leitura em comprimento de onda 595 nm.

#### 4.4.10 Sistema antioxidante

A atividade da superóxido dismutase (SOD) foi avaliada conforme o método proposto por Crouch *et al.* (1981) modificado. O princípio desta análise consiste em quantificar o complexo formado entre superóxido e azul de tetrazolium (NBT), mensurado a 600 nm durante 1,5 hora. Uma alíquota de 0,75 mg/mL de proteína em etanol 25% foi preparada em volume de 800 µL e centrifugada a 13680 g (4 °C) durante 20 minutos. A partir do sobrenadante, o meio de reação foi preparado em microplaca de 96 poços. Em triplicatas, em volume final de 200µL contendo 0,1 mg de proteína. mL<sup>-1</sup>, 0,09 mM de NBT, 0,015 mM de EDTA, 34,78 mM de sulfato de hidroxilamina, 79 mM de tampão carbonato de sódio pH 10,2 e a placa lida a 22°C. Uma unidade de SOD em nmol. min<sup>-1</sup>.mg de proteína<sup>-1</sup>.

A atividade da catalase (CAT) foi acompanhada pelo decréscimo da absorbância a 240 nm (AEBI, 1984) em sistema de reação constituído de tampão Tris-HCl 1,0 M, EDTA 5,0 mM, pH 8,0, água desionizada e 180 µl de H<sub>2</sub>O<sub>2</sub> (30%, d=1,1 g.ml<sup>-1</sup>, MM = 34 g.mol<sup>-1</sup>; concentração final no ensaio = 30 mM). A unidade para expressão da atividade da catalase foi mmol de H<sub>2</sub>O<sub>2</sub>. min<sup>-1</sup>. mg de proteína<sup>-1</sup>.

Foi também realizada a avaliação da atividade da enzima glutathione S-transferase (GST) através da metodologia de Keen & Jakoby (1976). A atividade dessa enzima foi mensurada durante 5 minutos, em intervalos de 30 segundos,

avaliando-se o aumento da absorvância devido à formação de um tioéter, a uma absorvância de 340nm. A composição de reação é tampão fosfato de potássio pH 6,5, GSH 1,5MM, CDNB 2mM em 1mL de etanol. A atividade da GST é expressa em  $\mu\text{moles de tioéter. min}^{-1} \cdot \text{mg de proteína}^{-1}$ .

A atividade da glutathione redutase (GR) foi avaliada de acordo com a técnica proposta por Sies *et al.* (1979). O sistema de reação foi constituído de tampão fosfato 100 mmol. L<sup>-1</sup> (pH 7,0) EDTA 1 mmol. L<sup>-1</sup>, GSSG 0,66 mmol. L<sup>-1</sup>, NADPH 0,075 mmol.L<sup>-1</sup>. A reação foi iniciada pela adição de GSSG e acompanhada durante 5 minutos a 340 nm. Os resultados foram expressos em NADPH oxidado.  $\text{min}^{-1} \cdot \text{mg de proteína}^{-1}$ .

#### 4.4.11 Estresse oxidativo

O estresse oxidativo foi avaliado por meio da mensuração da peroxidação lipídica, analisando-se o produto da reação de ácido tiobarbitúrico com o malondialdeído, sendo mensurado em espectrofotômetro a 535 nm e expresso em nmol de MDA.mg<sup>-1</sup> de proteína (BUEGE; AUST, 1978).

#### 4.4.12. Análises estatísticas

Em uma primeira etapa de análises, as variáveis quantitativas referentes às análises clínicas foram avaliadas quanto a normalidade (Teste de Shapiro-Wilk) e homocedasticidade (Teste de Bartlett), considerando os grupos Controle (sem Periodontite e sem PAV), Periodontite e Periodontite+PAV. Visto que os dados não se encontravam em acordo com tais pressupostos, as variáveis foram comparadas entre os grupos por meio do teste não paramétrico de Kruskal-Wallis, seguido do teste de acompanhamento de Dunn. As variáveis qualitativas foram comparadas entre os grupos por meio do teste de Qui Quadrado para Independência, seguido do teste de Resíduos Ajustados.

Em seguida foram realizadas as avaliações de normalidade e homoscedasticidade dos dados das análises bioquímicas da secreção traqueal e da saliva, considerando pacientes com e sem periodontite. Uma vez que os dados não se encontravam em acordo com tais pressupostos, as variáveis foram comparadas



entre os grupos (Normal – sem periodontite, e Periodontite) por meio do teste não paramétrico de Mann-Whitney-U. O mesmo procedimento foi realizado, porém, considerando apenas os pacientes com periodontite e comparando-os entre os pacientes que desenvolveram PAV e os que não desenvolveram (normal).

Por fim, as matrizes de variáveis da secreção traqueal e da saliva dos grupos Controle (sem Periodontite e sem PAV), Periodontite e Periodontite+PAV foram separadas, padronizadas pelo escore z e submetidas a análise multivariada de componentes principais (PCA). As cargas fatoriais dos três primeiros componentes principais foram comparadas entre os grupos por meio da Análise da Variância Fator Único, seguido do teste de Tukey-HSD, visto que os dados se encontravam em normalidade e homocedasticidade.

Todas as análises realizadas assumiram um nível de significância de 0,05, sendo realizadas no programa R (R Core Team, 2023).

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Artigo científico de acordo com as normas da revista (Anexo B).

## INFLAMMATINON

### **Occurrence of oxidative stress resulting from periodontal inflammation in the development of pneumonia associated with mechanical ventilation: clinical, observational, cross-sectional study.**

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### ABSTRACT

**Objective:** The objective of this study was to evaluate the occurrence of oxidative stress resulting from periodontal inflammation in the development of VAP in patients admitted to the Intensive Care Unit (ICU). **Method:** Clinical, cross-sectional and observational study. Subjects were assessed for the presence or absence of periodontitis and VAP. 117 patients were evaluated, however, 53 patients were included in the study. Divided into 3 groups: Control (n=17), periodontitis and without VAP (n=30), Periodontitis and with VAP (n=6). Clinical periodontal examination, collection and quantification of gingival crevicular fluid, collection of saliva and tracheal secretion for analysis of the antioxidant system and oxidative stress, hematological tests and other patient data were performed. **Results:** in the clinical periodontal examination, patients with periodontitis, but without VAP, presented significantly higher values. Hematological tests, urea and creatinine showed significant differences. The variables related to the antioxidant system and oxidative stress, in saliva, were different in the group of control patients and in the group with periodontitis, the SOD enzyme was inhibited and LPO was increased. And in the saliva of the groups of patients with periodontitis, there was an increase in SOD, CAT and GR in the Periodontitis + VAP group. In the integrative analysis of saliva, Dimension 3 (GST), there was a difference in the group with Periodontitis+VAP. In the analysis of tracheal secretion in patients with Periodontitis, GR and GST showed significant induction and inhibition. **Conclusion:** The increase in oxidative stress caused by the inflammation of periodontitis can alter the antioxidant status, facilitating the development of VAP.

**Keywords:** periodontitis; pneumonia associated with mechanical ventilation; oxidative stress; intensive care unit

## INTRODUCTION

Pneumonia is responsible for affecting the lung parenchyma and can be caused by bacteria, mycoplasmas, fungi, parasites and viruses, with the bacterial cause being the most common [1]. Ventilator-associated pneumonia (VAP) is responsible for inflammation of the lung parenchyma, occurring 48 to 72 hours after orotracheal intubation and the start of mechanical ventilation [2, 3]. These patients are under mechanical ventilation through a tracheal tube, undergoing tracheostomy, or may be in the process of disconnecting from the ventilator in the 48 hours prior to the onset of symptoms [3].

The high risk of developing VAP is due to the presence of the orotracheal tube, altered levels of consciousness, dry and open mouth, changes in the oropharyngeal flora, oral inflammation, impaired airway self-cleaning mechanism, inhibition of the cough reflex and microaspiration of secretions, allowing the oral cavity to be colonized by pathogenic bacteria [4]. Additionally, individual factors, such as age over 70 years, malnutrition, underlying diseases, depressed level of consciousness, tracheal intubation and reintubation, immunological status, use of immunosuppressive drugs, severity of the disease, aspiration of contaminated secretions, position of the patient in bed and insufficient elevation of the headboard, prolonged time on mechanical ventilation, also contribute to the development of VAP [5, 6].

The orotracheal tube provides an inert, non-scaling surface to which bacteria adhere and grow to form biofilms, from which the bacteria are shed and aspirated into the lower airway. Furthermore, the orotracheal tube induces mechanical abrasion, irritation of the respiratory mucosa, impairment of normal laryngeal function, and increased sedation – all leading to an increased risk of aspiration of secretions from the upper respiratory tract [7].

Raghavendram et al. (2007) also mention that the bacterial microbiota and/or gastric contents may also be important in the pathogenesis of postoperative pneumonia. Although ventilators alone are not considered a major source of bacterial dissemination, respiratory circuits can become heavily contaminated with microorganisms from the oropharynx and trachea [7].

The link between dental plaque/biofilm and VAP has also been studied. The incidence of respiratory tract infections in patients requiring an orotracheal tube is substantial, and the risk of acquiring VAP increases by 1-5% per day of hospital stay [7]. Studies have demonstrated the association between periodontitis and VAP, which is biologically realistic, as bacterial proliferation in individuals with periodontitis can result in colonization of the oropharynx, which facilitates direct aspiration of pathogens and sustains infection, mediated by inflammatory factors and immunological [8, 9, 10, 11].

Saliva is an important physiological component, useful for the diagnosis and monitoring of many oral and systemic pathological conditions. Oxidative stress (OS) markers in saliva have been shown to be a local indicator of the inflammation process, the progression of periodontitis and the amount of periodontopathic bacteria in periodontal pockets [12]. Furthermore, enzyme-laden saliva associated with periodontal disease can modify mucosal surfaces to promote adhesion and colonization by respiratory pathogens. Cytokines originating from periodontal tissues can promote inflammation of the lower airways after aspiration and thus alter the epithelium to promote infection by respiratory pathogens [7].

Oxidative stress is caused by the imbalance between the production of reactive oxygen species and the activity of local endogenous antioxidants [12]. A study by Brock et al. (2004) [13] assessed total antioxidant capacity as an indicator of tissue response in periodontitis and reported its decrease in the saliva of individuals with periodontitis, while SÁNCHEZ-VILLAMIL et al., (2020) [12] observed a greater capacity total salivary antioxidant.

In view of the above, it is believed that the imbalance in the activity of the antioxidant system in patients with periodontitis increases the risk of oxidative stress, thus facilitating the development of VAP.

## OBJECTIVE

The objective of this study is to evaluate the antioxidant system and the occurrence of oxidative stress in development of VAP in patients with and without periodontitis admitted to an ICU.

## METHODOLOGY

## **Type of Study**

This is a cross-sectional and observational clinical study, with quantitative analysis of data, including clinical and laboratory basis, aiming to assess the mechanisms by which inflammation caused by periodontitis can lead to the development of VAP in patients admitted to an ICU at a hospital in the macro-region of the city of Cascavel, state of Paraná, Brazil.

## **EXPERIMENTAL DESIGN**

### **Sample population**

The selected patients were evaluated with a complete extraoral and intraoral clinical examination, considering their hospitalization condition, with a focus on diagnosing periodontal changes. In addition to the clinical examination, analysis of hematological tests was carried out, as well as health history, considering the possible association with the periodontal changes evaluated and allowing the correlation of systemic, blood, and periodontal data. The collection of patient characterization data (age, sex, body temperature, exams, and health history) was carried out using the hospital's information system and electronic medical records – Tasy®.

The study was conducted from April 2022 to January 2023 with adult patients using MV for all orotracheal disorders. All patients selected for data collection must be within a period of up to 72 hours of orotracheal intubation (OTI). The effects of the factors presence and absence of periodontitis and presence and absence of VAP were evaluated. The interaction of these factors established 4 groups for comparison (Without Perio and without VAP, Without Perio and with VAP, Perio without VAP, and Perio with VAP); however, the group without periodontitis and with VAP had no patients, thereby resulting in a total of 3 groups for the study:

1. Control group (without periodontitis and without VAP): group of patients without periodontitis and who did not develop VAP;
2. Periodontitis without VAP: group of patients with periodontitis who did not develop VAP;
3. Periodontitis with VAP: group of patients with periodontitis who developed VAP.

### **Inclusion criteria**

Individuals over 18 years old, admitted into the ICU of a reference hospital in the macro-region of western Paraná and patients on mechanical ventilation for a period of 24 to 72 hours and presenting at least six teeth.

### **Exclusion criteria**

Patients with severe maxillofacial trauma, which made the possibility of examination difficult, using complete dentures (upper and lower), pregnant women, immunosuppressed patients, patients using any anti-inflammatory and antibiotic for more than three days, and patients with serious clinical or surgical complications.

### **Sample calculation**

This sample calculation was carried out a posteriori, as we obtained a sample consisting of 53 subjects. We assumed a t-Student family for the calculation, with an effect size of 0.57 (medium) and a significance level of 0.05, reaching an analysis power of 0.80. The sample calculation was performed using the G-power software.

## **DATA COLLECTION METHOD**

### **Clinical periodontal examination**

The clinical examination was carried out by a previously trained examiner under appropriate ergonomic and lighting conditions in relation to the positioning and inclination of the millimeter probe, as well as in relation to the probe pressure (approximately 25 grams). Training was maintained during data collection and, to carry it out, the examiner used a periodontal mannequin, positioned on the tray of the AX200 precision digital scale (Shimadzu®, Japan), simulating periodontal probing, applying force until reaching the value of 25 grams, using a millimeter periodontal probe, North Carolina model (Millennium®, São Paulo), with a modified pen-shaped handle and keeping the active tip perpendicular to the manikin's tooth until 10 repetitions are reached [14].

### Parameters evaluated

In sequential order and for convenience, the following were evaluated:

1. Clinical probing depth (CPD): distance from the gingival margin (GM) to the bottom of the sulcus/pocket with presence or absence recorded and measured in mm.
2. Bleeding on probing (BOP): every three teeth, with an interval of approximately 30 seconds, to record the parameter that corresponds to the probing time, with registration of presence or absence.
3. Clinical attachment loss (CAL): distance from the cemento-enamel junction (CEJ) to the bottom of the sulcus/pocket, with presence or absence recorded and measured in millimeters. Obtained from the sum of gingival recession and clinical probing depth (CAL = GR + CPD) – method used in a clinical study [15].

### Dental groups and sites evaluated

Considering that the study population were patients with possible hematological, oral and systemic alterations, who needed practical and minimally invasive examinations, the mouth examination was carried out, excluding third molars. At least 6 teeth were evaluated, with three sites evaluated for each tooth: mesio-buccal (MB), buccal (B) and disto-buccal (DB). The palatal and lingual surfaces were not evaluated, considering that the patients were on invasive mechanical ventilation via an orotracheal tube, thereby making the evaluation difficult and time-consuming [16].

### Hematological tests for analysis

The results of the laboratory tests were collected on the same day as the other collections. Changes in laboratory tests may also be associated with the emergence of changes in the oral cavity. Therefore, this study analyzed the following parameters:

1. Leukocytes
2. Urea
3. Creatinine
4. C-Reactive Protein (CRP)

### Quantification of Gingival Crevicular Fluid (GCF)

Three deeper sites, in millimeters (mm), were selected in different teeth, according to clinical periodontal examination, on the buccal surfaces and with previously detected gingival inflammation, using a #40 absorbent paper cone. The supragingival plaque was carefully removed in accordance with the standard operating procedure (SOP) established in the ICU in question. After the sites were isolated with cotton rolls and dried, the paper cones were then inserted below the gingival margin for 30 seconds and immediately placed in an alcoholic solution of 0.2% ninhydrin (2,2-dihydroxy -hydridene-1,3-dione) for 1 minute. They were then photographed from a distance of 10 cm and analyzed with software (Image Pro Plus® 4.5.0.29, Media Cybernetics, Silver Spring, MD, United States) to determine the amount of fluid absorbed in square millimeters (mm<sup>2</sup>) [17].

### Saliva collection

Saliva was collected immediately prior to the periodontal clinical examination, using the vacuum system by suction in a clean vacuum tube. Subsequently, the contents were aspirated with a syringe and placed in a single sterile Eppendorf tube. This sample was used to evaluate the antioxidant system (SOD, CAT, GR, GST), as well as the occurrence of oxidative stress. The samples were stored and frozen in a freezer at -70°C.

### Endotracheal collection

The collection of tracheal secretion was carried out after the periodontal clinical examination and after the patient's aspiration, by a professional trained for this procedure and using recommended personal protective equipment (PPE). A probe with an appropriate diameter was introduced, connected to the vacuum system via the endotracheal tube, until resistance was encountered. Once the probe reached the ideal amount of secretion, it was clamped and disconnected from the suction extender. Subsequently, the collected material was placed in a single sterile Eppendorf tube and frozen in a freezer at -70°C, for subsequent analysis of the antioxidant system (SOD, CAT, GR, GST), as well as oxidative stress.

### Diagnostic criteria for VAP

Notifications for the diagnosis of VAP were provided through a report from the Hospital Infection Control Commission (*Comissão de Controle de Infecção Hospitalar – CCIH*), based on the book *Crerios Diagnsticos de Infecção Relacionada à Assistncia à Saude* (“Diagnostic Criteria for HealthCare-Related Infection”) from the Brazilian Health Regulatory Agency (*Agncia Nacional de Vigilncia Sanitria – ANVISA*), referring to the year 2017 (*Agncia Nacional de Vigilncia Sanitria, 2017*) and 2021 (*Agncia Nacional de Vigilncia Sanitria, 2021*) [18, 19].

### Sample preparation

Saliva and tracheal secretion samples were homogenized in Tris-HCl buffer pH 7.4, centrifuged at 13,680 g and 4°C for 10 minutes and then placed in an ultra-low temperature freezer at -80°C. At the time of analysis, the samples were thawed, but kept cold during all procedures, as described below. Protein determination followed the Bradford method (1976), using bovine serum albumin as a standard, with reading at a wavelength of 595 nm [20].

### Antioxidant system

Superoxide dismutase (SOD) activity was evaluated according to the modified method proposed by Crouch et al., (1981) [21]. The principle of this analysis consists of quantifying the complex formed between superoxide and tetrazolium blue (NBT), measured at 600 nm for 1.5 hours. An aliquot of 0.75 mg/mL of protein in 25% ethanol was prepared in a volume of 800  $\mu$ L and centrifuged at 13680 g (4°C) for 20 minutes. From the supernatant, the reaction medium was prepared in a 96-well microplate. In triplicates, in a final volume of 200  $\mu$ L containing 0.1 mg of protein.  $\text{mL}^{-1}$ , 0.09 mM NBT, 0.015 mM EDTA, 34.78 mM hydroxylamine sulfate, 79 mM sodium carbonate buffer pH 10.2 and the plate read at 22°C. One unit of SOD in  $\text{nmol min}^{-1}.\text{mg protein}^{-1}$ .

Catalase (CAT) activity was accompanied by a decrease in absorbance at 240 nm [22] in a reaction system consisting of 1.0 M Tris-HCl buffer, 5.0 mM EDTA, pH 8.0, deionized water and 180  $\mu$ L of H<sub>2</sub>O<sub>2</sub> (30%,  $d=1.1 \text{ g.mL}^{-1}$ ,  $\text{MM} = 34 \text{ g.mol}^{-1}$ ; final concentration in the assay = 30 mM). The unit for expression of catalase activity was  $\text{mmol of H}_2\text{O}_2 \text{ min}^{-1} \text{ mg of protein}^{-1}$ .

The activity of the enzyme glutathione S-transferase (GST) was also evaluated using the methodology of Keen & Jakoby (1976) [23]. The activity of this enzyme was measured for 5 minutes, at intervals of 30 seconds, with an assessment of the increase in absorbance due to the formation of a thioether, at an absorbance of 340 nm. The reaction composition is potassium phosphate buffer pH 6.5, 1.5MM GSH, 2mM CDNB in 1 mL of ethanol. GST activity is expressed in  $\mu\text{moles of thioether min}^{-1} \text{ mg of protein}^{-1}$ .

Glutathione reductase (GR) activity was evaluated according to the technique proposed by Sies et al., (1979) [24]. The reaction system consisted of phosphate buffer 100  $\text{mmol L}^{-1}$  (pH 7.0), EDTA 1  $\text{mmol L}^{-1}$ , GSSG 0.66  $\text{mmol L}^{-1}$ , NADPH 0.075  $\text{mmol L}^{-1}$ . The reaction will be initiated by the addition of GSSG and monitored for 5 minutes at 340 nm. The results will be expressed as NADPH oxidized  $\text{min}^{-1} \text{ mg protein}^{-1}$ .

### Oxidative stress

Oxidative stress was assessed by measuring lipid peroxidation, analyzing the product of the reaction of thiobarbituric acid with malondialdehyde, measured in a spectrophotometer at 535 nm and expressed in  $\text{nmol of MDA.mg}^{-1} \text{ of protein}$  [25].

### Statistical analysis

In the first stage of analysis, the quantitative variables referring to clinical analysis were evaluated for normality (Shapiro-Wilk Test) and homoscedasticity (Bartlett Test), considering the Control groups (without Periodontitis and without VAP), Periodontitis, and Periodontitis + PAV. Since the data failed to agree with these assumptions, the variables were compared between the groups using the Kruskal-Wallis non-parametric test, followed by Dunn’s follow-up test. Qualitative variables were compared between groups using the Chi Square test for Independence, followed by the Adjusted Residuals test.

Next, assessments of normality and homoscedasticity of data from biochemical analysis of tracheal secretion and saliva were carried out, considering patients with and without periodontitis. Since the data failed to

agree with these assumptions, the variables were compared between the groups (Normal – without periodontitis, and Periodontitis) using the non-parametric Mann-Whitney-U test. Afterwards, the same procedure was performed, considering, however, only patients with periodontitis and comparing them between patients who developed VAP and those who did not (normal).

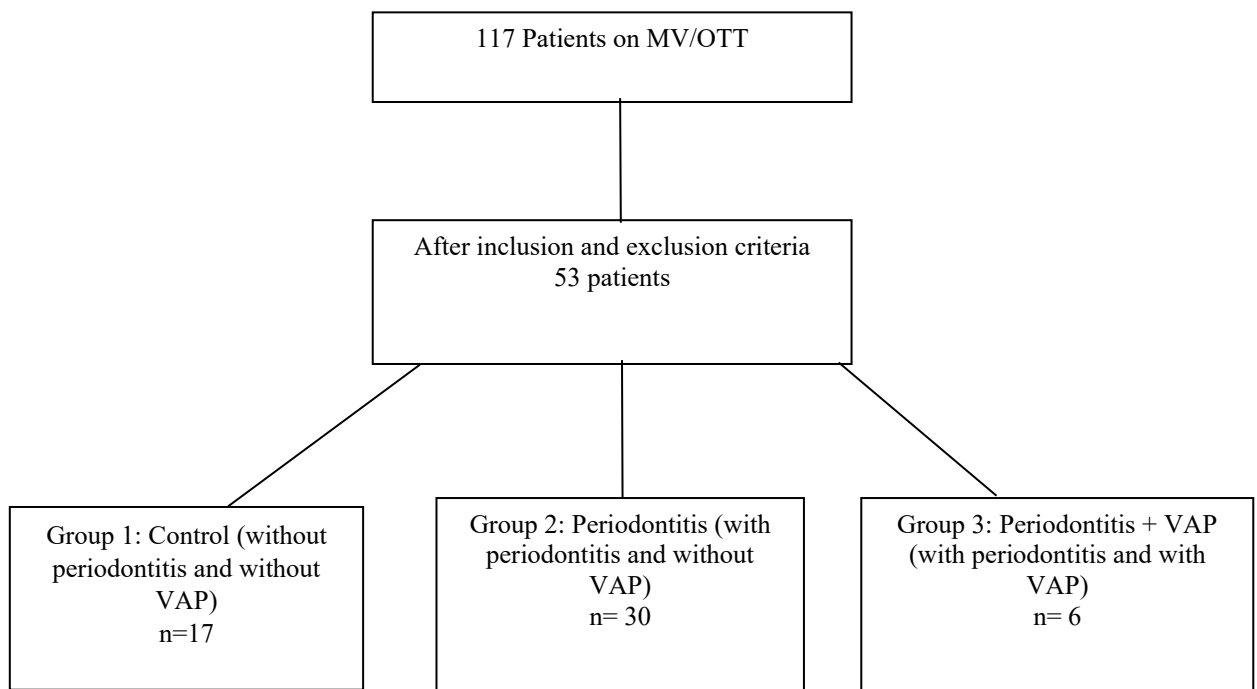
Finally, the matrices of tracheal secretion and saliva variables from the Control (without Periodontitis and without VAP), Periodontitis and Periodontitis + VAP groups were separated, standardized by the Z-score and subjected to multivariate principal component analysis (PCA). The factor loadings of the first three main components were compared between the groups using Single Factor Variance Analysis, followed by the Tukey-HSD test, as the data were within normality and homoscedasticity.

All analyses carried out assumed a level of significance of 0.05, being carried out in the R program (R Core Team, 2023).

## RESULTS

A total of 117 patients on mechanical ventilation via orotracheal tube (MV/OTT) were evaluated during the period from April 2022 to January 2023 and after applying the inclusion and exclusion criteria, with 53 patients were included in the study (Figure 1), and excluded patients being for reasons relating to: edentulism, being hemodynamically unstable during the examination period, or being underage.

Given that no patient was included in the group without periodontitis and with VAP, patients were divided into 3 groups, with a total of 23 women and 30 men with a mean age of 49.8 years, with no differences between the mean ages. between the 3 groups (Table 1).



**Fig 1** Selection and distribution of patients according to groups

**Table 1** Absolute frequencies and percentages (in parentheses) for sex and medians and interquartile ranges for age of patients in the groups. P-value of the Kruskal-Wallis test

Variable	Control Group (n= 17)	Periodontitis Group (n=30)	Perio + VAP Group (n=6)	p-value
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<b>Sex</b>	Female	9 (52.94%)	11 (36.67%)	3 (50.0%)	0,5640
	Male	8 (47.06%)	19 (63.33%)	3 (50.0%)	
<b>Age</b>		53,0 [43.0 – 64.0]	49.0 [37.0 – 63.8]	37.5 [25.3 – 52.0]	0.3511

Regarding clinical variables, it was possible to verify significant statistical differences in CPD, CAL, and BOP ( $p < 0.05$ ). The other variables did not show significant statistical differences between the groups ( $p > 0.05$ ) (Table 2).

Regarding CPD, CAL and BOP, it was found that the group of patients with periodontitis, but without VAP, presented significantly higher values than the Control group, but similar to the group with periodontitis and VAP ( $p = 0.0004$ ;  $p = 0.0003$ ;  $p = 0.0072$ ) (Table 2).

**Table 2** Medians and interquartile ranges for clinical parameters between the groups studied. Cascavel, 2022

<b>Variables</b>	<b>Control (n=17)</b>	<b>Periodontitis (n=30)</b>	<b>Periodontitis + VAP (n=6)</b>	<b>p-value</b>
CPD	2.1 <sup>b</sup> [2.0 - 2.3]	2.6 <sup>a</sup> [2.3 - 2.8]	2.2 <sup>ab</sup> [2.1 - 2.3]	<b>0.0004</b>
CAL	2.2 <sup>b</sup> [2.0 - 2.3]	2.7 <sup>a</sup> [2.4 - 3.6]	2.3 <sup>ab</sup> [2.1 - 2.5]	<b>0.0003</b>
BOP	0.0 <sup>b</sup> [0.0 - 2.6]	6.1 <sup>a</sup> [1.8 - 16.3]	7.1 <sup>ab</sup> [0.4 - 15.6]	<b>0.0072</b>
GCF Quantification	229.3 [129.5 - 342.9]	268.1 [200.2 - 431.9]	620.3 [239.9 - 918.2]	0.2524
Temperature	36.3 [36.0 - 36.7]	36.8 [36.0 - 37.2]	36.7 [36.0 - 37.1]	0.4491

<sup>a,b,ab</sup> different letters indicate statistical differences between groups. P-value obtained using the Kruskal-Wallis test.

Regarding hematological tests, it was possible to verify significant statistical differences only in creatinine and urea ( $p < 0.05$ ) ( $p = 0.036$ ;  $p = 0.033$ ). The other variables did not present significant statistical differences between the groups ( $p > 0.05$ ), making it possible to verify that the values were higher in the Control group (without periodontitis and without VAP), intermediate in the Periodontitis group and reduced in the group with Periodontitis and with VAP (Table 3).

**Table 3** Medians and interquartile ranges of the hematological tests of the groups studied. Cascavel, 2022

<b>Variables</b>	<b>Control (n=17)</b>	<b>Periodontitis without VAP (n=30)</b>	<b>Periodontitis with VAP (n=6)</b>	<b>p-value</b>
Creatinine	1.4 <sup>a</sup> [1.1 - 3.9]	1.1 <sup>ab</sup> [0.8 - 1.5]	0.8 <sup>b</sup> [0.6 - 1.2]	<b>0.0360</b>
Urea	65.0 <sup>a</sup> [43.0 - 93.0]	39.0 <sup>ab</sup> [27.0 - 65.5]	27.0 <sup>b</sup> [15.3 - 37.3]	<b>0.0330</b>
Leukocytes	15300 [12800 – 19700]	12700 [8975 – 17200]	10870 [8510 – 19950]	0.1773
CRP	22.0 [8.2 - 31.3]	14.5 [6.6 - 21.3]	27.2 [22.4 - 32.7]	0.2385

<sup>a,b,ab</sup> Different letters indicate statistical differences between groups. P-value obtained using the Kruskal-Wallis test.

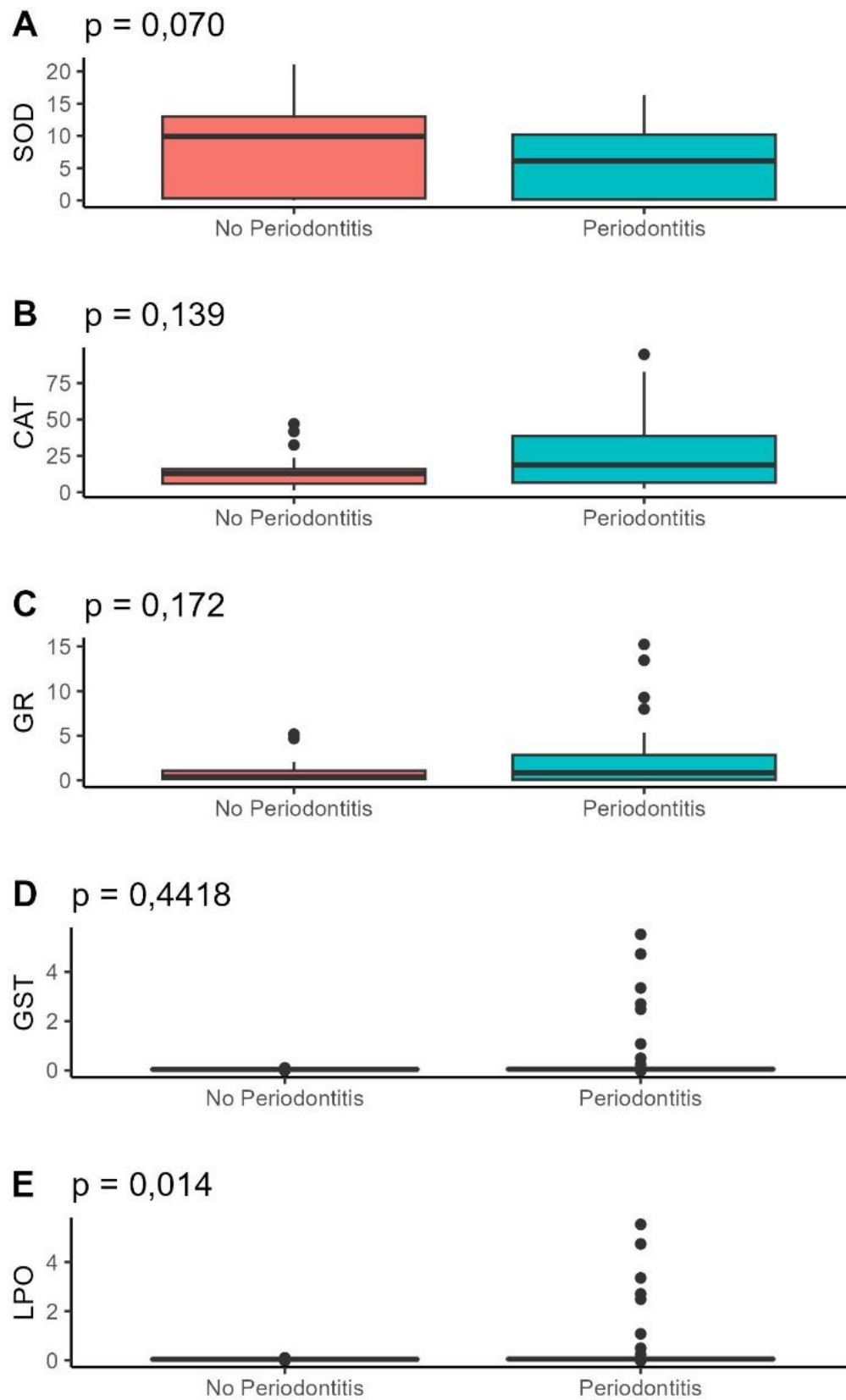
When evaluating the variables related to the antioxidant system and oxidative stress in the saliva of patients without and with Periodontitis, it was possible to see a tendency towards inhibition of SOD activity ( $p=0.070$ ) and an increase in oxidative stress expressed by an increase in the lipoperoxidation reaction ( $p=0.014$ ; Figures 2a and 6e).

When considering only patients with Periodontitis, it was possible to observe that there was a significant induction of the activity of the enzymes SOD, CAT and GR ( $p=0.022$ ,  $p=0.048$ ,  $p=0.060$ , respectively; Figures 3A-C), in patients with Periodontitis-Associated VAP, which possibly led to a reduction in the lipoperoxidation reaction ( $p=0.034$ ; Figure 3e).

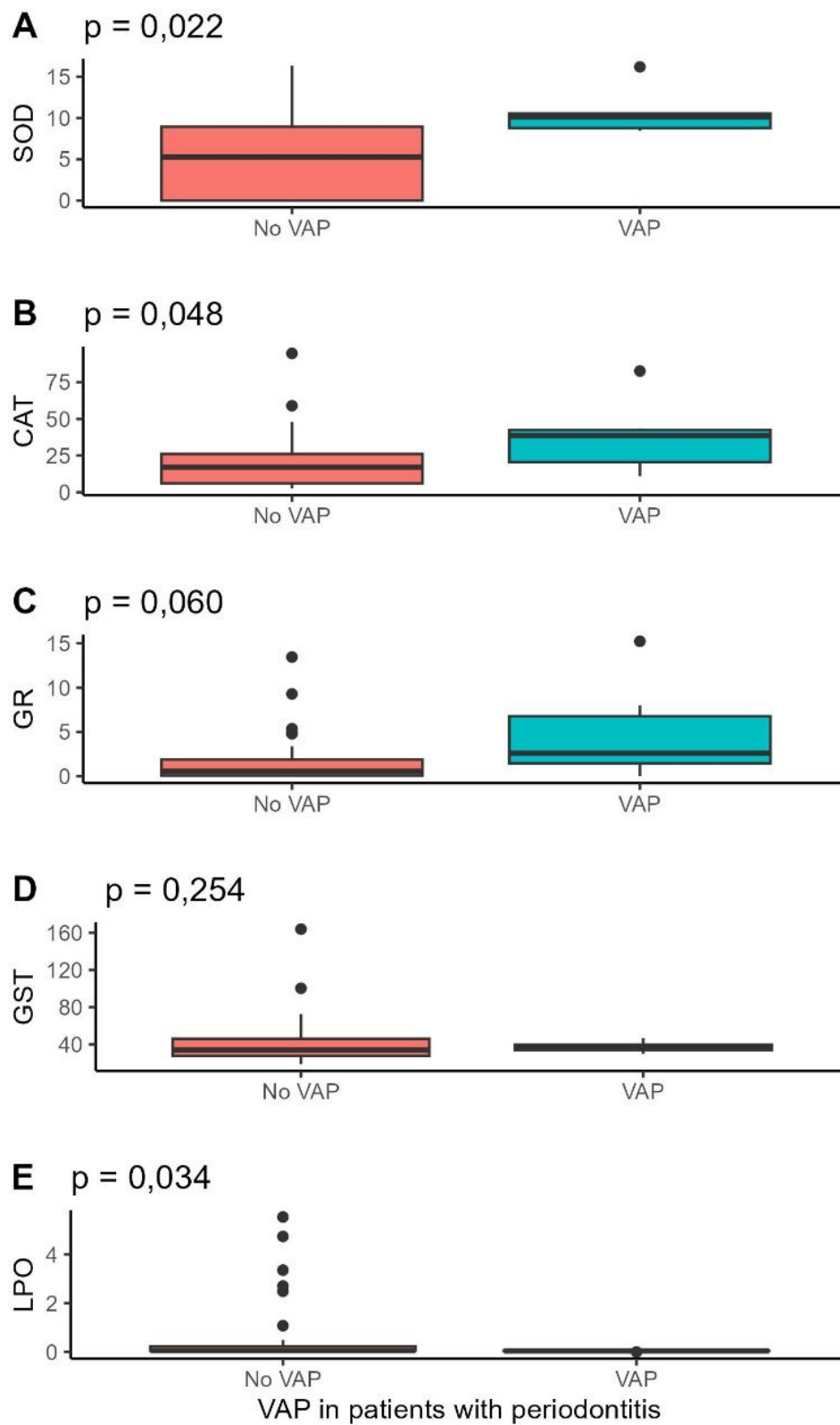
Since systems in an organism act in an integrated manner, it is necessary to carry out a correlational assessment between the variables analyzed through Principal Component Analysis (PCA). In the integrative analysis of saliva (Figure 4), the first principal component (Dimension 1 – Dim.1) presented the greatest contribution associated with the activity of LPO, SOD, and GR (Eigenvalue = 1.64; Variability = 32.78%). The second main component (Dimension 2 – Dim.2) presented a greater contribution from the CAT enzyme (Eigenvalue = 1.04; Variability = 20.87%). The third main component (Dimension 3 – Dim.3) presented a greater contribution from the GST enzyme (Eigenvalue = 0.94; Variability = 18.70%).

When comparing the factor loadings of the dimensions, Dimension 1 did not show a statistically significant difference between the groups ( $p > 0.05$ ). Dimension 2 (CAT) showed a statistically significant difference between the groups ( $F=3.43$ ;  $p=0.040$ ), with the group with Periodontitis + VAP showing significantly higher positive scores when compared to the Control group, which represents the induction of CAT activity ( $p<0.05$ ). The group with Periodontitis presented intermediate values, being statistically equivalent to both the group with Periodontitis + VAP and the Control group ( $p>0.05$ ).

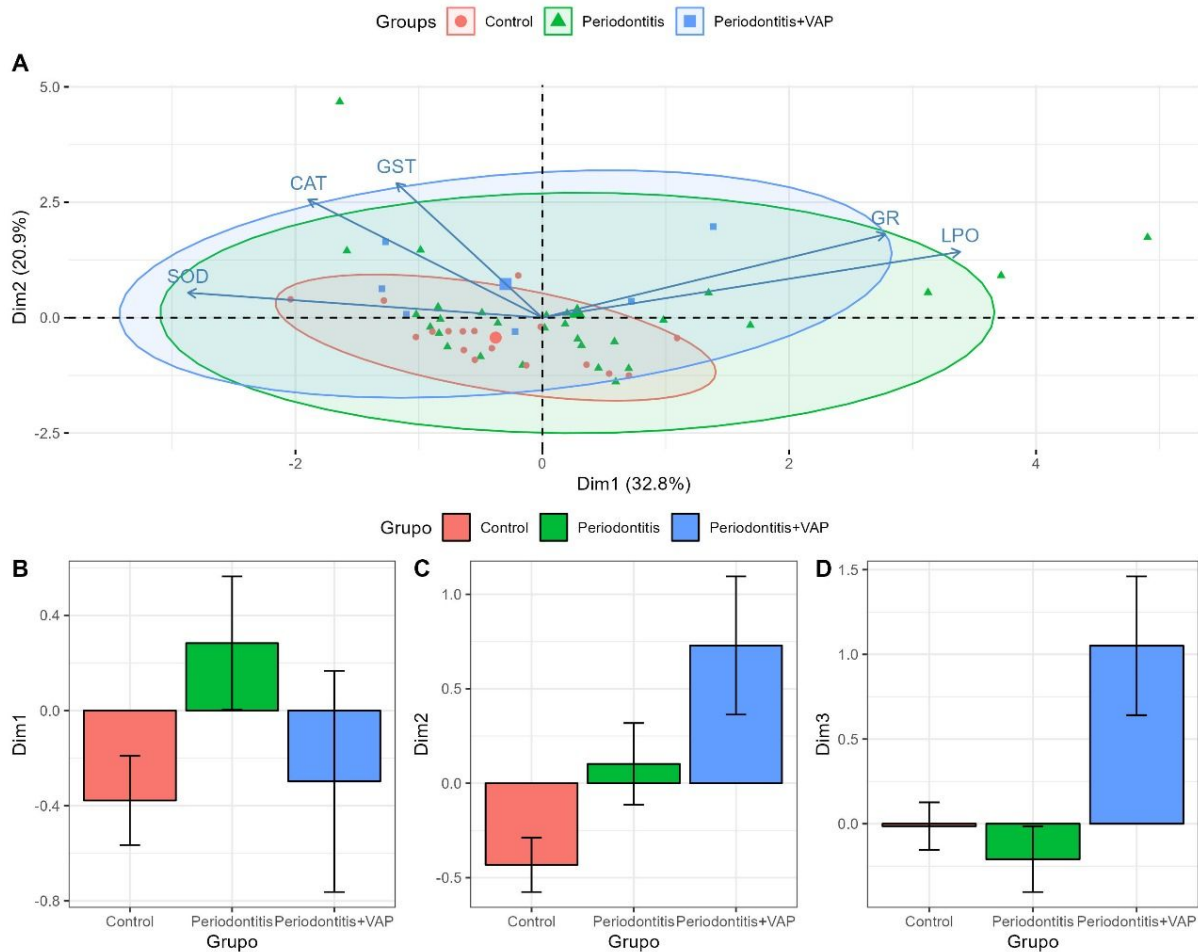
In relation to Dimension 3 (GST), it was possible to observe a significant statistical difference between the groups ( $F=4.75$ ;  $p=0.013$ ), with the group with Periodontitis + VAP presenting significantly higher positive scores when compared to the Control and Periodontitis groups, representing the induction of GST activity ( $p<0.05$ ).



**Fig 2** Boxplots of variables related to the antioxidant system and oxidative stress in the saliva of patients without or with Periodontitis. A) Superoxide Dismutase (SOD); B) Catalase (CAT); C) Glutathione reductase (GR); D) Glutathione-S-Transferase (GST); and E) Lipoperoxidation (LPO) Without Periodontitis; With Periodontitis



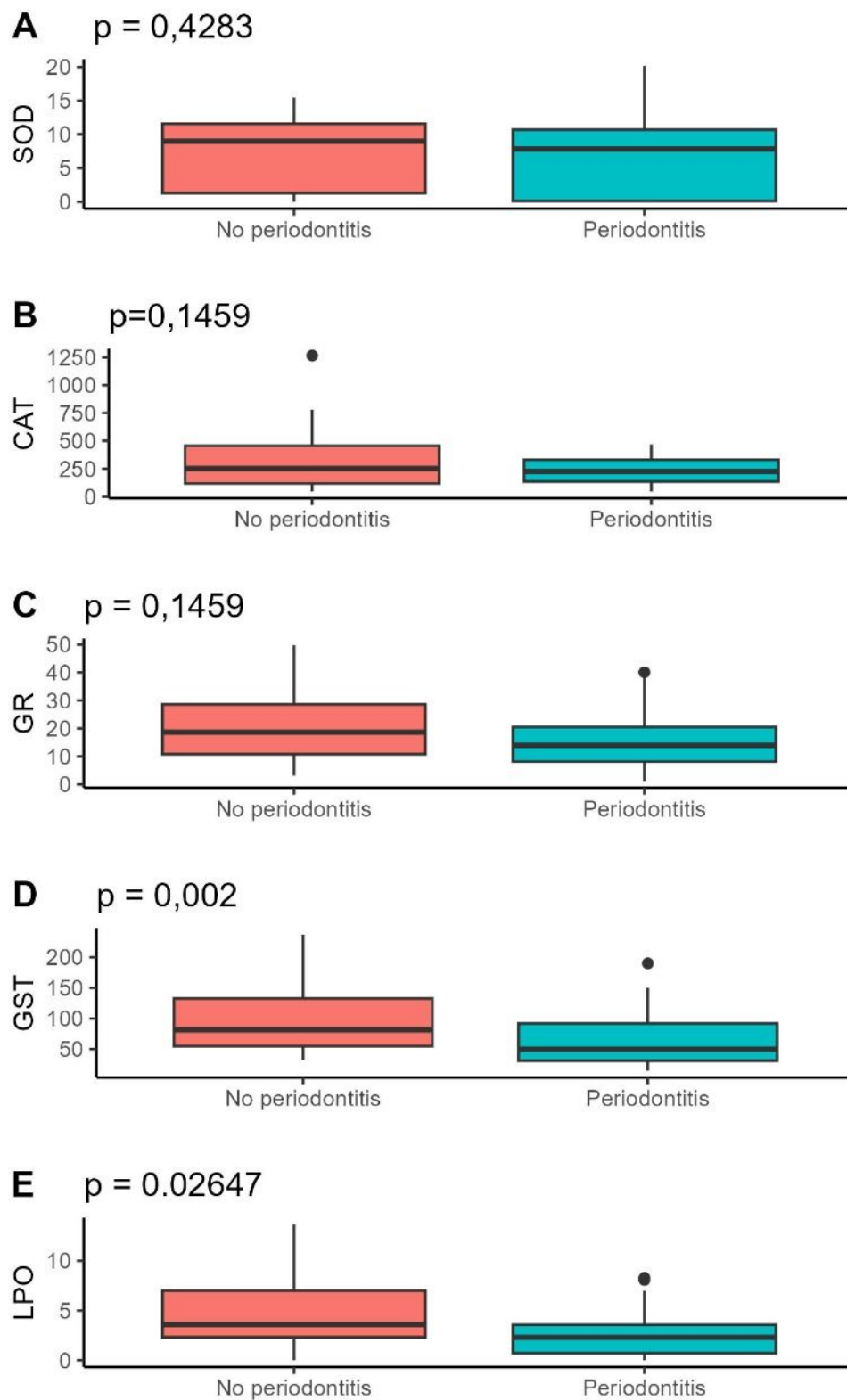
**Fig 3** Boxplots of variables related to the antioxidant system and oxidative stress in the saliva of patients with Periodontitis. A) Superoxide Dismutase (SOD); B) Catalase (CAT); C) Glutathione reductase (GR); D) Glutathione-S-Transferase (GST); and E) Lipoperoxidation (LPO) Without VAP; With VAP; VAP in patients with periodontitis



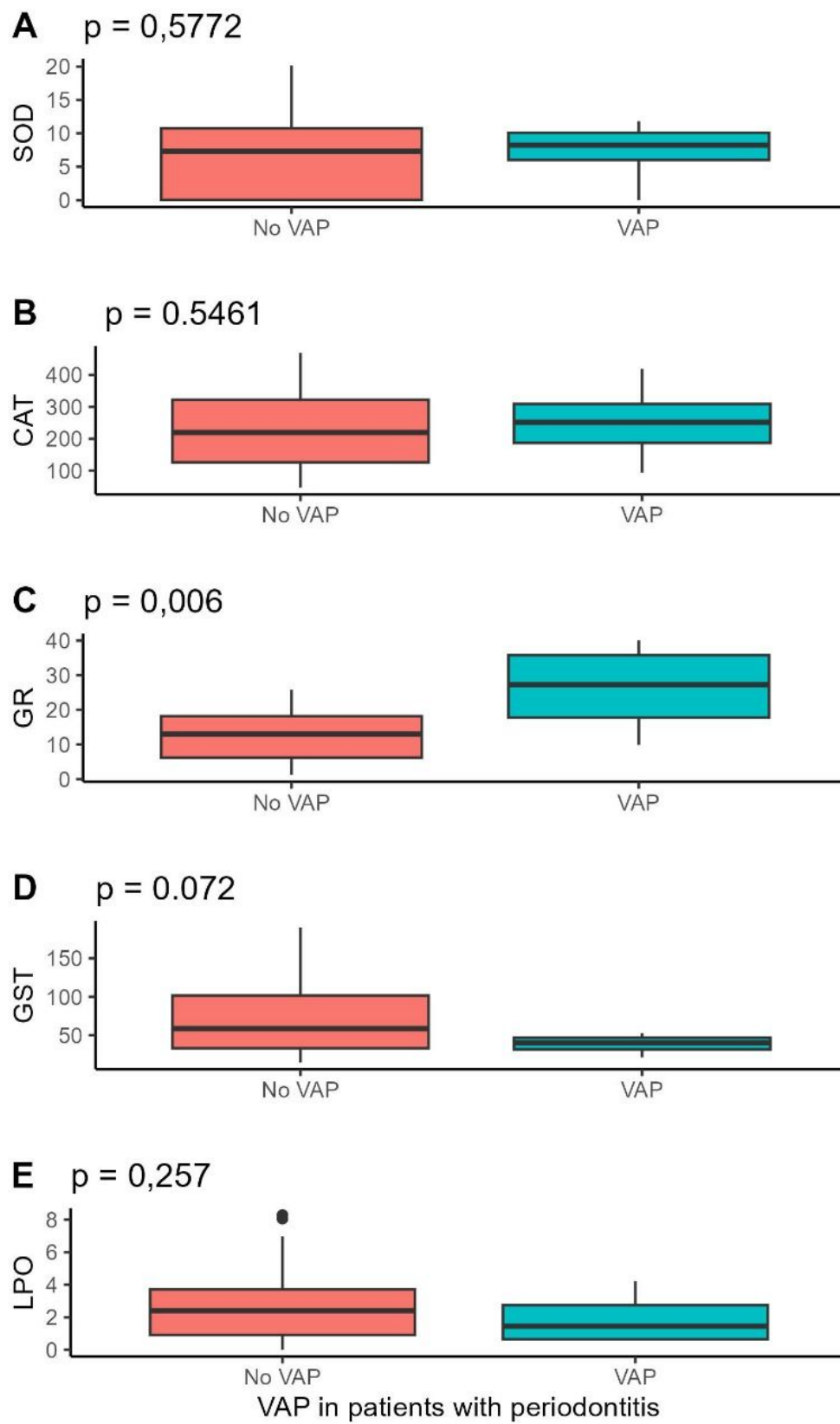
**Fig 4** Integrative assessment using the Principal Component Analysis (PCA) of saliva Group; Control; Periodontitis; Periodontitis + VAP

When evaluating the variables related to the antioxidant system and oxidative stress of the tracheal secretion of patients without and with Periodontitis, it was possible to verify that the enzymatic system (SOD and CAT) and GR, belonging to the auxiliary system of the antioxidant system, did not present statistically significant differences ( $p > 0.05$ ; Figures 5a-c). Conversely, GST, belonging to the auxiliary antioxidant system and responsible for detoxification processes, as well as lipoperoxidation showed statistically significant reductions between the groups studied ( $p=0.002$  and  $p=0.026$ ; Figures 5d-e).

When considering only patients with Periodontitis, it was possible to notice that the enzymatic system composed of SOD and CAT did not present statistically significant differences ( $p>0.05$ ; Figures 6a-b), as well as lipoperoxidation ( $p=0.257$ ; Figure 6e). Conversely, the auxiliary enzymes, GR and GST, presented significant induction and inhibition, respectively ( $p=0.006$ ;  $p=0.072$ ), when comparing the VAP group with the group without VAP.

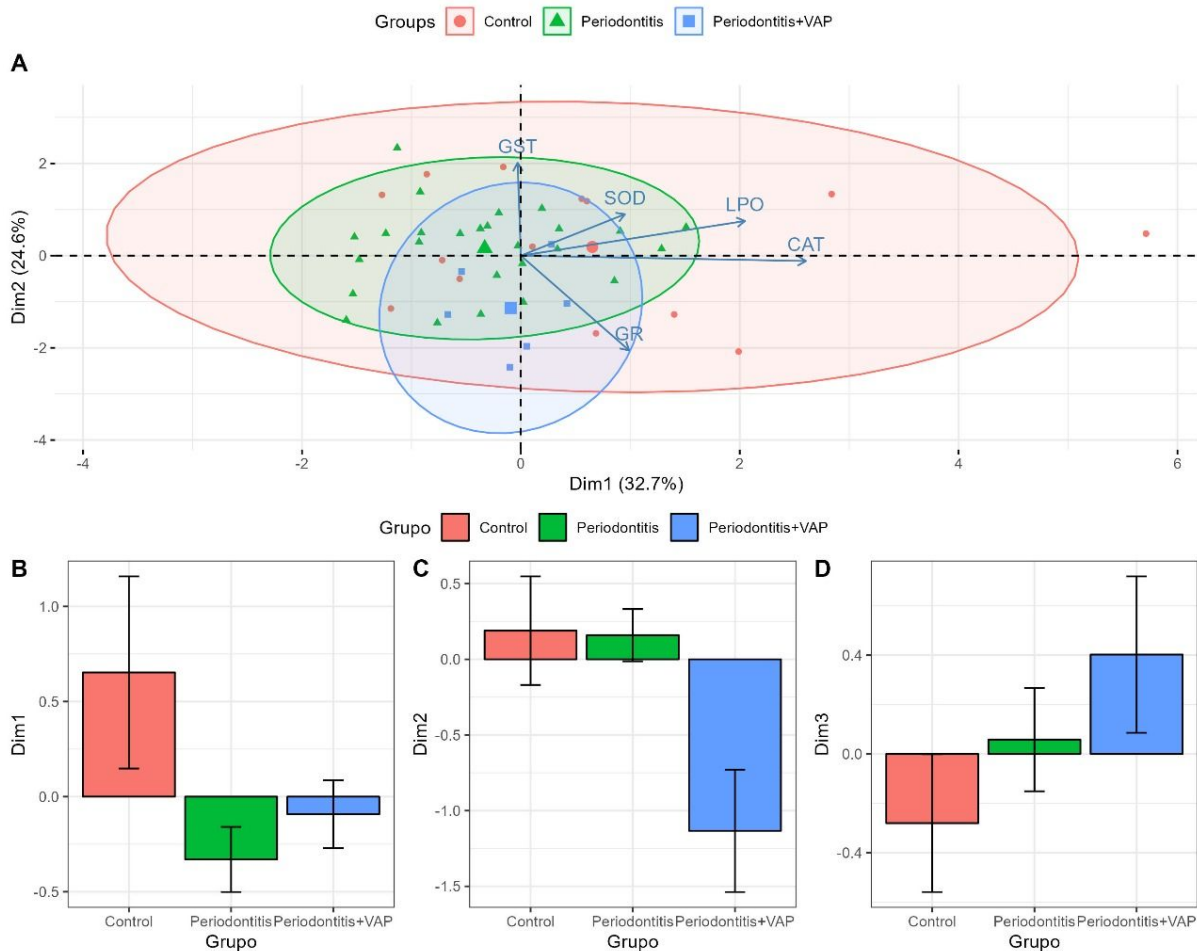


**Fig 5** Boxplots of variables related to the antioxidant system and oxidative stress in tracheal secretion of patients without or with Periodontitis. A) Superoxide Dismutase (SOD); B) Catalase (CAT); C) Glutathione reductase (GR); D) Glutathione-S-Transferase (GST); and E) Lipoperoxidation (LPO) Without Periodontitis; With Periodontitis; Periodontitis



**Fig 6** Boxplots of variables related to the antioxidant system and oxidative stress in the tracheal secretion of patients with Periodontitis. A) Superoxide Dismutase (SOD); B) Catalase (CAT); C) Glutathione reductase (GR); D) Glutathione-S-Transferase (GST); and E) Lipoperoxidation (LPO) Without VAP; With VAP; VAP in patients with periodontitis

Among the tracheal secretion samples (Figure 7), the first main component (Dimension 1 – Dim.1) presented the greatest contribution associated with CAT activity and lipoperoxidation reaction (Eigenvalue = 1.64; Variability = 32.71%). The second main component (Dimension 2 – Dim.2) presented a greater contribution from the enzymes of the auxiliary system GR and GST (Eigenvalue = 1.23; Variability = 24.58%). The third main component (Dimension 3 – Dim.3) presented a greater contribution from the SOD enzyme (Eigenvalue = 1.04; Variability = 20.75%). When comparing the factor loadings of the dimensions, only Dimension 2 (GR and GST) showed a statistically significant difference between the groups ( $F=4.00$ ;  $p=0.026$ ). Positive Dim.2 scores indicate higher GST enzymatic activity and lower GR activity. The group of patients with Periodontitis and VAP were significantly different from the other groups, characterized by negative scores, thus indicating greater GR activity and lower GST activity.



**Fig 7** Integrative assessment using Principal Component Analysis (PCA) of tracheal secretion

## DISCUSSION

Most critically ill patients require MV to facilitate survival. An OTT provides the interface between the patient and the ventilator and after insertion of the tube, with the possibility of changes occurring in the oral microenvironment and microbiome. The mechanisms underlying this “microbial displacement” are unclear, but may, in part, be due to the physical presence of OTT, which affects plaque removal, saliva flow, and mucosal dryness, in addition to interventions and medications related to the management of the underlying condition during critical illness [26].

When evaluating the oral health condition of the patients this present study, it was possible to observe that the BOP was higher in the groups with periodontitis, when compared to the control group, although it was not significant between the Periodontitis + VAP and control groups. Similarly, there were no significant differences in the periodontal parameters of CPD and CAL in control patients and patients with periodontitis +



VAP, corroborating the study by Almondes et al., 2017 [16], which also found no significant differences in periodontal parameters between the case groups (patients with VAP) and control (patients without VAP). In fact, this author showed that the control group presented worse periodontal conditions in relation to probing depth and loss of clinical attachment.

According to Scannapieco and Ho (2001) [27] and Hayes et al. (1998) [28], there is a tendency for attachment loss to increase when lung function is reduced, which did not occur in this study, which showed that there was no significant difference in CAL between the periodontitis and periodontitis + VAP groups.

The occurrence of lung injury may also affect the kidneys. Ventilator-induced lung injury is the most studied example of lung-kidney interaction. MV causes hemodynamic abnormalities, which can, in turn, affect renal perfusion by reducing cardiac output, as well as hormonal and sympathetic pathway stimulation [29]. In this study, a significant increase in urea and creatinine values was observed in the control group.

Abreu et al., 2013 [29], when examining the factors associated with acute kidney injury and outcome in patients with lung disease, observed that the comparison between survivors and non-survivors showed that those who did not survive had a higher frequency of need for MV, levels more than lower PEEP (positive end-expiratory pressure) on admission, higher urea levels on admission and more frequently required dialysis. It is known that high PEEP values decrease urinary flow, urinary sodium excretion and creatinine clearance. This study did not evaluate PEEP levels or the presence or development of acute kidney injury in patients.

Su et al., 2012 [30], when evaluating the levels of Triggering Receptor expressed on myeloid cells 1 (TREM-1), procalcitonin and the pulmonary infection score in the diagnosis and prognosis of VAP, observed that comparing the non-VAP group with the group VAP, had significant changes in temperature, oxygenation index, sTREM-1 levels, procalcitonin levels, leukocyte count, CRP concentration. In this study, there were no differences in temperature or leukocyte counts and CRP in the groups evaluated.

It should be noted that no study has compared these blood parameters in patients on MV with and without periodontitis, with and without VAP, nor the mechanisms by which periodontal inflammation may contribute to the development of VAP in patients on MV due to OTT.

Increasing evidence suggests that reactive oxygen species (ROS) are involved in the pathogenesis and progression of periodontitis. Low ROS levels are essential in several biochemical processes. They may, however, cause tissue damage through multiple mechanisms, including DNA damage, lipid peroxidation (LPO), and protein damage, as well as enzymatic oxidation. Oxidative stress (OS) occurs when cellular antioxidant defense is inadequate to completely inactivate ROS generated due to excessive production, loss of antioxidant defense, or both [31].

When performing saliva analysis, it was observed in the comparison between patients with and without periodontitis that the SOD enzyme was inhibited and the LPO reaction was increased among patients with this inflammation, thereby indicating the inhibition of the activity of the antioxidant system and a state of oxidative stress when compared to the Control group (without periodontitis). Other similar studies obtained similar results, with increased levels of oxidative stress in saliva in patients with periodontitis when compared to the control group [12, 32, 31, 33]. Ellis et al (1998) [34] analyzed gingival tissues from patients with severe periodontal disease and showed that the activity of SOD and CAT in these tissues was reduced.

When analyzing only patients with Periodontitis and comparing those who did or did not develop VAP, it was found that the SOD, CAT and GR enzymes were more active in the group of patients with VAP, which may have led to a reduction in OLP. GR is present in high concentrations in bronchoalveolar lavage, providing protection to the lung against oxidative injury. Its importance is confirmed in studies where its depletion has been related to a greater risk of lung disease [35], unlike this study, in which antioxidant enzymes in the lung were increased, not even being altered by the oxidative stress of the periodontitis.

Considering that the entire enzymatic framework of a cell is interrelated, integrative analyses become relevant to enable an understanding of the functioning of the cellular machinery. In the integrative analysis of saliva, the group with Periodontitis + VAP showed a significant induction of CAT and GST activity when compared to the Control group (without periodontitis and without VAP). To combat ROS formed in the extracellular space or of exogenous origin, the respiratory tract relies on antioxidant defenses present in the fluid that covers the surface of its epithelium. The extracellular antioxidants present in this fluid include catalase, SOD, GSHPx, GSH [36], which may justify the induction of these enzymes.

Additionally, studies report that oxidative stress resulting from periodontal disease has effects on systemic inflammation, such as respiratory disease [37, 8]. Thus, in this study, when evaluating the variables related to the antioxidant system and oxidative stress of tracheal secretion considering only Control patients (without periodontitis) and with Periodontitis, a reduction in GST activity and a lower state of oxidative stress

was observed, characterized by a reduction from LPO. GST comprises a group of enzymes capable of detoxifying a variety of compounds, including xenobiotics derived from pathogenic microorganisms, catalyzing their conjugation with GSH [38]. An increase in the action of GST is able to combat the effects of ROS produced in the endogenous detoxification process or from exogenous sources [39], which differs from this study, or because periodontitis would not have reached a degree of severity sufficient to alter the lung's antioxidant enzymes.

When evaluating only patients with Periodontitis, it was possible to notice that the enzymatic system composed of SOD and CAT did not present significant statistical differences, as did lipoperoxidation. Conversely, the auxiliary enzymes, GR and GST, showed significant induction and inhibition, respectively ( $p=0.006$ ;  $p=0.072$ ), when comparing the VAP group with the group without VAP. Glutathione (GSH) has a vital protective function, at the intra- and extra-cellularly level, against oxidative stress in the lungs [40]. Changes in glutathione (GSH) metabolism in alveoli and lung tissue are widely recognized as a key feature of a number of lung diseases [41]. GSTs do not use hydrogen peroxide as a substrate but are able to catalyze the GSH-dependent reduction of non-physiological hydroperoxides [42], thereby demonstrating that there may be an imbalance in the antioxidant system in these patients.

Although the number of patients in the Periodontitis + VAP group was reduced ( $n=6$ ), these results may strengthen the hypothesis that the existing imbalance in the antioxidant system of patients with periodontitis may be a risk factor for the development of VAP, as the inflammatory profile of periodontitis causes the release of lymphocytes, which when activated, produce a large amount of ROS [37], thereby justifying these results and the objective of this study, considering that, to date, no other study has evaluated this mechanism in relationship between the two diseases.

VAP results found in this study may have occurred due to the Saúde em Nossas Mãos (“Health in Our Hands”) project, which was implemented at HUOP in 2021. The project was developed collaboratively by the PROADI-SUS hospitals, and the technical teams of the Coordinator’s Office of the National Program for Patient Safety (*Coordenação do Programa Nacional de Segurança do Paciente – PNSP*), General Coordinator’s Office of Hospital and Emergency Care of the Hospital Care Department of the Health Care Secretariat (*Coordenação Geral de Atenção Hospitalar e de Urgência do Departamento de Atenção Hospitalar da Secretaria de Atenção à Saúde – CGHOSP/DAHU/SAS/MS*).

Aligned with the National Health Plan (*Plano Nacional de Saúde – PNS*), Saúde em Nossas Mãos (“Health in Our Hands”) hopes to reduce, in the medium term, the incidence of the main indicators of hospital infection, in addition to disseminating the improvement model to other units and hospitals, as well as demonstrating the financial impact from the prevention of infections. In the long term, the expectation is to contribute to changing the culture of healthcare organizations regarding patient safety. Between 2021 and 2023, Saúde em Nossas Mãos had the participation of 204 hospitals, including HUOP – Cascavel.

The main limitation of this study was the small sample size of the periodontitis + VAP group, due to the reduction in VAP during the collection period, probably due to the Saúde em Nossas Mãos project. Therefore, studies with a larger sample and longer collection time are necessary to confirm the imbalance of the antioxidant system and the occurrence of oxidative stress as a mechanism for the development of VAP in patients with and without periodontitis admitted to the ICU.

## CONCLUSION

According to the results obtained and based on the clinical significance of the results, it can be concluded that the increase in oxidative stress caused by the inflammation of periodontitis may lead to an imbalance in the antioxidant status, thereby facilitating the development of VAP. Clinical studies with larger samples and longer collection times are needed to confirm these findings.

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#### **Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

#### **Authors' Contribution**

All authors contributed to the study conception and design.

Bruna Belineli Gomes Frisso Silva, Rafaela Batista, Emily Cristina Ghiggi and Edson Oliveira Silva - clinical and experimental studies, data and statistical analysis; manuscript preparation, editing and review.

Ana Tereza Bittencourt Guimarães- experimental studies, data and statistical analysis; manuscript preparation.

Carlos Augusto Nassar, Patricia Oehlmeyer Nassar - definition of intellectual content; design; clinical and experimental studies, data and statistical analysis; manuscript preparation, editing and review.

All authors read and approved the final manuscript.

#### **Ethics approval**

All regulations for conducting studies with human beings were followed, with approval from the UNIOESTE Human Research Ethics Committee, under opinion N° 5.340.334.

#### **Consent to participate**

The objective and nature of the study were explained to all companions/guardians of the patients, and they were included as study participants after agreeing and signing the informed consent form (ICF).

## **CONCLUSÕES GERAIS**

De acordo com os resultados obtidos e com base na significância clínica dos resultados, pode-se concluir que a alteração do estresse oxidativo causado pela inflamação da periodontite com um desequilíbrio do estado antioxidante, pode ser um fator de risco para o desenvolvimento da PAV. Estudos clínicos com maior amostra e maior tempo de coleta são necessários para confirmar esses achados.

APÊNDICE A – PERIOGRAMA PARA COLETA DE DADOS

PERIOGRAMA												
DENTE	molar	Pré	13	12	11	21	22	23	Pré	Molar		
IP	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX		
IG	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX		
Sítio	D	V	M	M	V	D	M	V	D	M	V	D
Sangr.												
N.G.												
P.S.												
N.I.												
Sítio	D	L	M	L	M	L	D	M	L	D	M	L
Sangr.												
N.G.												
P.S.												
N.I.												

DENTE	molar	Pré	43	42	41	31	32	33	Pré	Molar		
IP	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX		
IG	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX		
Sítio	D	V	M	M	V	D	M	V	D	M	V	D
Sangr.												
N.G.												
P.S.												
N.I.												
Sítio	D	L	M	L	M	L	D	M	L	D	M	L
Sangr.												
N.G.												
P.S.												
N.I.												

Data inicial: / / Paciente: \_\_\_\_\_  
 NG – nível gengival; PS – profundidade de sondagem; NI – Nivel de Inserção; Sangr= sangramento à sondagem; IP- índice de placa; IG – índice gengival;

## APÊNDICE B – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - TCLE



### ANEXO I TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - TCLE

Título do Projeto: FISIOPATOLOGIA DA PERIODONTITE NO DESENVOLVIMENTO DA PNEUMONIA ASSOCIADA À VENTILAÇÃO MECÂNICA. ESTUDO CLÍNICO OBSERVACIONAL DO TIPO TRANSVERSAL.

Pesquisador responsável e colaboradores com telefones de contato: Profa. Dra. Patricia Oehlmeyer Nassar – (45) 99117 0903/ Prof. Dr. Carlos Augusto Nassar – (45) 99101 3369

Convidamos você como responsável pelo paciente, a participar de nossa pesquisa que tem o objetivo de avaliar a inflamação gengival de pacientes em ventilação mecânica invasiva (intubado); para isso será colocado um cone de papel absorvente bem fino entre o dente e a gengiva em 3 dentes diferentes por 30 segundos para avaliar a quantidade de inflamação desta gengiva e também um rolete de algodão para avaliar a quantidade de saliva. Este procedimento será realizado na UTI do Hospital Universitário do Oeste do Paraná (HUOP/UNIOESTE) - Cascavel – PR, por um profissional qualificado e treinado, sob responsabilidade da cirurgiã-dentista e professora do Curso de Odontologia, Dra. Patricia Oehlmeyer Nassar, sem nenhum risco ou constrangimento para o(a) paciente.

Para algum questionamento, dúvida ou relato de algum acontecimento os pesquisadores poderão ser contatados a qualquer momento. O TCLE será entregue em duas vias, sendo que uma ficará com o sujeito da pesquisa. Os dados obtidos por meio desta pesquisa serão confidenciais e não serão divulgados em nível individual e serão usadas somente com fins estatísticos, visando assegurar o sigilo da sua participação. As informações coletadas no histórico clínico ou fichas clínicas serão identificadas apenas através do código, sem nenhuma identificação pessoal.



Os dados e os termos de consentimento serão mantidos em total segurança e apenas a coordenação da pesquisa terá acesso a essas informações. Os dados de identificação serão mantidos em sigilo e só serão utilizados para estudos estatísticos, no nível coletivo.

Se ao final da pesquisa for observado que a presença desta inflamação na gengiva pode minimizar os efeitos da pneumonia, será realizado um controle periodontal de pacientes em ventilação mecânica invasiva.

O sujeito poderá cancelar sua participação a qualquer momento; o telefone do comitê de ética é 3220-3092, caso o sujeito necessite de maiores informações;

Declaro estar ciente do exposto e autorizo .....a participar da pesquisa (no caso de responsável por menor ou pessoa considerada legalmente incapaz).

Nome do sujeito de pesquisa ou responsável:

Assinatura:

Eu, **Patricia Oehlmeyer Nassar**, declaro que forneci todas as informações do projeto ao participante e/ou responsável.

Cascavel, \_\_\_\_\_ de \_\_\_\_\_ de 20\_\_\_\_.

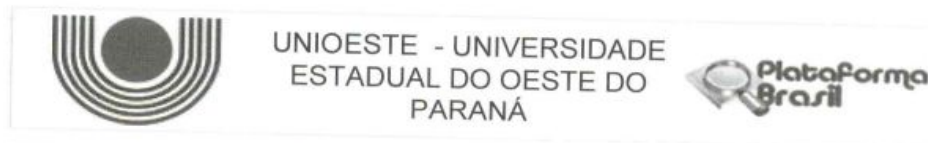
Cascavel, 10 de fevereiro de 2022



---

Patricia Oehlmeyer Nassar

## ANEXO A – PARECER CONSUBSTANCIADO DO CEP



### PARECER CONSUBSTANCIADO DO CEP

#### DADOS DO PROJETO DE PESQUISA

**Título da Pesquisa:** PERIODONTITE NO DESENVOLVIMENTO DA PNEUMONIA ASSOCIADA À VENTILAÇÃO MECÂNICA

**Pesquisador:** Patricia Oehlmeyer Nassar

**Área Temática:**

**Versão:** 2

**CAAE:** 56642221.3.0000.0107

**Instituição Proponente:** Centro de Ciências Biológicas e da Saúde CCBS - UNIOESTE

**Patrocinador Principal:** Financiamento Próprio

#### DADOS DO PARECER

**Número do Parecer:** 5.340.334

#### **Apresentação do Projeto:**

Saneamento de pendências da pesquisa:

**Título da Pesquisa:** PERIODONTITE NO DESENVOLVIMENTO DA PNEUMONIA ASSOCIADA À VENTILAÇÃO MECÂNICA

**Pesquisador Responsável:** Patricia Oehlmeyer Nassar

**Área Temática:**

**Versão:** 2

**CAAE:** 56642221.3.0000.0107

**Submetido em:** 08/04/2022

**Instituição Proponente:** Centro de Ciências Biológicas e da Saúde CCBS - UNIOESTE

**Situação da Versão do Projeto:** Em relatoria

**Localização atual da Versão do Projeto:** UNIOESTE - Universidade Estadual do Oeste do Paraná

#### **Objetivo da Pesquisa:**

Vide descrição anteriormente apresentada.

#### **Avaliação dos Riscos e Benefícios:**

Vide descrição anteriormente apresentada.

#### **Comentários e Considerações sobre a Pesquisa:**

Vide descrição anteriormente apresentada.

**Endereço:** RUA UNIVERSITARIA 2069

**Bairro:** UNIVERSITARIO

**CEP:** 85.819-110

**UF:** PR

**Município:** CASCAVEL

**Telefone:** (45)3220-3092

**E-mail:** cep.prppg@unioeste.br



Continuação do Parecer: 5.340.334

**Considerações sobre os Termos de apresentação obrigatória:**

Vide descrição anteriormente apresentada.

**Conclusões ou Pendências e Lista de Inadequações:**

Os possíveis riscos foram descritos no TCLE, no entanto, é necessário que se acrescente que se houver danos não previstos e, comprovadamente decorrente da participação na pesquisa, a pesquisadora providenciará o atendimento imediato, integral e gratuito.

**Considerações Finais a critério do CEP:**

Apresentar o Relatório Final na Plataforma Brasil até 30 dias após o encerramento desta pesquisa.

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1860575.pdf	08/04/2022 11:08:52		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	ModelodeTCLE2.pdf	08/04/2022 11:08:26	Patricia Oehlmeyer Nassar	Aceito
Outros	AnexoIV.pdf	01/04/2022 17:22:51	Patricia Oehlmeyer Nassar	Aceito
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Declaração de Instituição e Infraestrutura	termodecienciaidoresponsavelpelocampo deestudo1.pdf	10/02/2022 14:04:51	Patricia Oehlmeyer Nassar	Aceito
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## ANEXO B – NORMAS DA REVISTA

### INSTRUCTIONS FOR AUTHORS

#### General

The Council of Biology Editors Style Manual should be used as the style guide for the preparation of manuscripts, particularly with respect to such matters as the use of abbreviations, numbers, and symbols.

*Inflammation* will not consider studies on the effects of plant extracts on inflammation.

*Inflammation* considers Original Articles and Reviews. Authors of unsolicited Review articles are encouraged to send a pre-submission enquiry and abstract before submitting their full article.

#### Manuscript Submission

##### **Manuscript Submission**

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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### **Online Submission**

Please follow the hyperlink “Submit manuscript” and upload all of your manuscript files following the instructions given on the screen.

### **Source Files**

Please ensure you provide all relevant editable source files at every submission and revision. Failing to submit a complete set of editable source files will result in your article not being considered for review. For your manuscript text please always submit in common word processing formats such as .docx or LaTeX.

### **Authorship Policy**

Authorship should incorporate and should be restricted to those who have contributed substantially to the work in one or more of the following categories:

- Conceived of or designed study.
- Performed research.
- Analyzed data.
- Contributed new methods or models.
- Wrote the paper.

### **Page Charges**

The journal makes no page charges. Color can be used without charge for the electronic edition of the journal but will appear in the printed version of the journal at the author’s expense. The cost for color reproduction in the printed journal is \$1,150.00 per article, charged to the author.

## Title Page

Please make sure your title page contains the following information.

### Title

The title should be concise and informative.

### Author information

- The name(s) of the author(s);
- The affiliation(s) of the author(s), i.e. institution, (department), city, (state), country;
- A clear indication and an active e-mail address of the corresponding author;
- If available, the 16-digit ORCID of the author(s);

If address information is provided with the affiliation(s) it will also be published.

For authors that are (temporarily) unaffiliated we will only capture their city and country of residence, not their e-mail address unless specifically requested.

Large Language Models (LLMs), such as ChatGPT, do not currently satisfy our authorship criteria. Notably an attribution of authorship carries with it accountability for the work, which cannot be effectively applied to LLMs. Use of an LLM should be properly documented in the Methods section (and if a Methods section is not available, in a suitable alternative part) of the manuscript.

### Abstract

Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

*For life science journals only (when applicable)*

- Trial registration number and date of registration for prospectively registered trials
- Trial registration number and date of registration, followed by “retrospectively registered”, for retrospectively registered trials.

## Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

## Text

### Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX. We recommend using Springer Nature's LaTeX template.

## Headings

Please use no more than three levels of displayed headings.

## Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

## Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

### **Acknowledgments**

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

### **References**

#### **Citation**

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

#### **Reference list**

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text.

The entries in the list should be numbered consecutively.



Journal names and book titles should be *italicized*.

If available, please always include DOIs as full DOI links in your reference list (e.g. “<https://doi.org/abc>”).

- Journal article

Alber, John, Daniel C. O’Connell, and Sabine Kowal. 2002. Personal perspective in TV interviews. *Pragmatics* 12: 257–271.

- Article by DOI

Suleiman, Camelia, Daniel C. O’Connell, and Sabine Kowal. 2002. ‘If you and I, if we, in this later day, lose that sacred fire...’: Perspective in political interviews. *Journal of Psycholinguistic Research*. <https://doi.org/10.1023/A:1015592129296>.

- Book

Cameron, Deborah. 1985. *Feminism and linguistic theory*. New York: St. Martin’s Press.

- Book chapter

Cameron, Deborah. 1997. Theoretical debates in feminist linguistics: Questions of sex and gender. In *Gender and discourse*, ed. Ruth Wodak, 99-119. London: Sage Publications.

- Online document

Frisch, Mathias. 2007. Does a low-entropy constraint prevent us from influencing the past? PhilSci archive. <http://philsci-archive.pitt.edu/archive/00003390>. Accessed 26 June 2007.

## Statements & Declarations

The following statements must be included in your submitted manuscript under the heading 'Statements and Declarations'. This should be placed after the References section. Please note that submissions that do not include required statements will be returned as incomplete.

## Funding

Please describe any sources of funding that have supported the work. The statement should include details of any grants received (please give the name of the funding agency and grant number).

Example statements:

*“This work was supported by [...] (Grant numbers [...] and [...]). Author A.B. has received research support from Company A.”*

*“The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.”*

### **Competing Interests**

Authors are required to disclose financial or non-financial interests that are directly or indirectly related to the work submitted for publication. Interests within the last 3 years of beginning the work (conducting the research and preparing the work for submission) should be reported. Interests outside the 3-year time frame must be disclosed if they could reasonably be perceived as influencing the submitted work.

Example statements:

*“Financial interests: Author A and B declare they have no financial interests. Author C has received speaker and consultant honoraria from Company M. Dr. C has received speaker honorarium and research funding from Company M and Company N. Author D has received travel support from Company O. Non-financial interests: Author D has served on advisory boards for Company M and Company N.”*

*“The authors have no relevant financial or non-financial interests to disclose.”*

Please refer to the “Competing Interests” section below for more information on how to complete these sections.

### **Author Contributions**

Authors are encouraged to include a statement that specifies the contribution of every author to the research and preparation of the manuscript.

Example statement:

*“All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.”*

Please refer to the “Authorship Principles ” section below for more information on how to complete this section.

In addition to the above, manuscripts that report the results of studies involving humans and/or animals should include the following declarations:

### **Ethics approval**

Authors of research involving human or animal subjects should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee and reference number, if available). For research involving animals, their data or biological material, authors should supply detailed information on the ethical treatment of their animals in their submission. If a study was granted exemption or did not require ethics approval, this should also be detailed in the manuscript.

*“This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No....).”*

*“This is an observational study. The XYZ Research Ethics Committee has confirmed that no ethical approval is required.”*

For detailed information on relevant ethical standards and criteria, please refer to the sections on “Research involving human participants, their data or biological material”, “Research involving animals, their data or biological material”.

### **Consent to participate**

For all research involving human subjects, freely-given, informed consent to participate in the study must be obtained from participants (or their parent or legal

guardian in the case of children under 16) and a statement to this effect should appear in the manuscript.

Example statement:

*“Informed consent was obtained from all individual participants included in the study.”*

*“Written informed consent was obtained from the parents.”*

Please refer to the section on “Informed Consent” for additional help with completing this information.

### **Consent to publish**

Individuals may consent to participate in a study, but object to having their data published in a journal article. If your manuscript contains any individual person’s data in any form (including any individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. This is in particular applicable to case studies. A statement confirming that consent to publish has been received from all participants should appear in the manuscript.

Example statement:

*“The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.”*

Please refer to the section on “Informed Consent” for additional help with completing this information.

### **Tables**

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.

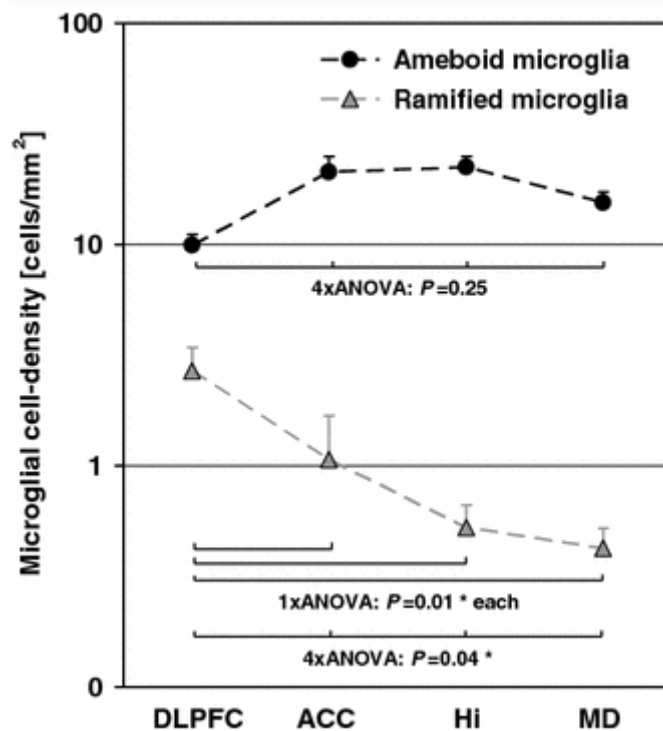
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

## Artwork and Illustrations Guidelines

### Electronic Figure Submission

- Supply all figures electronically.
- Indicate what graphics program was used to create the artwork.
- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

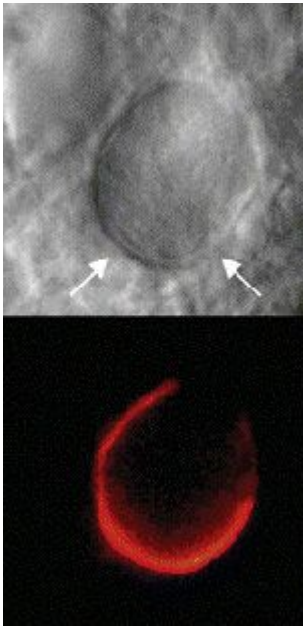
### Line Art



- Definition: Black and white graphic with no shading.
- Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

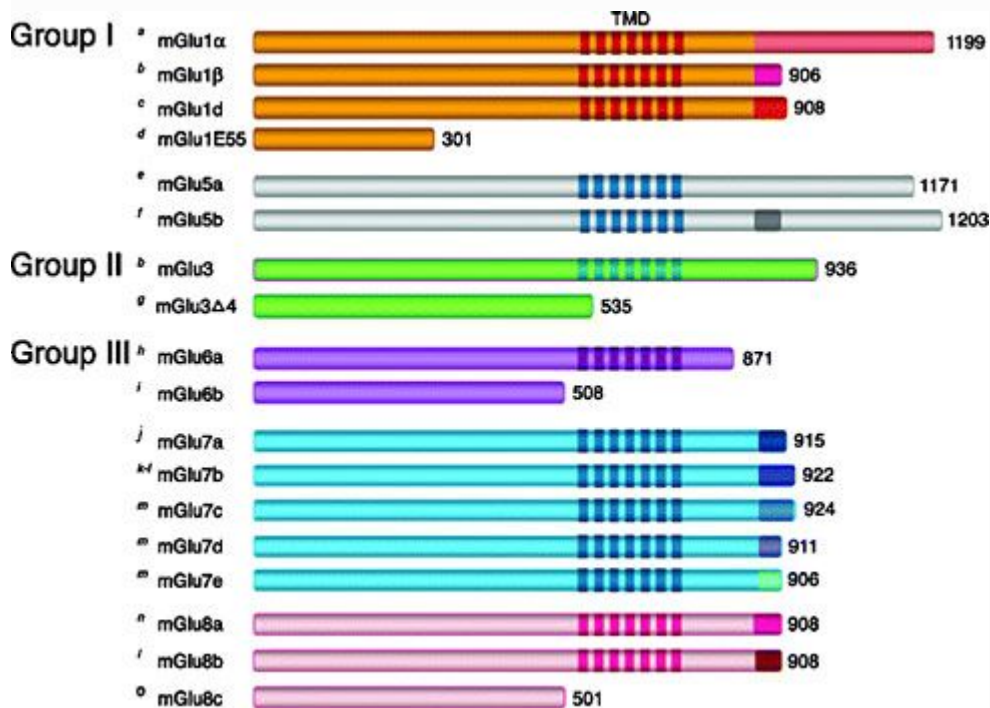
- All lines should be at least 0.1 mm (0.3 pt) wide.
- Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.
- Vector graphics containing fonts must have the fonts embedded in the files.

### **Halftone Art**



- Definition: Photographs, drawings, or paintings with fine shading, etc.
- If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.
- Halftones should have a minimum resolution of 300 dpi.

## Combination Art



- Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.
- Combination artwork should have a minimum resolution of 600 dpi.

## Color Art

- Color art is free of charge for online publication.
- If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.
- If the figures will be printed in black and white, do not refer to color in the captions.
- Color illustrations should be submitted as RGB (8 bits per channel).

## Figure Lettering

- To add lettering, it is best to use Helvetica or Arial (sans serif fonts).

- Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).
- Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.
- Avoid effects such as shading, outline letters, etc.
- Do not include titles or captions within your illustrations.

### **Figure Numbering**

- All figures are to be numbered using Arabic numerals.
- Figures should always be cited in text in consecutive numerical order.
- Figure parts should be denoted by lowercase letters (a, b, c, etc.).
- If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices [Supplementary Information (SI)] should, however, be numbered separately.

### **Figure Captions**

- Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.
- Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.
- No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.
- Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.
- Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

### **Figure Placement and Size**



- Figures should be submitted within the body of the text. Only if the file size of the manuscript causes problems in uploading it, the large figures should be submitted separately from the text.
- When preparing your figures, size figures to fit in the column width.
- For large-sized journals the figures should be 84 mm (for double-column text areas), or 174 mm (for single-column text areas) wide and not higher than 234 mm.
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In order to give people of all abilities and disabilities access to the content of your figures, please make sure that.

- All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware);
- Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements);
- Any figure lettering has a contrast ratio of at least 4.5:1.

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- Supply all supplementary material in standard file formats.
- Please include in each file the following information: article title, journal name, author names; affiliation and e-mail address of the corresponding author.
- To accommodate user downloads, please keep in mind that larger-sized files may require very long download times and that some users may experience other problems during downloading.
- High resolution (streamable quality) videos can be submitted up to a maximum of 25GB; low resolution videos should not be larger than 5GB.

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- Aspect ratio: 16:9 or 4:3.
- Maximum file size: 25 GB for high resolution files; 5 GB for low resolution files.
- Minimum video duration: 1 sec.
- Supported file formats: avi, wmv, mp4, mov, m2p, mp2, mpg, mpeg, flv, mxf, mts, m4v, 3gp.

### **Text and Presentations**

- Submit your material in PDF format; .doc or .ppt files are not suitable for long-term viability.
- A collection of figures may also be combined in a PDF file.

### **Spreadsheets**

- Spreadsheets should be submitted as .csv or .xlsx files (MS Excel).

### **Specialized Formats**

- Specialized format such as .pdb (chemical), .wrl (VRML), .nb (Mathematica notebook), and .tex can also be supplied.

### **Collecting Multiple Files**

- It is possible to collect multiple files in a .zip or .gz file.

### **Numbering**

- If supplying any supplementary material, the text must make specific mention of the material as a citation, similar to that of figures and tables.
- Refer to the supplementary files as “Online Resource”, e.g., “... as shown in the animation (Online Resource 3)”, “... additional data are given in Online Resource 4”.
- Name the files consecutively, e.g. “ESM\_3.mpg”, “ESM\_4.pdf”.

### **Captions**

- For each supplementary material, please supply a concise caption describing the content of the file.

### **Processing of supplementary files**

- Supplementary Information (SI) will be published as received from the author without any conversion, editing, or reformatting.

### **Accessibility**

In order to give people of all abilities and disabilities access to the content of your supplementary files, please make sure that

- The manuscript contains a descriptive caption for each supplementary material.
- Video files do not contain anything that flashes more than three times per second (so that users prone to seizures caused by such effects are not put at risk).

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Upon request authors should be prepared to send relevant documentation or data in order to verify the validity of the results presented. This could be in the form of raw data, samples, records, etc. Sensitive information in the form of confidential or proprietary data is excluded.

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  - an expression of concern may be placed with the article.
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All authors whose names appear on the submission

- 1) made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; or the creation of new software used in the work;
- 2) drafted the work or revised it critically for important intellectual content;
- 3) approved the version to be published; and
- 4) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

\* Based on/adapted from:

ICMJE, Defining the Role of Authors and Contributors,  
Transparency in authors' contributions and responsibilities to promote integrity in scientific publication, McNutt et al, PNAS February 27, 2018.

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All authors are requested to include information regarding sources of funding, financial or non-financial interests, study-specific approval by the appropriate ethics committee for research involving humans and/or animals, informed consent if the research involved human participants, and a statement on welfare of animals if the research involved animals (as appropriate).

The decision whether such information should be included is not only dependent on the scope of the journal, but also the scope of the article. Work submitted for publication may have implications for public health or general welfare and in those cases it is the responsibility of all authors to include the appropriate disclosures and declarations.

## Data transparency

All authors are requested to make sure that all data and materials as well as software application or custom code support their published claims and comply with field standards. Please note that journals may have individual policies on (sharing) research data in concordance with disciplinary norms and expectations.

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**One author** is assigned as Corresponding Author and acts on behalf of all co-authors and ensures that questions related to the accuracy or integrity of any part of the work are appropriately addressed.

The Corresponding Author is responsible for the following requirements:

- ensuring that all listed authors have approved the manuscript before submission, including the names and order of authors;
- managing all communication between the Journal and all co-authors, before and after publication;\*



- providing transparency on re-use of material and mention any unpublished material (for example manuscripts in press) included in the manuscript in a cover letter to the Editor;
- making sure disclosures, declarations and transparency on data statements from all authors are included in the manuscript as appropriate (see above).

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### **Author contributions**

In absence of specific instructions and in research fields where it is possible to describe discrete efforts, the Publisher recommends authors to include contribution statements in the work that specifies the contribution of every author in order to promote transparency. These contributions should be listed at the separate title page.

#### **Examples of such statement(s) are shown below:**

- Free text:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [full name], [full name] and [full name]. The first draft of the manuscript was written by [full name] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Example: CRediT taxonomy:

- Conceptualization: [full name], ...; Methodology: [full name], ...; Formal analysis and investigation: [full name], ...; Writing - original draft preparation: [full name, ...]; Writing - review and editing: [full name], ...; Funding acquisition: [full name], ...; Resources: [full name], ...; Supervision: [full name],....

For **review articles** where discrete statements are less applicable a statement should be included who had the idea for the article, who performed the literature search and data analysis, and who drafted and/or critically revised the work.

For articles that are based primarily on the **student's dissertation or thesis**, it is recommended that the student is usually listed as principal author:

A Graduate Student's Guide to Determining Authorship Credit and Authorship Order, APA Science Student Council 2006.

### **Affiliation**

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### **Summary of requirements**

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Funding' and/or 'Competing interests'. Other declarations include Ethics approval, Consent, Data, Material and/or Code availability and Authors' contribution statements.

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**Research involving human participants, their data or biological material**

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When reporting a study that involved human participants, their data or biological material, authors should include a statement that confirms that the study was approved (or granted exemption) by the appropriate institutional and/or national research ethics committee (including the name of the ethics committee) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. If doubt exists whether the research was conducted in accordance with the 1964 Helsinki Declaration or comparable standards, the authors must explain the reasons for their approach, and demonstrate that an independent ethics committee or institutional review board explicitly approved the doubtful aspects of the study. If a study was granted exemption from requiring ethics approval, this should also be detailed in the manuscript (including the reasons for the exemption).

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### Summary of requirements

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Ethics approval'.

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- This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).
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- The questionnaire and methodology for this study was approved by the Human Research Ethics committee of the University of D (Ethics approval number: ...).

Examples of statements to be used for a retrospective study:

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- This research study was conducted retrospectively from data obtained for clinical purposes. We consulted extensively with the IRB of XYZ who determined that our study did not need ethical approval. An IRB official waiver of ethical approval was granted from the IRB of XYZ.
- This retrospective chart review study involving human participants was in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Human Investigation Committee (IRB) of University B approved this study.

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### **Summary of requirements**

The above should be summarized in a statement and placed in a 'Declarations' section before the reference list under a heading of 'Consent to participate' and/or 'Consent to publish'. Other declarations include Funding, Competing interests, Ethics approval, Consent, Data and/or Code availability and Authors' contribution statements.

Please see the various examples of wording below and revise/customize the sample statements according to your own needs.

**Sample statements for "Consent to participate":**

Informed consent was obtained from all individual participants included in the study.

Informed consent was obtained from legal guardians.

Written informed consent was obtained from the parents.

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The authors affirm that human research participants provided informed consent for publication of the images in Figure(s) 1a, 1b and 1c.

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