UNIVERSIDADE FEDERAL DE MINAS GERAIS
Faculdade de Odontologia
Colegiado de Pós-Graduação em Odontologia

Sicília Rezende Oliveira

PERIODONTITE ASSOCIADA À ARTRITE REUMATOIDE: *O PAPEL DAS REDES EXTRACELULARES DE NEUTRÓFILOS (NETs)*

Belo Horizonte
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Orientadora: Profa. Dra. Tarcília Aparecida Silva
Coorientador: Prof. Dr. Lucas Guimarães Abreu

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FOLHA DE APROVAÇÃO

PERIODONTITE ASSOCIADA À ARTRITE REUMATOIDE: O PAPEL DAS REDES EXTRACELULARES DE NEUTRÓFILOS (NETS)

SICÍLIA REZENDE OLIVEIRA

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ODONTOLOGIA, como requisito para obtenção do grau de Doutor em ODONTOLOGIA, área de concentração PATOLOGIA BUCAL.

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“É mais importante conhecer a pessoa que tem a doença do que a doença que a pessoa tem.”

Hipócrates.

RESUMO

As redes extracelulares de neutrófilos (NETs) são apontadas como um dos mecanismos relevantes na patogênese da periodontite e artrite reumatoide (AR). No entanto, permanece pouco compreendido a participação das NETs como mecanismo de ligação entre as duas doenças. Os principais objetivos do trabalho foram: 1) Investigar a concentração das NETs na saliva, no plasma e *in vitro* em indivíduos com AR e controles saudáveis e a associação com a periodontite e atividade da AR; 2) Avaliar o impacto do tratamento periodontal não cirúrgico na concentração das NETs na saliva e no plasma; 3) Investigar a associação entre a presença de polimorfismos de nucleotídeo único no gene codificador da enzima peptidil arginina deaminase 4 (PAD4) com a AR, a produção de NETs *in vitro* e a periodontite; 4) Sistematizar as evidências disponíveis na literatura sobre o efeito do tratamento periodontal não cirúrgico sobre os principais parâmetros clínicos e laboratoriais da AR e *score* de atividade da doença 28 (DAS28). Para atender aos objetivos 1 e 2, a concentração de NETs na saliva, no plasma e na cultura de neutrófilos isolados do sangue periférico foi determinada por meio da identificação do complexo mieloperoxidase (MPO)-DNA com o uso do kit PicoGreen®. Para atender ao objetivo 3 foi realizada a extração do DNA genômico das células mononucleares do sangue periférico de indivíduos com AR e controles e foi realizada a genotipagem para os polimorfismos de nucleotídeo único *PADI4_89*, *PADI4_90*, *PADI4_92* e *PADI_104*. Para atender ao objetivo 4 foi realizada uma *overview* incluindo revisões sistemáticas que avaliaram o efeito do tratamento periodontal não cirúrgico sobre os parâmetros da AR. A busca foi realizada nas principais bases de dados, sem restrição de idioma ou data de publicação. Foi realizada ainda uma meta-análise incluindo dados dos estudos primários identificados nas revisões sistemáticas analisadas. Entre os principais resultados observados: 1) e 2) Indivíduos com AR e com periodontite apresentaram maior concentração de NETs na saliva, no plasma e *in vitro*. O tratamento periodontal não cirúrgico reduziu a concentração de NETs na saliva e plasma de indivíduos com AR. 3) Não foi observada associação entre a presença de genótipos polimórficos e a AR. A presença de um haplótipo homozigoto para o polimorfismo foi associada a uma maior produção de NETs *in vitro* e piores parâmetros periodontais. 4) Foram incluídas na *overview* nove revisões sistemáticas. Os principais desfechos avaliados foram DAS28; proteína C-Reativa e/ou velocidade de hemossedimentação. A meta-análise mostrou que o tratamento periodontal não cirúrgico resultou em diminuição significativa do DAS28. A concentração das NETs na saliva, no plasma e na cultura de neutrófilos de sangue periférico está associada a AR e a periodontite, podendo representar o elo entre as duas doenças. O tratamento periodontal não cirúrgico leva à redução da atividade da AR. Polimorfismos no gene *PADI4* estão associados a produção de NETs *in vitro* e à presença de periodontite.

Palavras-chave: redes extracelulares de neutrófilos; periodontite; artrite reumatoide.

ABSTRACT

Periodontitis associated with rheumatoid arthritis: the role of neutrophil extracellular traps (Nets)

Neutrophil extracellular traps (NETs) are recognized as one of the relevant mechanisms in the pathogenesis of periodontitis and rheumatoid arthritis (RA). However, the participation of NETs as a linking mechanism between the two diseases remains poorly understood. The main objectives of the work were:1) To investigate the concentration of NETs in saliva, plasma, and *in vitro* in individuals with RA and healthy controls and the association of NETs with periodontitis and RA activity; 2) To evaluate the impact of non-surgical periodontal treatment on the concentration of NETs in saliva and plasma; 3) To investigate the association between the presence of single nucleotide polymorphisms in the gene coding for the enzyme peptidyl arginine deaminase 4 (PAD4) with RA, *in vitro* production of NETs, and periodontitis; 4) To systematize the evidence available in the literature on the effect of non-surgical periodontal treatment on the main clinical and laboratory parameters of RA and disease activity score 28 (DAS28). To accomplish Objectives 1 and 2, the concentration of NETs in saliva, plasma, and culture of neutrophils isolated from peripheral blood was determined by identifying the myeloperoxidase (MPO)-DNA complex using the PicoGreen kit®. To accomplish Objective 3, genomic DNA was extracted from peripheral blood mononuclear cells of individuals with RA and healthy controls, and genotyping was performed for single nucleotide polymorphisms *PADI4*_89, *PADI4*_90, *PADI4_92*, and *PADI_104*. To accomplish the Objective 4, an overview, including systematic reviews that evaluated the effect of non-surgical periodontal treatment on RA parameters, was performed. The search was carried out in the main databases, with no restriction on language or date of publication. A meta-analysis, including data from the primary studies identified in the analyzed systematic reviews, was also performed. For Objectives 1 and 2, individuals with RA and periodontitis showed a higher concentration of NETs in saliva, plasma, and *in vitro*. Non-surgical periodontal treatment reduced the concentration of NETs in saliva and plasma of individuals with RA. For Objective 3, no association between the presence of polymorphic genotypes and RA was observed. The presence of a homozygous haplotype for the polymorphism was associated with a higher production of NETs *in vitro* and worse periodontal parameters. For Objective 4, nine systematic reviews were included in the overview. The main outcomes evaluated were DAS28, C-Reactive protein, and/or erythrocyte sedimentation rate. The meta-analysis showed that non-surgical periodontal treatment resulted in a significant decrease in DAS28. The concentration of NETs in saliva, plasma and culture of peripheral blood neutrophils is associated with RA and periodontitis and may represent the link between the two diseases. Non-surgical periodontal treatment leads to reduced RA activity. Polymorphisms in the *PADI4* gene are associated with the *in vitro* production of NETs and with presence of periodontitis.

Keywords: neutrophil extracellular traps; periodontitis; rheumatoid arthritis.

LISTA DE ABREVIATURAS E SIGLAS

ACPAs Anticorpos Anti-proteínas Citrulinadas

AR Artrite Reumatoide

DAS28 Pontuação de Atividade da Doença em 28 Articulações

DMARDs Drogas Antirreumáticas Modificadoras da Doença

EVA Escala Visual Analógica

FR Fator Reumatoide

GM-CSF Fator Estimulador de Colônias de Granulócitos-Macrófagos

H3 Histona Citrulinadas

IL-6 Interleucina-6

IL-28A Interleucina-28A

IL-10 Interleceucina-10

IL-17E Interleucina-17E

IL-25 Interleucina-25

K_2-EDTA Ácido Etileno-Diamino-Tetra Acético Dipotássico

LPS Lipopolissacarídeo

MPO Mieloperoxidase

MTX Metrotexato

NETs Redes Extracelulares de Neutrófilos

PAD4 Peptidilarginina Deiminase 4 (PAD4).

PCR Proteína C Reativa

PCR Reação em Cadeia da Polimerase

SNPs Polimorfismos de Nucleotídeo Único

SPSS Pacote Estatístico para as Ciências Sociais

TNF Fator de Necrose Tumoral

UFMG Universidade Federal de Minas Gerais

VHS Velocidade de Hemossedimentação

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

A artrite reumatoide (AR) é uma doença autoimune de natureza inflamatória que apresenta uma prevalência global de 5 a cada 1000 habitantes, com variações devido à localização geográfica (ALMUTAIRI *et al*., 2021). A doença atinge 2 a 3 vezes mais mulheres do que homens podendo afetar adultos de qualquer idade, com o pico de incidência na sexta década de vida (SHI *et al.,* 2023). A AR é caracterizada principalmente por inflamação da membrana sinovial, dano progressivo da cartilagem e erosão óssea, podendo apresentar manifestações extra articulares resultando em incapacidade funcional (SMOLEN; ALETAHA; MCINNES, 2016).

Uma revolução terapêutica no tratamento da AR na última década marcada principalmente, pelo advento de novas terapias e introdução de terapia precoce modificou os desfechos articulares e sistêmicos da doença (MCINNES; SCHETT, 2017). Tratamentos com drogas antirreumáticas modificadoras da doença (DMARDs) são adotados com o objetivo de reduzir a inflamação e previr ou retardar a progressão do dano articular (SMOLEN *et al.,* 2018). Os DMARDs incluem medicamentos classificadas em: DMARDs sintéticos (por exemplo, o Metotrexato (MTX) e a Leflunomida); DMARDs biológicos que são agentes biológicos direcionados à inibição de alvos específicos como por exemplo, inibidores de fator de necrose tumoral (TNF) e inibidores do receptor de interleucina-6 (IL-6); e DMARDs sintéticos direcionados a vias moleculares específicas como os inibidores de Janus quinase (KERSCHBAUMER *et al.,* 2023). Atualmente a terapia de primeira linha para AR consiste em DMARDs sintéticos em combinação com glicocorticoides. Caso não seja observada melhora em no máximo 3 meses após o início do tratamento ou se a meta terapêutica não for alcançada em 6 meses, terapias combinadas entre DMARDs biológicos e sintéticos devem ser consideradas (SMOLEN *et al.,* 2023).

A patogênese da AR envolve modificações pós-traducionais de proteínas, como a conversão da arginina em resíduos de citrulina pela enzima peptidilarginina deiminase 4 (PAD4). Este processo contribui para a formação de anticorpos anti-proteínas citrulinadas (ACPAs) e para quebra da tolerância imunológica (SMOLEN *et al*., 2018). Em humanos, cinco isoformas de PAD foram descritas, PAD1, PAD2, PAD3, PAD4 e PAD6, distribuídas em diversas células e tecidos (MONDAL; THOMPSON, 2019). Entre estas, a PAD4 que é expressa principalmente em neutrófilos, eosinófilos e monócitos representa a isoforma mais importante para a

autoimunidade e não é ativa em condições de homeostase (NAKASHIMA; HAGIWARA; YAMADA, 2002; VOSSENAAR *et al.,* 2004). A AR apresenta etiologia complexa que envolve contribuições genéticas e ambientais ainda não completamente compreendidas (DEANE *et al.,* 2017). Fatores genéticos como polimorfismos de nucleotídeo único (SNPs) no gene *PADI4* têm sido associados ao aumento da susceptibilidade à AR (BANG *et al.,* 2010; MATUZ-FLORES *et al.*, 2022; SUZUKI *et al.,* 2003; KANG *et al.,* 2006). Além disso, fatores ambientais como tabagismo, disbiose microbiana e presença de periodontite, também podem estar associados à AR (MÖLLER *et al.,* 2020; POTEMPA; MYDEL; KOZIEL, 2017; TONG *et al.,* 2020). Indivíduos com AR apresentam uma incidência aumentada de periodontite, e ambas as doenças compartilham características fisiopatológicas importantes (BOLSTAD; FEVANG; LIE, 2023; DE PABLO *et al.,* 2008; POTEMPA; MYDEL; KOZIEL, 2017; XIAO *et al.,* 2021).

A periodontite é uma doença infecciosa crônica multifatorial que apresenta um impacto na saúde sistêmica (HAJISHENGALLIS *et al.,* 2015). Suas características primárias incluem a perda de suporte tecidual periodontal, manifestada clinicamente como perda de inserção clínica, perda óssea alveolar, presença de bolsas periodontais e sangramento gengival (PIHLSTROM; MICHALOWICZ; JOHNSON, 2005). A periodontite é reconhecida como uma das doenças bucais mais prevalentes em toda a população (EKE; BORGNAKKE; GENCO, 2020; KWON; LAMSTER; LEVIN, 2021) e foi relatada como a condição dentária mais comumente associada a doenças sistêmicas crônicas, como a AR (SEITZ *et al.,* 2019; KAPILA, 2021). Dessa forma, a doença representa um grande problema de saúde pública, pois além de resultar na perda dentária, pode também afetar negativamente a função mastigatória e estética, ser fonte de desigualdade social e prejudicar a qualidade de vida (PERES *et al.*, 2019). O tratamento inicial adequado para periodontite com o objetivo de desmantelar o biofilme bacteriano, envolve principalmente tratamento periodontal não cirúrgico por meio de raspagem e alisamento radicular, além de orientações sobre higiene oral (HEITZ-MAYFIELD; LANG, 2013; KWON; LAMSTER; LEVIN, 2021). Assim como na AR, a presença de uma comunidade microbiana disbiótica também está associada à patogênese da periodontite, via estimulação de resposta inata e ativação de células residentes a produzirem mediadores inflamatórios (HAJISHENGALLIS; LAMONT, 2012; POTEMPA; MYDEL; KOZIEL, 2017). Essa cascata de eventos resulta por fim no estabelecimento de uma inflamação crônica

com impactos sistêmicos, no aumento significativo de moléculas inflamatórias e no recrutamento de neutrófilos hiper-reativos (HAJISHENGALLIS *et al.,* 2015; POTEMPA; MYDEL; KOZIEL, 2017).

A inter-relação entre a AR e a periodontite foi proposta baseada principalmente na ação de microrganismos periodontopatogênicos na AR (KRIAUCIUNAS *et al.,* 2019); na propagação da inflamação pelo modelo *two-hit* (GOLUB *et al.,* 2006); e na produção de autoanticorpos (ROSENSTEIN *et al.,* 2004). Bactérias associadas à periodontite, como a *Porphyromonas gingivalis,* são capazes de promover a formação de neoantígenos por meio da ativação de PADs específicas (PPADs) que induzem modificações pós-traducionais como citrulinação (VITKOV *et al.,* 2018; WEGNER *et al.,* 2010). Estudos comprovam que proteínas citrulinadas geralmente detectadas na sinóvia, também são expressas na mucosa gengival inflamada (LEE *et al.,* 2021). Desta forma, a inflamação persistente em tecidos periodontais doentes pode perpetuar a geração de antígenos e, assim, servir como reservatório de autoanticorpos (EEZAMMUDDEEN; VAITHILINGAM; HASSAN, 2023; GONZALEZ *et al.,* 2015). Estudos recentes mostraram que a periodontite participa ativamente da quebra da tolerância imunológica via ativação de células inflamatórias e geração de autoanticorpos (BREWER *et al.,* 2023). Outro mecanismo importante compartilhado pela AR e pela periodontite é a geração das redes extracelulares de neutrófilos (NETs) (WANG *et al.,* 2021; WRIGHT; MOOTS; EDWARDS, 2014).

A ativação da PAD4 participa da formação das NETs por meio da catalisação da citrulinação de histonas e outras moléculas, gerando descondensação da cromatina e posterior liberação das NETs (LI *et al.,* 2010). As NETs são caracterizadas por redes fibrosas secretadas por neutrófilos ativados compostas principalmente por DNA e proteínas como histonas, elastase de neutrófilos e mieloperoxidase (MPO) (BRINKMANN *et al.,* 2004). Muitos estímulos podem induzir a formação de NETs como citocinas inflamatórias, microrganismos e lipopolissacarídeos (LPS) (AQUINO-MARTINEZ *et al.,* 2020). Embora as NETs exerçam função antimicrobiana, a formação exacerbada e/ou a eliminação retardada das mesmas podem perturbar a homeostase e resultar em danos teciduais relacionados a progressão tanto da periodontite (JIANG *et al.,* 2021; MAGÁN-FERNÁNDEZ *et al.,* 2020) quanto da AR (KHANDPUR *et al.,* 2013; PRATESI *et al.,* 2014). Dessa forma, em indivíduos com periodontite, as NETs podem representar uma

importante fonte de autoantígenos citrulinados que contribuem para a progressão da AR (O'NEIL; KAPLAN, 2019; POTEMPA; MYDEL; KOZIEL, 2017).

O desenvolvimento desse trabalho se justifica pela necessidade de compreensão do papel das NETs na AR e na periodontite, possibilitando assim o desenvolvimento de terapias adjuvantes baseadas na inibição da produção das NETs. Além disso, investigamos a associação entre polimorfismos do gene *PADI4* e a susceptibilidade à AR, assim como a influência da presença de genótipos polimorfos na produção das NETs e consequentemente na periodontite. Por fim, devido a inter-relação entre a AR e a periodontite, realizamos uma busca sistemática na literatura a fim de avaliar se o tratamento periodontal não cirúrgico influencia na atividade da doença, podendo representar uma terapia adjuvante ao tratamento antirreumático.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a associação entre as NETs e a periodontite em indivíduos com AR.

2.2 Objetivos específicos

a) Comparar a concentração das NETs na saliva e no plasma de indivíduos controles saudáveis e indivíduos com AR de acordo com o status periodontal e com a classificação da AR;

b) Avaliar a associação entre a concentração das NETs na saliva e no plasma e os parâmetros clínicos periodontais e a atividade da doença;

c) Avaliar efeito do tratamento periodontal não cirúrgico em indivíduos com AR sobre a concentração das NETs e dos mediadores inflamatórios na saliva e no plasma;

d) Investigar a produção das NETs na cultura de neutrófilos isolados do sangue periférico de indivíduos controles saudáveis e indivíduos com AR e determinar se a presença do LPS de *Porphyromonas gingivalis* influencia na produção das NETs;

e) Investigar a associação entre a produção das NETs na cultura de neutrófilos de indivíduos com AR, a presença da periodontite e a atividade da doença;

f) Caracterizar a presença das NETs em tecido gengival parafinizado através da marcação por imunofluorescência de células MPO/histonas citrulinas H3 positivas e comparar o número de células positivas de acordo com o status periodontal;

g) Investigar a relação entre SNPs no gene *PADI4* e o risco de AR;

h) Investigar a associação entre a presença de SNPs no gene *PADI4,* a produção de NETs na cultura de neutrófilos e os parâmetros clínicos periodontais de indivíduos com AR;

i) Realizar uma avaliação geral das revisões sistemáticas disponíveis na literatura com o objetivo de identificar as evidências sobre o efeito do tratamento periodontal não cirúrgico nos parâmetros clínicos e laboratoriais da AR, principalmente sobre a atividade da doença.

3 METODOLOGIA

3.1 Aspectos éticos

Trata-se de um estudo desenvolvido em dois serviços: o Ambulatório de Reumatologia do Hospital das Clínicas da Universidade de São Paulo, Ribeirão Preto e o Ambulatório de Reumatologia do Hospital das Clínicas da Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brasil. O estudo foi aprovado pelo Comitê de Ética em Pesquisa da UFMG (Protocolo CAAE: 03128012.0.0000.5149/2012) e pelo Comitê de Ética em Pesquisa da Universidade de São Paulo (Protocolo: 05934818.3.0000.5440). Além disso, o trabalho seguiu todos os princípios éticos para o desenvolvimento de pesquisas médicas envolvendo seres humanos recomendados na Declaração de Helsinque. Os pacientes que aceitaram participar assinaram um termo de consentimento livre e esclarecido.

3.2 População do estudo

Os indivíduos foram selecionados nos dois serviços de Reumatologia no período de agosto de 2018 a dezembro de 2021. O principal critério de inclusão correspondia a indivíduos maiores de 18 anos com diagnóstico de AR de acordo com as diretrizes do *American College of Rheumatology* e do Comitê Executivo da Liga Europeia contra o Reumatismo (ALETAHA *et al.,* 2010).

Após a definição do diagnóstico, os seguintes critérios de exclusão foram aplicados:

- Indivíduos portadores de outras doenças reumáticas;
- Indivíduos portadores de diabetes descompensada;
- Diagnóstico e/ou tratamento de neoplasias malignas nos últimos 5 anos;
- Realização de tratamento para periodontite nos últimos seis meses;
- Uso de aparelhos ortodônticos;
- Uso de antibióticos nos últimos três meses;
- Necessidade de profilaxia antibiótica para endocardite infecciosa durante a realização de procedimentos;
- Gravidez ou lactação;
- Presença de menos de oito dentes naturais.

Os participantes que atenderam aos critérios de elegibilidade foram convidados a participar. Os que aceitaram foram classificados de acordo com o tempo

de sintomas da AR: o grupo AR inicial foi composto pelos indivíduos que apresentavam 24 meses de sintomas da doença ou menos; e o grupo AR estabelecida foi composto pelos indivíduos que apresentavam mais de 24 meses de sintomas (MITCHELL; PISETSKY, 2007). Além disso, voluntários controles saudáveis foram selecionados após entrevista a respeito do histórico médico e os mesmos critérios de exclusão foram aplicados. Os indivíduos que aceitaram participar, compuseram o grupo controle.

3.3 Avaliação reumatológica

Através de uma revisão dos prontuários médicos dos pacientes incluídos, os seguintes dados foram registrados: níveis de fator reumatoide (FR, U/mL), ACPA, (U/mL), proteína C reativa (PCR) (mg/L) e velocidade de hemossedimentação (VHS) (mm/h). Dados sobre a dor avaliada através de uma escala visual analógica (EVA), também foram coletados (BIRD; DICKSON, 2001). O *score* da atividade da doença em 28 articulações (DAS28), que é um índice composto pela contagem de articulações sensíveis, contagem de articulações inchadas, pontuação na EVA e concentração de PCR ou VHS, também foi registrado (INOUE *et al.*, 2007). A atividade da doença foi então categorizada segundo a gravidade em: remissão, baixa, moderada e alta (PREVOO *et al.,* 1995). Além disso, a resposta ao tratamento antirreumático avaliada pelo médico reumatologista de acordo com critérios pré-estabelecidos, também foi registrada (ALETAHA; SMOLEN, 2018).

3.4 Avaliação periodontal

3.4.1 Parâmetros periodontais

Todas as avaliações periodontais foram realizadas por examinadores devidamente treinados e calibrados com o auxílio de sondas periodontais (Modelo PCP15, Hu-Friedy®, Chicago, Illinois, EUA), espelho clínico e gaze. Terceiros molares, dentes com cárie dentária extensa, dentes que apresentavam procedimentos restauradores iatrogênicos e presença excessiva de cálculo que impediam a sondagem periodontal foram excluídos da análise (COSTA *et al.,* 2009). Os parâmetros clínicos foram obtidos através da medida circunferencial dos seguintes sítios em cada dente (distal, mesial, vestibular, palatino/lingual):

- Profundidade de sondagem: A profundidade de sondagem foi obtida mensurando-se a distância entre margem gengival ao fundo do sulco gengival ou bolsa periodontal.
- Nível de inserção clínica: O nível de inserção clínica foi determinado pela distância entre a junção amelocementária e o fundo do sulco gengival ou bolsa periodontal.
- Índice de sangramento gengival: O teste de sangramento foi realizado durante o exame de sondagem, mediante a introdução cuidadosa da sonda no sulco gengival até o limite de sua base. A interpretação do sangramento foi realizada 30 segundos após a sondagem.
- Índice de placa: O índice de placa foi analisado através da inspeção do acúmulo de debris moles e mineralizados em duas superfícies dentais (vestibular e lingual/palatina). Para o cálculo do índice, o número de faces positivas para a presença de placa foi dividido pelo número de dentes presentes multiplicado por dois (modificado de LÖE, 1967).

3.4.2 Classificação da periodontite

Após a realização do exame periodontal, os indivíduos foram classificados de acordo com os seguintes critérios (EKE *et al.,* 2012):

- Periodontite leve: pelo menos dois sítios interproximais com perda de inserção clínica ≥ 3 mm e dois ou mais sítios interproximais com profundidade de sondagem ≥ 4 mm em dentes diferentes; ou um sítio com profundidade de sondagem ≥ 5 mm;
- Periodontite moderada: pelo menos dois sítios interproximais com perda de inserção clínica ≥ 4mm em dentes diferentes ou dois sítios interproximais com profundidade de sondagem ≥ 5 mm também em dentes diferentes.
- Periodontite severa: pelo menos dois sítios interproximais com perda de inserção clínica ≥ 6 mm em dentes diferentes, e pelo menos 1 sítio interproximal com profundidade de sondagem ≥ 5mm.

Os participantes foram classificados também de acordo com a classificação de 2018 para periodontite e divididos em dois grupos: indivíduos sem periodontite (saúde periodontal e estágio I) e indivíduos com periodontite (estágios II, III ou IV), segundo o critério (TONETTI; GREENWELL; KORNMAN, 2018):

- Perda de inserção clínica interproximal em 2 ou mais dentes não adjacentes ou perda de inserção clínica vestibular ou lingual ≥ 3 mm com profundidade de sondagem > 3 mm em dois ou mais dentes.

Entretanto é importante salientar que, a perda de inserção clínica observada não pode ser atribuída a causas não periodontais, como:

a) recessão gengival de origem traumática;

b) cárie dentária que se estenda até a região cervical do dente;

c) presença de perda de inserção clínica na face distal de um segundo molar associada ao mau posicionamento ou extração de um terceiro molar;

d) lesão endodôntica drenando através do periodonto marginal; e

e) ocorrência de fratura radicular vertical.

3.5 Tratamento periodontal não cirúrgico

O tratamento periodontal não cirúrgico (raspagem de boca inteira e alisamento radicular) foi realizado com curetas periodontais manuais e dispositivos ultrassônicos em uma única sessão nos indivíduos com AR. Além disso, instruções a respeito da manutenção da saúde bucal também foram fornecidas aos participantes (HEITZ-MAYFIELD; LANG, 2013; KWON; LAMSTER; LEVIN, 2021). Os dados clínico periodontais, assim como as amostras de saliva e plasma, foram coletados antes e 45 dias após a realização do tratamento periodontal não cirúrgico.

3.6 Coleta de material biológico

3.6.1 Amostra de saliva

A coleta de saliva foi realizada sempre no período da manhã. Os indivíduos foram orientados a não comer ou beber por pelo menos uma hora antes da coleta, e não realizar movimentos de deglutição e fala por pelo menos 5 minutos antes da coleta. A saliva não estimulada foi coletada pelo método de cuspir, por meio do qual os participantes foram instruídos a cuspir em um tubo de coleta estéril durante cinco minutos (KHURSHID *et al.,* 2016). Em seguida, todas as amostras foram divididas em alíquotas e armazenadas a uma temperatura de -70°C.

3.6.2 Amostra de sangue periférico

Amostras de sangue periférico (15 ml) foram coletadas em tubos contendo ácido etileno-diamino-tetra acético dipotássico (K_2-EDTA) (BD VacutainerTM, Franklin

Lakes, Nova Jersey, EUA) e centrifugadas a 450 *g* por 10 minutos em temperatura ambiente para obtenção do plasma. Em seguida, as amostras de plasma foram alíquotadas e armazenadas a uma temperatura de -70°C. As amostras de sangue periférico contidas no tubo (que foi separado do plasma) foram utilizadas, então, para o isolamento de neutrófilos (protocolo a seguir).

3.7 Experimento *in vitro*

3.7.1 Isolamento dos neutrófilos humanos para a produção das NETs na cultura estimulada e não estimulada por *Porphyromonas gingivalis*

Os neutrófilos foram isolados do sangue periférico através da separação por gradiente de densidade. Resumidamente, 2 ml de Percoll® (Sigma-Aldrich, San Luis, Missouri, EUA) de densidade 72%, 63%, 54% e 45% foram cuidadosamente colocados um sobre o outro em ordem decrescente de densidade em um tubo falcon estéril de 15 ml. Em seguida, 3 ml de sangue periférico total diluído 1:1 em meio *Hanks balanced* (Sigma-Aldrich, San Luis, Missouri, EUA) foi adicionado sobre os gradientes e centrifugado por 30 minutos, a 650 *g*, 22°C. Após a centrifugação, os leucócitos polimorfonucleares acumularam-se como uma banda entre os gradientes 72% e 63%. Essas células foram então coletadas e as hemácias foram lisadas através da utilização de um tampão de lise celular. O número total de células foi contado usando microscopia de luz e a porcentagem de neutrófilos viáveis foi determinada microscopicamente através da coloração azul Turky com o auxílio de uma câmara de *Neubauer.* Em uma placa de poliestireno de 48 poços, um milhão de neutrófilos foram incubados em meio *Hanks* com o objetivo de analisar a produção não estimulada das NETs pelos neutrófilos. Em outro poço, contendo o mesmo número de neutrófilos, foi adicionado LPS de *Porphyromonas gingivalis* (Heat Killed *P. Gigivalis*; Invivo Gen, San Diego, Ca, EUA) na concentração de 1 µg/µL (CHEN *et al.,* 2022) com o objetivo de analisar a produção das NETs estimulada. A placa foi então encubada a 25°C, 5% de dióxido de carbono (CO_2) por 4 horas. Em seguida, o sobrenadante foi recolhido e armazenado a -70°C para posterior quantificação das NETs (SCHNEIDER *et al.,* 2021).

3.8 Análises laboratoriais

3.8.1 Análise da concentração das NETs na saliva, no plasma e no sobrenadante da cultura de neutrófilos

Para a quantificação da concentração das NETs na saliva, no plasma e na cultura de neutrófilos, 50 µL de cada amostra foram pipetados em uma placa preta de fundo transparente de 96 poços coberta com anticorpo anti-MPO (PA5-16672, diluição 1:500, Invitrogen, Carlsbad, CA, EUA). A concentração das NETs foi determinada por fluorescência usando o kit Quant-iT™ PicoGreen®, através da quantificação do complexo MPO-DNA (CZAIKOSKI *et al.,* 2016; SCHNEIDER *et al.,* 2021). A intensidade de fluorescência (excitação a 488 nm e emissão a 525 nm de comprimento de onda) foi determinada por um leitor de microplacas *FlexStation 3* (Molecular Devices, San Jose, CA, EUA).

3.8.2 Análise da concentração de mediadores inflamatórios na saliva e no plasma

As concentrações dos mediadores inflamatórios TNF-α, lL-28A, IL-6, IL-10, IL-17E/IL-25 e fator estimulador de colônias de granulócitos-macrófagos (GM-CSF) foram determinadas usando o Milliplex® MAP *Human cytokine/chemokine Magnetic Bead Panel* (Merck, Darmstadt, Alemanha) através de citometria de fluxo (Luminex® 200™ FLEXMAP 3D™, Austin, TX, EUA). Os testes foram realizados de acordo com as instruções do fabricante.

3.9 Identificação das NETs em tecido gengival parafinizado

Amostras de tecido gengival foram obtidas de indivíduos com AR que necessitaram de cirurgia para realização de exodontias. As amostras foram fixadas com paraformaldeído tamponado a 10% por 48 horas, processadas e incluídas em parafina. Os blocos de parafina foram seccionados em série usando um micrótomo em seções de 4 µm. As lâminas foram então desparafinizadas em xilol e reidratadas. Para a recuperação antigênica, as lâminas foram imersas em solução de citrato (pH:6,0), e incubadas em banho maria a 95 °C por 20 minutos. O bloqueio das proteínas foi realizado em temperatura ambiente com albumina de soro bovino 2% e Triton® X-100 (Sigma-Aldrich®, Saint Louis, MO, EUA) por 2 horas.

Posteriormente, as lâminas foram incubadas durante a noite com anticorpos primários anti-histona citrulinada H3 fabricado em coelho (1:100) (Thermo Fisher Scientific®, Waltham, MA, EUA) e com anti-MPO fabricado em camundongo (1:50) (Thermo Fisher Scientific®, Waltham, MA, EUA). A detecção foi realizada utilizando fluoróforos associados a anticorpos secundários anti-coelho (AlexaFluor 488) (1:2000) e anti-camundongo (AlexaFluor 594) (1:1250) (Thermo Fisher

Scientific®, Waltham, MA, EUA). Os núcleos foram corados com 4',6-diamidino-2-fenilindol (DAPI) (Abcam®, Cambridge, Reino Unido) e os cortes foram analisados em um microscópio de fluorescência ZEISS Axioscope 5 (ZEISS®, Oberkochen, Alemanha). Cada campo foi fotografado com um laser a 405 nm (DAPI—azul), 488 nm (FITC—verde) e 594 nm (RHOD—vermelho), e as imagens foram processadas e combinadas em uma imagem mesclada. A presença das NETs foi confirmada através da co-localização entre DAPI, MPO e histona citrulinada H3. As células positivas foram contadas em 10 campos consecutivos em ampliação de 400x.

3.10 Genotipagem para polimorfismos *PADI4*

O DNA genômico foi extraído de células mononucleares do sangue periférico usando o *QIAamp® DSP DNA Blood Mini Kit* (QIAGEN®, Hilden, Alemanha). A qualidade e a concentração do DNA extraído foram determinadas usando um espectrofotômetro *Nano Drop* (Nano Drop 2000, Thermo Scientific, EUA). Todos os participantes com AR e voluntários controle foram genotipados para 4 SNPs: *PADI4_89* (rs11203366), *PADI4_90* (rs11203367), *PADI4_92* (rs874881) e *PADI4_104* (rs1748033) usando *TaqMan™ SNP Genotyping Assay* (Thermo Fisher Scientific®, Waltham, MA, EUA) pelo sistema *StepOnePlus Real-Time PCR* (Applied Biosystems, Foster City, EUA). Para realizar a amplificação por reação em cadeia da polimerase (PCR), cada mistura continha 1 µl de DNA extraído (com concentração de 20 ng/µl), 10 µl de *TaqMan Master Mix*, 0,5 µl *TaqMan Genotyping Assay mix* (contendo primers pré-concebidos e sondas marcadas com FAM ou VIC; Applied Biosystems, Foster City, EUA) e água destilada até o volume final de 20 µl. As condições de termociclagem para amplificação por PCR foram: desnaturação inicial a 94°C por 3 min seguida de 35 ciclos de 30 s a 94°C, 30 s a 56°C e 30 s a 72°C; em seguida, foi realizada uma extensão final de 1 min a 72°C. A determinação dos alelos em cada amostra foi realizada por meio da análise dos gráficos de discriminação alélica por meio do software *TaqMan® Genotyper* (Thermo Fisher Scientific®, Waltham, MA, EUA).

3.11 Análise estatística

O *Statistical Package for the Social Sciences* (SPSS) (versão 25.0, Armonk, EUA) e o *GraphPad Prism* (versão 7.00, La Jolla, CA, EUA) foram empregados para as análises estatísticas. A análise descritiva foi aplicada aos dados clínico-

demográficos. O teste Shapiro-Wilk foi utilizado para verificar a normalidade da distribuição dos dados. Quando a distribuição normal foi observada, testes paramétricos foram adotados (teste *t* de *Student,* teste *t* pareado e ANOVA), e os resultados das análises das variáveis contínuas foram relatados como média (± desvio padrão). Quando observada distribuição não normal, testes não paramétricos foram utilizados (Mann-Whitney, teste Wilcoxon), e os resultados foram descritos como mediana (intervalo interquartil). O teste do qui-quadrado de Pearson foi utilizado para avaliar as diferenças entre os grupos quanto às variáveis categóricas. A regressão logística foi adotada para calcular a *odds ratio* entre os genótipos observados e os grupos controles saudáveis e AR. Para todas as análises, o nível de significância adotado foi $p < 0,05$.

3.12 Desenho do estudo e critérios de elegibilidade

Trata-se de uma avaliação geral de revisões sistemáticas *(overview)* realizada com o objetivo de construir evidências mais robustas sobre o efeito do tratamento periodontal não cirúrgico na AR. Foram incluídas revisões sistemáticas com e sem meta-análise avaliando o efeito do tratamento periodontal não cirúrgico nos parâmetros clínicos e bioquímicos da AR, bem como na atividade da doença.

A definição de revisões sistemáticas foi realizada com base principalmente nos seguintes requisitos (GREEN *et al.,* 2011):

• um conjunto claro de objetivos com critérios de elegibilidade pré-definidos para os estudos;

• uma metodologia explícita e reproduzível;

• uma busca sistemática em múltiplas bases que tem como objetivo identificar todos os estudos que atenderiam aos critérios de elegibilidade;

• uma apresentação sistemática e síntese das características e achados dos estudos incluídos.

A pergunta do PICOS adotada foi a seguinte:

P (População): Indivíduos com AR;

I (Intervenção): Tratamento periodontal não cirúrgico;

C (Comparação): a) Grupo que não realizou tratamento periodontal não cirúrgico; b) avaliação antes do tratamento periodontal não cirúrgico (linha de base);

O (Resultado): atividade da doença (DAS28), contagem de articulações sensíveis, contagem de articulações inchadas, EVA, rigidez matinal, VHS e PCR;

S (Estudos): Revisões sistemáticas com ou sem meta-análises.

Os critérios de exclusão foram estudos em animais e estudos *in vitro*, revisões críticas e narrativas, cartas ao editor, opiniões de especialistas e resumos de conferências ou reuniões.

3.13 Fontes de busca

As buscas eletrônicas foram realizadas em abril de 2023 e atualizadas em junho de 2023 nas seguintes bases de dados: *PubMed (National Library of Medicine), Scopus (Elsevier), Embase (Elsevier)* e *Web of Science (Thomson Reuters*). A literatura cinza (*Open Grey*) e o *Google Scholar* também foram examinados, com buscas limitadas aos 200 primeiros resultados (HADDAWAY *et al.,* 2015). Também foram realizadas buscas manuais na lista de referências dos artigos incluídos.

Referências duplicadas em diferentes bancos de dados foram encontradas e removidas usando o programa *EndNote* (EndNote®, Clarivate Analytics, Toronto, Canadá). Não foram impostas restrições quanto ao idioma, data de publicação ou localização geográfica.

3.14 Estratégia de busca

As estratégias de busca adotadas nas bases de dados e os operadores booleanos que ligam termos e palavras-chave são detalhadas na Tabela 1:

Tabela 1- Estratégias de busca empregadas para identificar os artigos nas bases de dados

Base de dados	**Estratégia de pesquisa**
PubMed	Periodontite OU Doença periodontal OU Gengivite OU Infecção periodontal OU Doença da gengiva OU Inflamação periodontal OU Inflamação gengival OU Condição periodontal OU Profundidade de sondagem OU Sondagem periodontal OU Sangramento à sondagem OU Perda de inserção clínica OU Nível clínico de inserção OU Placa E Terapia periodontal OU Tratamento periodontal OU Raspagem OU Alisamento radicular OU Tratamento periodontal não cirúrgico OU Terapia periodontal não cirúrgica OU Tratamento bucal completo OU Raspagem bucal completa OU Desinfecção bucal completa E Artrite reumatoide OU Artrite
Web of Science	Periodontite OU Doença periodontal OU Gengivite OU Infecção periodontal OU Doença da gengiva OU Inflamação periodontal OU Inflamação gengival OU Condição periodontal OU Profundidade de sondagem OU Sondagem periodontal OU Sangramento à sondagem OU Perda de inserção clínica OU Nível clínico de inserção OU Placa E Terapia periodontal OU Tratamento periodontal OU Raspagem OU Alisamento radicular OU Tratamento periodontal não cirúrgico OU Terapia periodontal não cirúrgica OU Tratamento bucal completo OU Raspagem bucal completa OU Desinfecção bucal completa E Artrite reumatoide OU Artrite

Embase	Periodontite OU “Doença periodontal” OU Gengivite OU “Infecção periodontal” OU “Doença da gengiva” OU “Inflamação periodontal” OU “Inflamação gengival” OU “Condição periodontal” OU “Profundidade de sondagem” OU “Sondagem periodontal” OU “Sangramento à sondagem” OU “Perda clínica de inserção” OU “Nível clínico de inserção” OU Placa E “Terapia periodontal” OU “Tratamento periodontal” OU Raspagem OU “Alisamento radicular” OU “Tratamento periodontal não cirúrgico” OU “Terapia periodontal não cirúrgica” OU “Tratamento bucal completo” OU “Raspagem bucal completa” OU “Desinfecção bucal completa” E “Artrite Reumatoide” OU Artrite
Scopus	Periodontite OU “Doença periodontal” OU Gengivite OU “Infecção periodontal” OU “Doença da gengiva” OU “Inflamação periodontal” OU “Inflamação gengival” OU “Condição periodontal” OU “Profundidade de sondagem” OU “Sondagem periodontal” OU “Sangramento à sondagem” OU “Perda clínica de inserção” OU “Nível clínico de inserção” OU Placa E “Terapia periodontal” OU “Tratamento periodontal” OU Raspagem OU “Alisamento radicular” OU “Tratamento periodontal não cirúrgico” OU “Terapia periodontal não cirúrgica” OU “Tratamento bucal completo” OU “Raspagem bucal completa” OU “Desinfecção bucal completa” E “Artrite Reumatoide” OU Artrite
Google Scholar	(Periodontite OU “Doença periodontal” OU Gengivite OU “Infecção periodontal” OU “Doença da gengiva” OU “Inflamação periodontal” OU “Inflamação gengival” OU “Condição periodontal” OU “Profundidade de sondagem” OU “Sondagem periodontal” OU “Sangramento à sondagem” OU “Perda clínica de inserção” OU “Nível clínico de inserção” OU Placa) E (“Terapia periodontal” OU “Tratamento periodontal” OU Raspagem OU “Alisamento radicular” OU “Tratamento

	periodontal não cirúrgico” OU “Terapia periodontal não cirúrgica” OU “Tratamento bucal completo” OU “Raspagem bucal completa” OU “Desinfecção bucal completa”) E (“Artrite Reumatoide” OU Artrite)

Fonte: Elaborado pela autora, 2023.

3.15 Seleção dos estudos

A seleção dos estudos foi realizada em duas fases. Na fase 1, os títulos/resumos de todas as referências recuperadas durante a busca foram lidos independentemente por dois autores (S.R.O. e T.A.S.). As referências cujos títulos/resumos atenderam aos critérios de elegibilidade foram incluídas imediatamente. Quando o título/resumo não estava disponível ou não trazia informações suficientes para a decisão de inclusão ou exclusão, o texto completo foi lido e avaliado, e os mesmos critérios de elegibilidade descritos acima foram aplicados na fase 2. Artigos que atenderam a esses critérios nesta fase também foram incluídos. Discordâncias entre os dois autores foram resolvidas através de discussão com um terceiro autor (L.G.A.) até o consenso.

3.16 Extração dos dados

As seguintes informações foram extraídas de cada revisão sistemática incluída: sobrenome do primeiro autor, ano de publicação, país onde o estudo foi realizado, bases de dados nas quais as buscas foram realizadas, período em que a busca foi realizada, restrição de idioma, critérios de inclusão e exclusão aplicados, realização de meta-análise, realização de análise de risco de viés, número de artigos incluídos, número de indivíduos incluídos, tempo de acompanhamento, principais achados após o tratamento periodontal não cirúrgico. A extração de dados foi realizada por dois autores de forma independente (S.R.O. e T.A.S.), e as divergências entre os autores foram resolvidas por meio de discussão.

3.17 Síntese dos resultados (análise quantitativa)

Primeiramente, os dados extraídos das revisões sistemáticas incluídas foram tabulados com o auxílio do *Microsoft Office Excel 2019* (software Microsoft®, Redmond, WA, EUA) e analisados descritivamente.

Meta-análises dos estudos primários incluídos nas revisões sistemáticas foram realizadas usando o *Review Manager 5.3* (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). O objetivo foi reunir dados de estudos primários incorporados em meta-análises anteriores e dados de estudos primários que não haviam sido previamente meta-analisados (HIGGINS *et al.,* 2022).

A realização de quatro meta-análises foram viáveis: 1) uma meta-análise comparando o DAS28 antes (linha de base) e no acompanhamento ≤3 meses após o

tratamento periodontal não cirúrgico; 2) uma meta-análise comparando o DAS28 antes (linha de base) e no acompanhamento 6 meses após o tratamento periodontal não cirúrgico; 3) uma meta-análise comparando a mudança no DAS28 após um acompanhamento ≤3 meses entre indivíduos que não realizaram nenhum tratamento periodontal não cirúrgico e indivíduos que realizaram o tratamento periodontal não cirúrgico e; 4) uma meta-análise comparando a mudança no DAS28 após um acompanhamento de 6 meses entre indivíduos que não realizaram nenhum tratamento periodontal não cirúrgico e indivíduos que realizaram o tratamento periodontal não cirúrgico.

Nas quatro meta-análises, foram utilizados os dados de média ($\bar{X}$) e desvio padrão (S) do DAS28, bem como tamanho da amostra. Quando os dados do DAS28 fornecidos pelos estudos foram expressos em mediana e intervalo interquartil, foram utilizadas as seguintes equações para obtenção de $\bar{X}$ e S (WAN *et al.,* 2014):

$$\bar{X} \approx \frac{q1 + m + q3}{3}$$

$$S \approx \frac{q3 - q1}{1.35}$$

$\bar{X}$= média
q1 = primeiro quartil
m = mediana
q3 = terceiro quartil
S= desvio padrão

Quando os dados do DAS28 fornecidos eram expressos em mediana e mínimo e máximo, foi utilizada a seguinte equação para obtenção dos dados de $\bar{X}$ e S (WAN *et al.,* 2014):

$$\bar{X} \approx \frac{a + 2m + b}{4}$$

$$S \approx \frac{b - a}{4}$$

$\bar{X}$= média
a = valor mínimo

m = median
b = valor máximo
S= desvio padrão

Nas duas meta-análises comparando indivíduos submetidos ao tratamento periodontal não cirúrgico e indivíduos não submetidos ao tratamento periodontal não cirúrgico, a diferença média e o S agrupado do DAS28 entre o acompanhamento (≤3 meses ou 6 meses) e a linha de base foram obtidos com as seguintes equações (COHEN, 1988):

$$\bar{X}_{diferença} \approx \bar{X}_{acompanhamento} - \bar{X}_{linha\ de\ base}$$

$$S\ agrupado = \sqrt{\frac{(S_{acompanhamento})^2 + (S_{linha\ de\ base})^2}{2}}$$

$\bar{X}$= média
S= Desvio padrão

Em todas as análises, foi utilizado o modelo de efeitos aleatórios (BORENSTEIN *et al.*, 2010). Os resultados das meta-análises foram relatados em diferença média e intervalo de confiança de 95%.

3.18 Avaliação crítica dos estudos

A avaliação da qualidade foi realizada de forma independente por dois autores (S.R.O. e T.A.S.), e as discordâncias foram resolvidas por discussão. A metodologia das revisões sistemáticas incluídas foi examinada utilizando a lista de verificação *A MeaSurement Tool* para avaliar revisões sistemáticas (AMSTAR-2) (SHEA *et al.,* 2017).

3.19 Protocolo e registro

O relato desta avaliação geral de revisões sistemáticas está em conformidade com os Itens Preferenciais de Relatórios para Revisões Sistemáticas e Meta-Análise (PRISMA) (PAGE *et al.,* 2021). Seguimos o extenso material novo sobre

métodos de revisões sistemáticas disponível na edição totalmente atualizada do Cochrane Handbook (HIGGINS *et al.,* 2022). Um protocolo foi registrado no Registro Prospectivo Internacional de Revisões Sistemáticas (PROSPERO) sob o número de registro (CRD42023414714).

4 RESULTADOS E DISCUSSÃO

4.1 Artigo 1

Os resultados e a discussão serão apresentados no formato de artigos científicos e seguirão as normas do periódico.

Os resultados relacionados aos objetivos específicos de comparar a concentração das NETs na saliva e no plasma de indivíduos controles saudáveis e indivíduos com AR de acordo com o status periodontal e com a classificação da AR; avaliar a associação entre a concentração das NETs na saliva e no plasma e os parâmetros clínicos periodontais e a atividade da doença; e por fim, avaliar efeito do tratamento periodontal não cirúrgico em indivíduos com AR sobre a concentração das NETs e dos mediadores inflamatórios salivares e plasmáticos; são apresentados no manuscrito intitulado *"Are neutrophil extracellular traps the link for the cross-talk between periodontitis and rheumatoid arthritis physiopathology?"* que foi publicado em Março de 2021 no periódico *Rheumatology* (Fator de impacto: 5.5).

Are neutrophil extracellular traps the link for the cross-talk between periodontitis and rheumatoid arthritis physiopathology?

Abstract

Objectives: Neutrophil extracellular traps (NETs) play a role in the pathogenesis of periodontitis and rheumatoid arthritis (RA). However, it remains poorly understood whether NETs participate in the cross-talk between periodontitis and RA. Herein, we investigated the production of NETs in individuals with periodontitis and RA and its association with clinical parameters. The impact of periodontal therapy on RA and NET release was also assessed.

Methods: The concentration of NETs and cytokines was determined in the saliva and plasma of individuals with early RA (n=24), established RA (n=64), and individuals without RA (n=76). The influence of periodontitis on the production of NETs and cytokines was also evaluated.

Results: Individuals with early RA had a higher concentration of NETs in saliva and plasma than individuals with established RA or without RA. Periodontitis resulted in an increase in the concentration of NETs of groups of individuals without RA and with early RA. The proportion of individuals with high concentrations of IL-6, IL-10 and GM-CSF was higher among individuals with periodontitis than among individuals without periodontitis. The concentrations of TNF-α, IL-6, IL-17/IL-25, and IL-28A were particularly high in individuals with early RA. Worse periodontal clinical parameters, RA onset and RA activity were significantly associated with circulating NETs. Periodontal therapy was associated with a reduction in the concentration of NETs and inflammatory cytokines and amelioration in periodontitis and RA.

Conclusion: This study reveals that NETs are a possible link between periodontitis and RA, with periodontal therapy resulting in a dramatic switch in circulating NET levels.

Keywords: Periodontitis; Rheumatoid arthritis; Neutrophils; Neutrophil extracellular traps.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes progressive articular damage and substantial burden on body functions [1, 2]. Treatment of an individual with RA is quite costly and has been estimated to range from 12,509 to 36,053 dollars annually [3].

RA and periodontitis share very much alike clinical and pathogenic characteristics [4] such as the sustained influx of neutrophils and lymphocytes into synovial cavities [1, 5] and periodontal tissues [6], as well as the release of pro-inflammatory cytokines and bone damage. Importantly, neutrophils contribute to the pathophysiology of periodontitis and RA [4]. Neutrophil activation culminates in the release of neutrophil extracellular traps (NETs) which are composed of DNA containing histones and enzymes derived from granules [7, 8].

NETs have emerged as a relevant antimicrobial mechanism in the pathogenesis of gingivitis and periodontitis [9-11]. Excessive NET release or delayed clearance may provide a source of autoantigens contributing to periodontal damage associated with Papillon-Lefèvre syndrome [12] and leukocyte adhesion deficiency type I [13]. Similarly, NET formation has implications for progression and, consequently, for the pathogenesis of RA [14]. Nevertheless, the understanding of the involvement of NETs in the pathogenesis of both chronic inflammatory diseases and of the contribution of periodontitis to NET formation during RA has yet to be fully clarified. Herein, we investigate NET production in individuals with periodontitis and RA and its association with clinical parameters, as well as the effect of periodontal therapy on NET release. Our hypothesis was that NETs are triggered by the deterioration of periodontal inflammation, contributing to worse RA outcomes.

Methods

Study design and ethical issues

This was a retrospective cross-sectional study shared by two services: the Rheumatology Outpatient Clinic of the Medical School Hospital, University of São Paulo, Ribeirão Preto and the University Hospital, Federal University of Minas Gerais, Belo Horizonte, Brazil. The report of this study conformed to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement [15]. The study was approved by the Ethics Committee (#05934818.3.0000.5440; #03128012.0.0000.5149).

Study population

From August 2018 to December 2019, RA individuals whose diagnosis had been made according to criteria of the American College of Rheumatology [16] were selected for this study. Exclusion criteria is provided in Supplementary Methods file. The participants were divided into two groups according to the time of symptoms of the disease: early RA group (≤24 months) and established RA group (>24 months) [17]. Control healthy volunteers without rheumatic disease were included in the study after an interview about their medical history.

Rheumatological assessment

Information on the rheumatoid factor, anti-citrullinated protein antibody, C-reactive protein (CRP), pain assessed with a visual analogue scale (VAS) [18], erythrocyte sedimentation rate, disease activity score 28 (DAS 28) [19], and response to methotrexate (MTX) treatment was collected. Disease activity was categorized, according to the following DAS 28 cut-off points: remission (≤2.4), low (>2.4-3.6), moderate (>3.6-5.5), and high (>5.5) [20].

Periodontal evaluation

Probing depth, clinical attachment loss (CAL) and bleeding on probing (BOP) were measured at six sites around each tooth with a periodontal probe by calibrated periodontists. The presence or absence of biofilm was evaluated at two sites per tooth [modified from 21]. The periodontitis was classified as mild, moderate, and severe [22]. Non-surgical periodontal therapy was performed with manual periodontal scalers, curettes, and ultrasonic devices in a single session. After 45 days, periodontal clinical data, and DAS 28 information were also collected from all participants.

NET quantification: saliva and plasma sampling

Paired samples of saliva and plasma were collected from the participants for NET quantification before and 45 days after non-surgical periodontal treatment. Unstimulated saliva was collected using the spitting method for five minutes [23]. Peripheral blood samples were collected into tubes containing K_2EDTA (BD Vacutainer™, Franklin Lakes, NJ, USA). Samples were centrifuged at 450× *g* for 10 minutes at room temperature in order to obtain plasma.

The myeloperoxidase-deoxyribonucleic acid complex (MPO-DNA) has been reported as an important component of NETs [7]. The Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to quantify NET levels by identifying the MPO-DNA complex in saliva and plasma [24, 25]. Additional detail is provided in Supplementary Methods file.

Analysis of inflammatory mediators

Salivary concentrations of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-28A, IL-6, IL-10, IL-17E/IL-25, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured with a flow cytometer. Moreover, salivary and plasma levels of IL-8, IL-1, IL-6, IL-10, and TNF-α were measured in individuals with RA before and 45 days after periodontal treatment. Additional detail is provided in Supplementary Methods file.

Data analysis

Statistics details is provided in Supplementary Methods file.

Results

Clinicodemographic data

The general clinicodemographic data of the participants are listed in **Table 1**. A total of 164 individuals were included in this study, 76 of them with no RA, 24 with early RA and 64 with established RA. As regards clinical rheumatologic data, individuals in the early RA group had significantly higher values on the VAS and higher DAS 28 (mean: 65.9 and 4.4, respectively) than individuals in the established RA group (mean: 34.1 and 3.1, respectively) ($p \leq 0.05$). There was also a significant difference between the groups of early RA and established RA regarding the categories of disease activity ($p \leq 0.05$). There was no significant difference between groups with respect to the other clinical rheumatological variables ($p > 0.05$). Regarding the treatment adopted, most individuals in both the early RA group and the established RA group started treatment with MTX, with no statistical difference in response to treatment between these two groups ($p > 0.05$) **(Table 1)**. For the established RA group, the use of other drugs such as anti-TNF (n=4) and Leflunomide (n=29) was also

reported. In the early RA group, no individual used anti-TNF and nine individuals used Leflunomide.

Data on the periodontal status of the participants are also presented in **Table 1**. The mean number of missing teeth was significantly higher ($p \leq 0.05$) among individuals in the early RA group (9.7) and established RA group (7.0) compared to the group of individuals with no RA (3.5). Regarding the status of periodontal disease, most individuals in the early RA (79.2%) and established RA groups (57.8%) had periodontitis. Conversely, most individuals in the group with no RA had no periodontitis (52.6%). A significant difference was observed between the group with early RA and the group with no RA ($p \leq 0.05$).

NET concentration in saliva and plasma was influenced by periodontitis and RA onset

Individuals in the early RA group had a significantly higher concentration of NETs in saliva compared to the group with no RA ($p=0.004$) and the group with established RA ($p=0.043$). The concentration of NETs in the saliva of individuals with established RA was similar to that of individuals with no RA ($p=0.311$) **(Figure 1A)**. The plasma concentration of NETs was also significantly higher in early RA compared to individuals with no RA ($p<0.001$) and individuals with established RA ($p<0.001$). There was no statistically significant difference between the groups with no RA and with established RA ($p=0.953$) **(Figure 1D)**.

In order to better evaluate the influence of periodontitis on NET concentration, individuals with no RA, individuals with early RA and individuals with established RA were subgrouped according to periodontal status. Periodontal status directly influenced NET concentration because individuals with periodontitis, both in the no RA, early RA, and established RA groups, had a higher NET concentration in their saliva compared to individuals without periodontitis ($p<0.001$, $p=0.011$, $p=0.001$, respectively). Individuals with early RA and periodontitis had a higher concentration of NETs in saliva compared to individuals without RA and with periodontitis ($p=0.020$). No significant difference was observed between the groups with early RA and with no RA when comparing individuals without periodontitis ($p=0.614$) **(Figure 1B)**. Individuals with established RA without periodontitis had a higher concentration of NETs in saliva compared to individuals without RA and without periodontitis ($p=0.020$).

There was no statistical difference between individuals with periodontitis of the no RA and established RA groups (p=0.519) **(Figure 1C)**.

Individuals with periodontitis, both in the groups with no RA (p<0.001) and with early RA (p=0.023), exhibited higher plasma NET concentrations than individuals without periodontitis. There was no statistical difference regarding periodontal status in individuals with established RA (p=0.712). Individuals in the early RA group, with and without periodontitis, had significantly higher plasma NET concentrations than individuals with no RA, with and without periodontitis (p<0.001, p=0.003, respectively) **(Figure 1E)**.

Individuals with established RA with and without periodontitis had a significantly higher NET concentration in plasma when compared with individuals without RA, with and without periodontitis (p=0.048, p=0.044, respectively) **(Figure 1F)**. When we analyzed individuals with early RA and established RA grouped together, we observed results similar to those observed when we analyzed these groups separately. Individuals with RA had higher NET concentrations in saliva (p=0.002) and plasma (p=0.030) than individuals without RA **(Supplementary Figure 1)**. Likewise, in the RA group, individuals with periodontitis showed a higher concentration of NETs in saliva (p<0.001) when compared to individuals without periodontitis. There was no statistical difference in the concentration of NETs in plasma and periodontal status in individuals in the RA group (p=0.192). Individuals with RA and without periodontitis had a higher concentration of NETs in both saliva and plasma when compared to individuals without periodontitis and without RA (p≤0.05) **(Supplementary Figure 1)**.

Association between clinical periodontal parameters and salivary NETs

An association between clinical periodontal parameters and NET concentration in saliva was observed in individuals with RA (early + established) **(Figure 2)**. Individuals with a higher concentration of NETs in saliva (median >4.75 ng/mL) had worse periodontal parameters represented by significantly greater probing depth and CAL compared to individuals with a lower concentration of NETs in saliva (median ≤4.75 ng/mL) (p=0.017, p=0.013, respectively) **(Figures 2A-B)**, and also had greater BOP (p=0.050) **(Figure 2C)**. When assessing tooth loss, individuals with no RA and individuals with RA who had four or less missing teeth (median) had lower NET concentrations in saliva than individuals who had more than four missing teeth

(p=0.014) (data not shown). There was no significant association between plasma NET concentration and probing depth, CAL, BOP, or tooth loss (p=0.503, p=0.0.567, p=0.311, p=0.332, respectively) (data not shown).

RA activity impacted NET concentrations in plasma

Concerning RA activity, individuals with high disease activity had elevated plasma NET concentrations compared to individuals with low disease activity (p=0.026). There was no statistically significant difference between individuals with moderate and high disease activity (p=0.329) or between individuals with moderate and low disease activity (p=0.199) **(Figure 2D)**.

Individuals with high DAS 28 (median >3.46) had a significantly higher plasma NET concentration (mean: 7.20±7.23 ng/mL) than individuals with low DAS 28 (median ≤3.46) (mean: 3.57±3.49 ng/mL) (p=0.017). There was no statistically significant association of NET concentration in saliva with disease activity (p>0.05) (data not shown).

The concentration of NETs in saliva and plasma was directly associated with periodontitis

Linear regression models were employed to investigate the association of NET concentrations with health status, periodontitis, tobacco smoking, and age in individuals without RA and in those with RA (early + established). Individuals with periodontitis showed a significantly higher NET concentration in saliva (p<0.001) and in plasma (p=0.047) than individuals with no periodontitis regardless of health status, tobacco smoking or age **(Table 2)**.

Supplementary Table 1 depicts the linear regression model used to analyze individuals with early RA and established RA separately. Individuals with periodontitis showed a significantly higher concentration of NETs in saliva than individuals without periodontitis (p≤0.05), regardless of the influence of health status, tobacco smoking or age, both in the early RA and established RA models. In the model including only the individuals in the early RA group and no RA, individuals with periodontitis also had a higher concentration of NETs in plasma when compared with individuals without periodontitis (p=0.037) and individuals with early RA had a higher concentration of NETs in plasma when compared to individuals with no RA (p<0.001), regardless of the tobacco smoking and age variables. There was no statistical

difference between the concentration of NETs in plasma and the independent variables in the model including only individuals with established RA and no RA ($p>0.05$).

Periodontal therapy significantly reduced NET and cytokine concentrations and improved periodontal and RA clinical parameters

There was a significant influence of periodontal condition on circulating NETs in individuals with RA. Thus, we investigated if non-surgical periodontal therapy would impact NET concentration and disease activity. Fifty-three individuals (early RA: n=16 *vs.* established RA: n=37) underwent periodontal therapy. Periodontal clinical data were collected before and after periodontal therapy **(Table 3)**. A reduction of probing depth and plaque index scores was observed in the early RA group after periodontal therapy ($p\leq0.05$). However, no significant difference was observed in the severity of periodontitis or in the analysis of periodontal parameters such as CAL and BOP ($p>0.05$). A reduction in the number of individuals with moderate periodontitis and severe periodontitis was observed in the established RA group after periodontal therapy ($p\leq0.05$). Significant reductions in the probing depth, CAL, BOP, and plaque index scores were also observed ($p\leq0.05$).

The concentration of NETs and of the inflammatory mediators IL-1, IL-8, IL-6, TNF-α, and IL-10 was analysed before and after periodontal therapy in the saliva and plasma of individuals with early and established RA **(Figure 3)**. There was a significant reduction in the concentrations of NETs ($p=0.031$) **(Figure 3A)**, IL-1 ($p=0.005$) **(Figure 3B)** and IL-8 ($p=0.05$) **(Figure 3C)** in saliva after periodontal therapy, whereas no significant difference was observed in the concentration of IL-6 ($p=0.980$) **(Figure 3D)**, TNF-α ($p=0.530$) or IL-10 ($p=0.930$) in saliva when comparing the levels before and after periodontal therapy (data not shown). After periodontal therapy, statistically significant reductions in plasma levels of NETs ($p<0.001$) **(Figure 3E)**, IL-1 ($p=0.030$) **(Figure 3F)**, IL-8 ($p=0.002$) **(Figure 3G)**, and IL-6 ($p=0.001$) **(Figure 3H)** were also observed. No significant difference in plasma levels of TNF-α ($p=0.720$) or IL-10 ($p=1.000$) was observed when comparing the levels before and after periodontal therapy (data not shown). A significant reduction in DAS 28 was also detected after periodontal therapy (mean: 2.97±0.79) compared to the period before periodontal therapy (mean: 3.29±1.17) ($p=0.023$).

RA onset and periodontitis influenced the concentration of salivary cytokines

Individuals in the early RA group had significantly higher concentrations of TNF-α, IL-17E/IL-25, IL-6, and IL-28A compared to individuals with no RA (p=0.025, p=0.001, p=0.019, p=0.044 respectively) and to individuals with established RA (p=0.011, p=0.002, p=0.001, p=0.014, respectively). There was no significant difference between the established RA group and the group of individuals with no RA (p=0.998, p=0.825, p=0.779, p=0.997, respectively). Individuals in the early RA group had significantly higher concentrations of IL-10 and GM-CSF compared to the established RA individuals (p=0.020, p=0.046, respectively). There was no statistically significant difference between individuals with early RA and individuals without RA regarding the levels of the salivary cytokines IL-10 (p=0.607) and GM-CSF (p=0.068). Also, there were no statistically significant differences between individuals with established RA and individuals without RA regarding levels of the salivary cytokines IL-10 (p=0.313) and GM-CSF (p=0.989) **(Supplementary Figure 2)**. There was no significant correlation between the tested cytokines and concentrations of NETs in saliva and plasma (Pearson's correlation test p>0.05).

Supplementary Figure 2 shows the concentrations of cytokines dichotomized by the median into low and high concentration in individuals with RA comparing those with periodontitis and those without periodontitis. The analyses showed that the proportion of high GM-CSF concentration was greater in RA individuals with periodontitis than in RA individuals without periodontitis (p=0.042). The proportion of a high IL-6 concentration was greater in RA individuals with periodontitis than in RA individuals without periodontitis (p=0.033). The proportion of a high IL-10 concentration was greater in RA individuals with periodontitis than in RA individuals without periodontitis (p=0.002). There were no statistically significant differences in the salivary concentrations of TNF-α, IL-28A and IL-17E/IL-25 (p>0.05) between individuals with and without periodontitis.

Discussion

We have noticed for the first time that NET levels in saliva were associated with the presence of periodontitis in individuals with RA. Moreover, NET concentration was impacted by non-surgical periodontal treatment. The production of inflammatory mediators in saliva was pronounced in individuals with periodontitis and early RA.

The decrease in plasma levels of NETs observed after non-surgical periodontal treatment is consistent with the features of other studies that examined

alterations in the production of NETs derived from neutrophils in blood before and after periodontal treatment [6, 26]. Indeed, neutrophils are predominant inflammatory cells involved in periodontitis and have previously been shown to exhibit hyperactivity and hyperreactivity in terms of reactive oxygen species (ROS) production in individuals with chronic periodontitis [27]. Based on this, non-surgical periodontal therapy has beneficial effects on individuals with periodontitis and RA, improving their clinical and inflammatory parameters [28]. In this scenario, individuals with RA have an increased risk of developing periodontitis and suffer the loss of teeth compared to the general population [28].

Evidence supports that both RA and periodontitis are characterized by an imbalance between levels of pro-inflammatory and anti-inflammatory mediators [29]. Herein, a higher concentration of inflammatory mediators was observed in the saliva of individuals with early RA and in individuals with periodontitis. Previous investigations have also demonstrated that the concentration of pro-inflammatory cytokines in the preclinical and early stages of RA is high [30, 31]. Treatment with disease-modifying antirheumatic drugs, MTX in particular, and also with non-steroidal anti-inflammatory drugs may lead to a reduction of the levels of circulating inflammatory mediators [32]. Thus, a longer treatment time with MTX in individuals with established RA may explain the lower concentration of inflammatory mediators observed in our patients. Furthermore, most individuals with early RA had not yet undergone any type of treatment at the time of collection, thus presenting the disease as highly active with a consequently higher concentration of inflammatory mediators.

The association between levels of salivary cytokines and periodontitis has been highlighted in the literature [33, 34]. This exaggerated production of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-17, has a pivotal role in the tissue damage [35]. By contrast, IL-4 and IL-10 may have an inhibitory effect on osteoclastogenesis [35]. We observed that the decrease in circulating levels of NETs after periodontal therapy was also accompanied by an important reduction in levels of IL-1, IL-8, and IL-6. Although controversial, the local anti-inflammatory effect of periodontal treatment has already been detected by others [36]. Despite the improvement in periodontal parameters after non-surgical periodontal therapy, residual gingival inflammation (gingival bleeding) may contribute to the expression of IL-6 in saliva. This finding may explain why there was no significant decrease in salivary IL-6 [37]. The systemic effect of periodontal therapy is generally reflected in the decrease

in disease activity, a finding that we observed in this study through the decrease in DAS 28 after non-surgical periodontal therapy, similarly to a previous investigation [38]. Currently, new biological therapies for RA, in particular TNF-α inhibitors, have led to a significant improvement in disease activity parameters and also to a sharp reduction of periodontal inflammation [39]. Moreover, solid proof has indicated that IL-6 receptor inhibition therapy affects the control of periodontal inflammation in individuals with RA [40]. Herein, we did observe that the IL-6 levels of RA patients are influenced by periodontal status. Furthermore, periodontal therapy caused a significant reduction of IL-6 in plasma, suggesting that periodontal therapy may contribute to the responsiveness to anti-IL6 therapy, a hypothesis that deserves further investigation.

The pro-inflammatory mediators and oral bacteria can also spark the activation of neutrophils, drive these cells to the sites of inflammation or infection and formation of NETs [7, 41]. The NET formation process is followed by the production of ROS through the action of the enzyme NADPH oxidase in a phenomenon recognized as MPO stimulation. This event not only triggers NET formation but, with the aid of the protein arginine deiminase 4, also promotes chromatin decondensation [42]. Notably, neutrophils of individuals with RA are more likely to produce NETs, and NET accumulation contributes substantially to tissue damage so that the antibodies of individuals with RA are prime targets for the citrullinated histones found in NETs [43]. Noteworthy, individuals with periodontitis and RA showed high levels of circulating NETs when compared to healthy individuals. Consistent with this finding, a large number of hyperreactive neutrophils have also been observed in inflamed periodontal tissues and these cells tend to release a significant amount of NETs [44]. Accordingly, NETs generated during chronic periodontitis may be an important source of autoantigens and, therefore, trigger the development or RA progression [4].

This study has some limitations inherent to its cross-sectional nature, including the fact that individuals without RA were not paired by risk factors. Since RA and periodontitis are complex conditions with multiple causal factors, the association between the microbiological profile and the production of NETs cannot be ruled out.

In summary, our findings revealed that NET concentrations are directly associated with periodontal and rheumatological clinical parameters and act on the progression of both chronic inflammatory diseases. Non-surgical periodontal treatment proved to be efficient in reducing NET concentrations, improving the clinical parameters of these illnesses. The assessment of circulating levels of NETs has

proven that a dialogue between periodontitis and RA exists, a result that sets ambitious targets for new therapies.

Table 1. Difference in the distribution of clinicodemographic data among individuals without rheumatoid arthritis (RA), individuals with early RA, and individuals with established RA

Variables	No RA (n=76)	Early RA (n=24)	Established RA (n=64)	*p* value
Age, mean (SD)	41.8 (±14.5)[a]	51.4 (±11.5)[b]	51.6 (±12.2)[b]	<0.001†
Sex (%)				
Female	56 (73.7)[a]	20 (83.3)[a,b]	58 (90.6)[b]	0.030‡
Male	20 (26.3)	4 (16.7)	6 (9.4)	
Tobacco smoking (%)				
Never	73 (96.1)[a]	10 (41.7)[b]	48 (75.0)[c]	<0.001‡
Still	2 (2.6)	8 (33.3)	5 (7.8)	
Stopped	1 (1.3)	6 (25.0)	11 (17.2)	
Alcohol consumption (%)				
Never	56 (73.7)[a]	18 (75.0)[b]	52 (81.3)[a,b]	<0.040‡
Still	14 (18.4)	2 (8.3)	11 (17.2)	
Stopped	-	3 (12.5)	1 (1.5)	
NR	6 (7.9)	1 (4.2)	-	
Time of RA symptoms (months, mean ± SD)	-	15.9 (±13.5)	79.93 (±124.8)	0.068§
RF (IU/mL, mean ± SD)	-	74.0 (±136.2)	294.4 (±509.7)	0.100§
ACPA (IU/mL, mean ± SD)	-	113.4 (±108.7)	108.6 (±113.0)	0.893§
CRP (mg/L, mean ± SD)	-	2.3 (±3.1)	12.1 (±24.9)	0.061§
VAS (mm, mean ± SD)	-	65.9 (±22.6)	34.1 (±35.3)	0.003§

ESR (mm/h, mean ± SD)	-	29.8 (±22.5)	17.3 (±20.2)	0.076§
DAS 28 (mean ± SD)	-	4.4 (±1.0)	3.1 (±1.3)	<0.001§
Disease activity (n, %)				
Remission	-	-	22 (34.4)	
Low	-	6 (25.0)[a]	19 (29.7)[b]	<0.001¶
Moderate	-	11 (45.8)	19 (29.7)	
High	-	5 (20.9)	4 (6.2)	
NR	-	2 (8.3)	-	
Methotrexate treatment (n, %)				
Failure	-	8 (33.3)[a]	9 (14.1)[a]	0.270‡
Responsive	-	13 (54.2)	34 (53.1)	
Adverse events	-	1 (4.2)	1 (1.5)	
NR	-	2 (8.3)	20 (31.3)	
Tooth loss (mean ± SD)	3.5 (±4.1)[a]	9.7 (±6.3)[b]	7.0 (±5.2)[b]	<0.001†
Periodontitis (n, %)				
Presence	36 (47.4)[a]	19 (79.2)[b]	37 (57.8)[a,b]	0.021⊥
Absence	40 (52.6)	5 (20.8)	27 (42.2)	

ACPA, anti-citrullinated protein antibody; CRP, C-reactive protein; DAS 28, disease activity score 28; ESR, erythrocyte sedimentation rate; NR, not reported; RF, rheumatoid factor; SD, standard deviation; VAS, visual analogue scale for pain.
†ANOVA; ‡Fisher's test; §t-test; ¶Linear by linear test; ⊥Pearson test.
Superscript letters indicate a difference between groups. Equal letters indicate no significant difference, while different letters indicate a significant difference.

Table 2. Regression analysis for the assessment of the association of neutrophil extracellular traps (NETs) in saliva and plasma with health status (no rheumatoid arthritis [RA]/early RA + established RA), periodontitis, tobacco smoking, and age

Variables	NETs in saliva (ng/mL)			NETs in plasma (ng/mL)		
	Coefficient	**t**	***p* value***	**Coefficient**	**t**	***p* value***
Health status						
No RA (Ref)	0.127	1.279	0.204	0.164	1.700	0.091
RA (early + established)						
Periodontal disease						
No (Ref)	0.400	4.242	<0.001*	0.171	2.009	0.047*
Yes						
Tobacco smoking						
Never (Ref)	-0.007	-0.077	0.939	0.049	0.538	0.591
Still/Stopped						
Age	-0.006	-0.066	0.947	-0.025	-0.269	0.788

Ref, reference category.
*Statistically significant at $p \leq 0.05$.

Table 3. Periodontal assessment of individuals with early rheumatoid arthritis (RA) and established RA before and after periodontal therapy

Variables	Early RA (n=16)			Established RA (n=37)		
	Before	**After**	***p* value**	**Before**	**After**	***p* value**
Classification of PD* n (%)						
Absent	5 (31.3)[a]	5 (31.3)[a]		5 (13.5)[a]	21 (56.8)[b]	
Mild	-	-	0.154†	-	1 (2.7)	0.024†
Moderate	7 (43.8)	7 (43.8)		20 (54.1)	12 (32.4)	
Severe	4 (25.0)	4 (25.0)		12 (32.4)	3 (8.1)	
Probing depth (mm, mean ± SD)	2.14 ± 0.45	1.64 ± 0.57	0.002‡	2.71 ± 1.06	1.87 ±0.50	<0.001‡
CAL (mm, mean ± SD)	2.60 ± 1.02	2.48 ± 1.46	0.562‡	3.07 ± 1.22	2.40 ± 0.87	<0.001‡
BOP, mean ± SD	16.38 ± 19.05	13.31 ± 9.38	0.439‡	40.27 ± 29.45	10.30 ± 9.29	<0.001‡
Plaque index, mean ± SD	39.19 ± 25.33	22.50 ± 12.08	0.010‡	40.21 ± 25.70	14.43 ± 6.38	0.001‡

BOP, bleeding on probing; CAL, Clinical attachment loss; PD, periodontitis; SD, standard deviation.
*Based on the study by Eke et al.[22]
†Fisher's test; ‡Paired t test.
Superscript letters indicate a difference between groups. Equal letters indicate no significant difference, while different letters indicate a significant difference.

Figure 1. Concentration of neutrophil extracellular traps (NETs) in saliva according to: **(A)** health status of individuals categorized as no rheumatoid arthritis (RA), early RA and established RA, **(B)** periodontal status (no PD/PD) of individuals without RA and individuals with early RA, and **(C)** periodontal status of individuals without RA and individuals with established RA. Concentration of NETs in plasma according to: **(D)** health status of individuals categorized as no RA, early RA and established RA, **(E)** periodontal status (no PD/PD) of individuals without RA and individuals with early RA and, **(F)** periodontal status of individuals without RA and individuals with established RA. Note: PD, periodontitis; (*) denotes a difference between individuals without RA and/or without PD ($p \leq 0.05$); (#) denotes a difference between early RA ($p \leq 0.05$); the *t*-test was employed to determine whether there was a difference between groups and one-way ANOVA followed by the Tukey *post hoc* test was used to determine whether the difference was statistically significant.

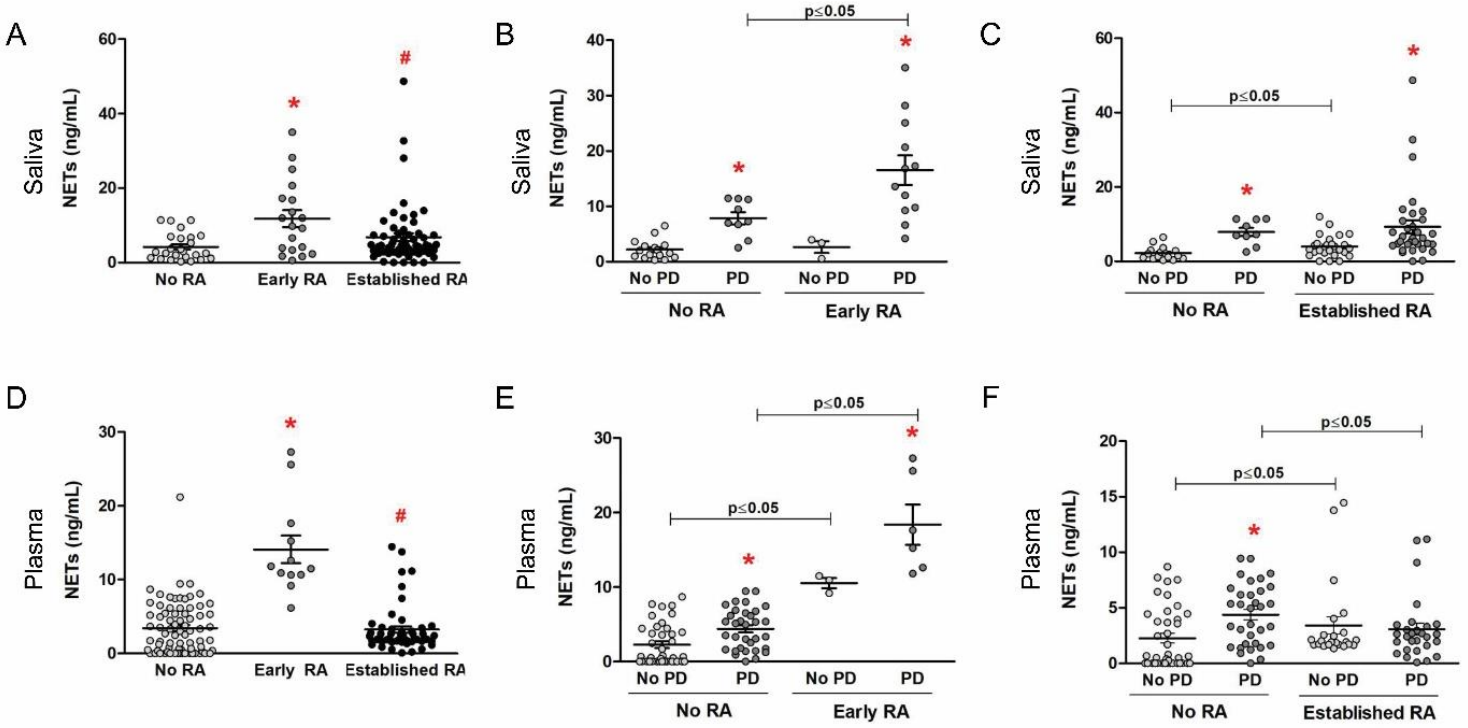


Figure 2. (A) Periodontal probing, **(B)** clinical attachment loss (CAL) and **(C)** bleeding on probing (BOP) of individuals with rheumatoid arthritis (RA) (early + established) according to the concentration of neutrophil extracellular traps (NETs) in saliva dichotomized by the median (4.75 ng/mL). **(D)** Concentration of NETs in plasma of individuals with RA according to disease activity. Note: (*) denotes $p \leq 0.05$; the *t*-test was employed to determine whether there was a difference between groups and one-way ANOVA followed by the Tukey *post hoc* test was used to determine whether the difference was statistically significant.

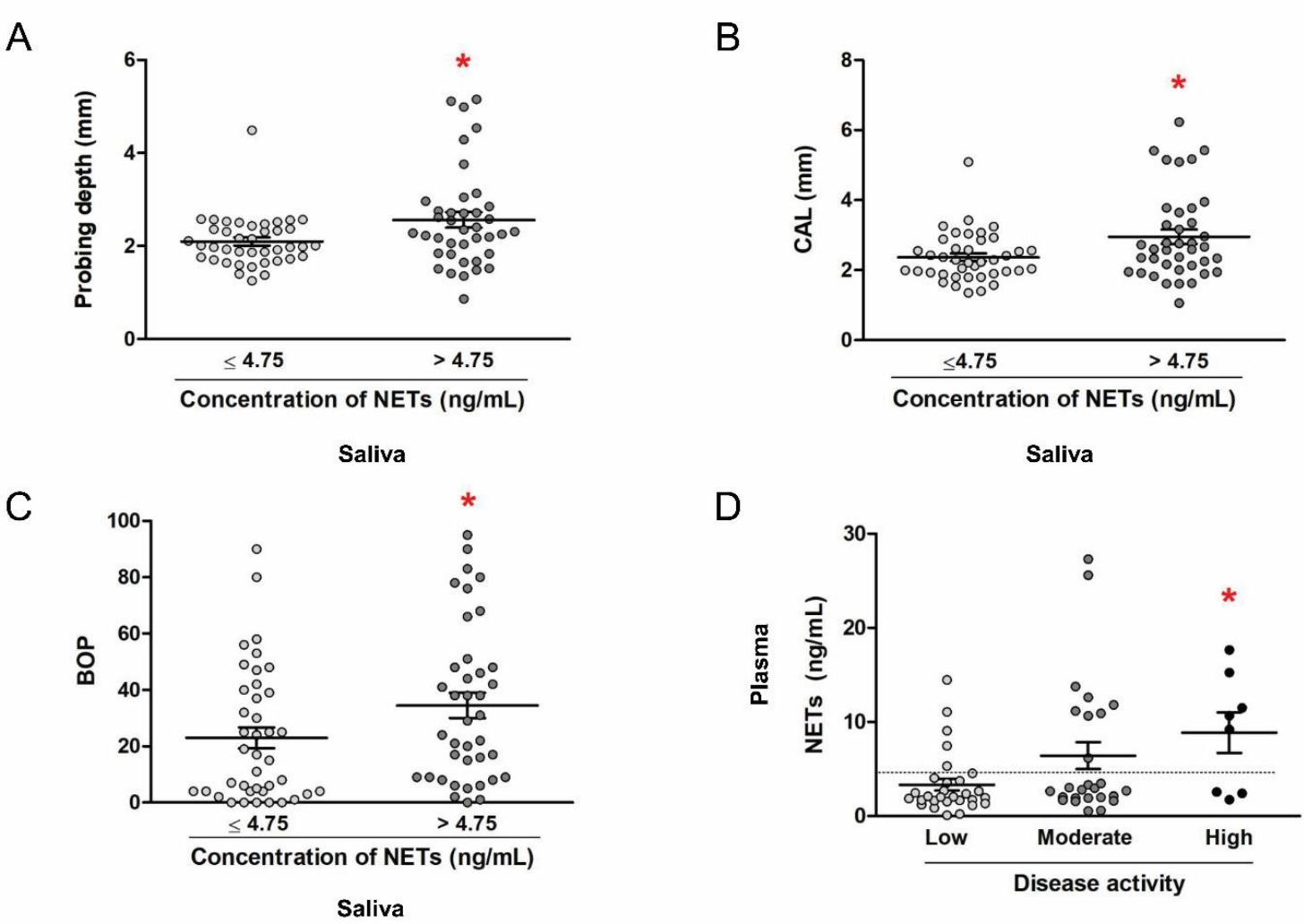
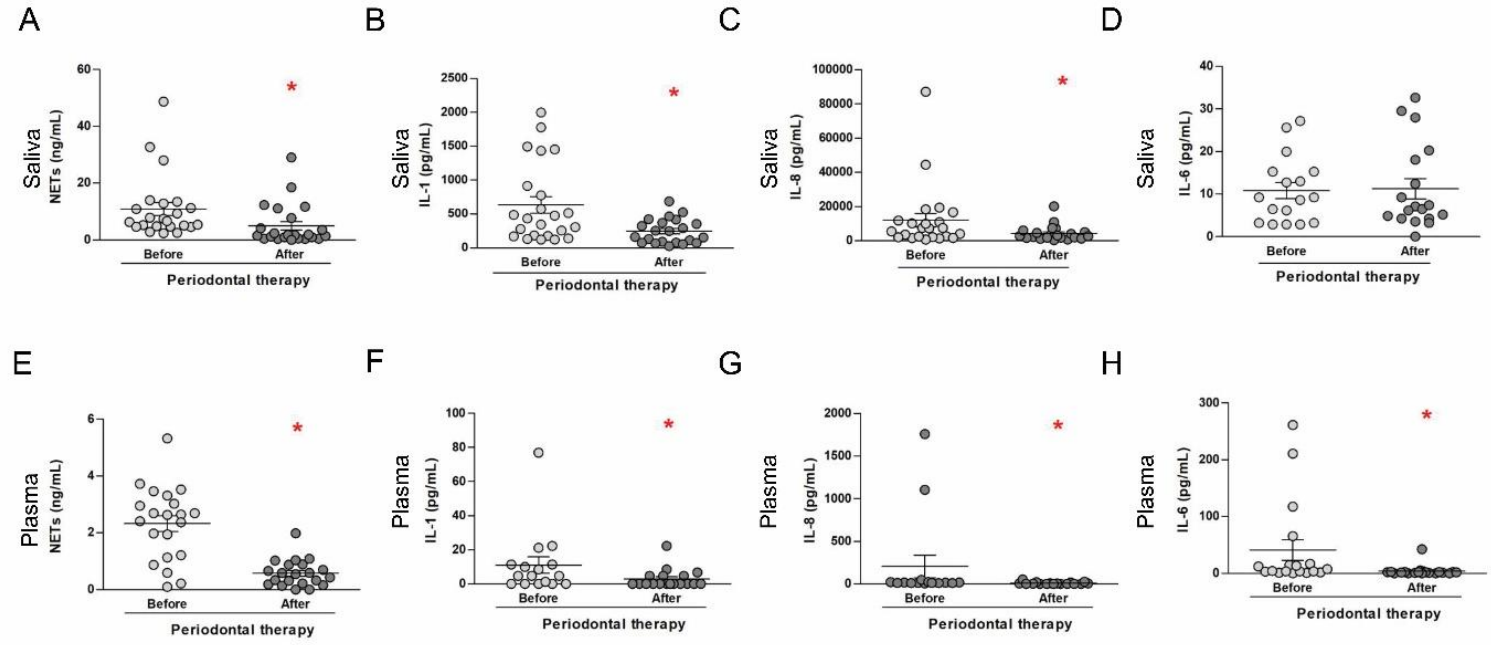


Figure 3. Salivary concentration of neutrophil extracellular traps (NETs) **(A)**, IL-1 **(B)**, IL-8 **(C)**, IL-6 **(D)** in the rheumatoid arthritis (RA) (early + established) group before and after periodontal therapy. Plasma concentration of NETs **(E)**, IL-1 **(F)**, IL-8 **(G)** and, IL-6 **(H)** in the RA (early + established) group before and after periodontal therapy. Note: (*) denotes $p \leq 0.05$; the t-test was employed to determine a statistically significant difference between groups.



Supplementary Methods

Study population

Exclusion criteria were as follows: other rheumatic disease, diabetes, malignant neoplasms, use of ≥5 mg/day of prednisone or equivalent, treatment for periodontal disease within the last six months, use of orthodontic appliances, use of antibiotics within the last three months, pregnancy or lactation, and the presence of less than eight teeth.

NET quantification: saliva and plasma sampling

Briefly, 50 µL of each sample were placed in a 96-well clear-bottom black plate covered with anti-MPO antibody (PA5-16672, dilution 1:500, Invitrogen, Carlsbad, CA, USA). The amount of DNA bound to MPO was quantified using the Quant-iT™ PicoGreen® kit according to the manufacturer's instructions. Fluorescence intensity (excitation at 488 nm and emission at 525 nm wavelength) was determined with a FlexStation 3 Microplate Reader (Molecular Devices, San Jose, CA, USA).

Analysis of inflammatory mediators

Salivary concentrations of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-28A, IL-6, IL-10, IL-17E/IL-25, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were determined with a flow cytometer (Luminex® 200™ FLEXMAP 3D™, Austin, TX, USA) using the Milliplex® MAP Human cytokine/chemokine Magnetic Bead Panel (Merck, Darmstadt, Germany). The tests were performed according to the manufacturer's instructions. Moreover, salivary and plasma levels of IL-8, IL-1, IL-6, IL-10, and TNF-α were measured in individuals with RA before and 45 days after periodontal treatment using the same method as described above. The detection ranges were as follows: TNF-α: 0.95-53 pg/mL, IL-28A: 10.00-1600 pg/mL, IL-6: 0.91-556 pg/mL, IL-10: 0.22-34 pg/mL, IL-17E/IL-25: 10.00- 3300 pg/mL, IL-1: 7.20- 1998 pg/mL, IL-8: 3.60-87067 pg/mL, and GM-CSF: 10.00-1600 pg/mL.

Data analysis

The Statistical Package for the Social Sciences (SPSS) (version 25.0, Armonk, USA) and the GraphPad Prism (version 7.00, La Jolla, USA) were used. Descriptive analysis was applied to clinicodemographic and periodontal evaluation data. Results of analyses of continuous variables are reported as mean (±SD). ANOVA

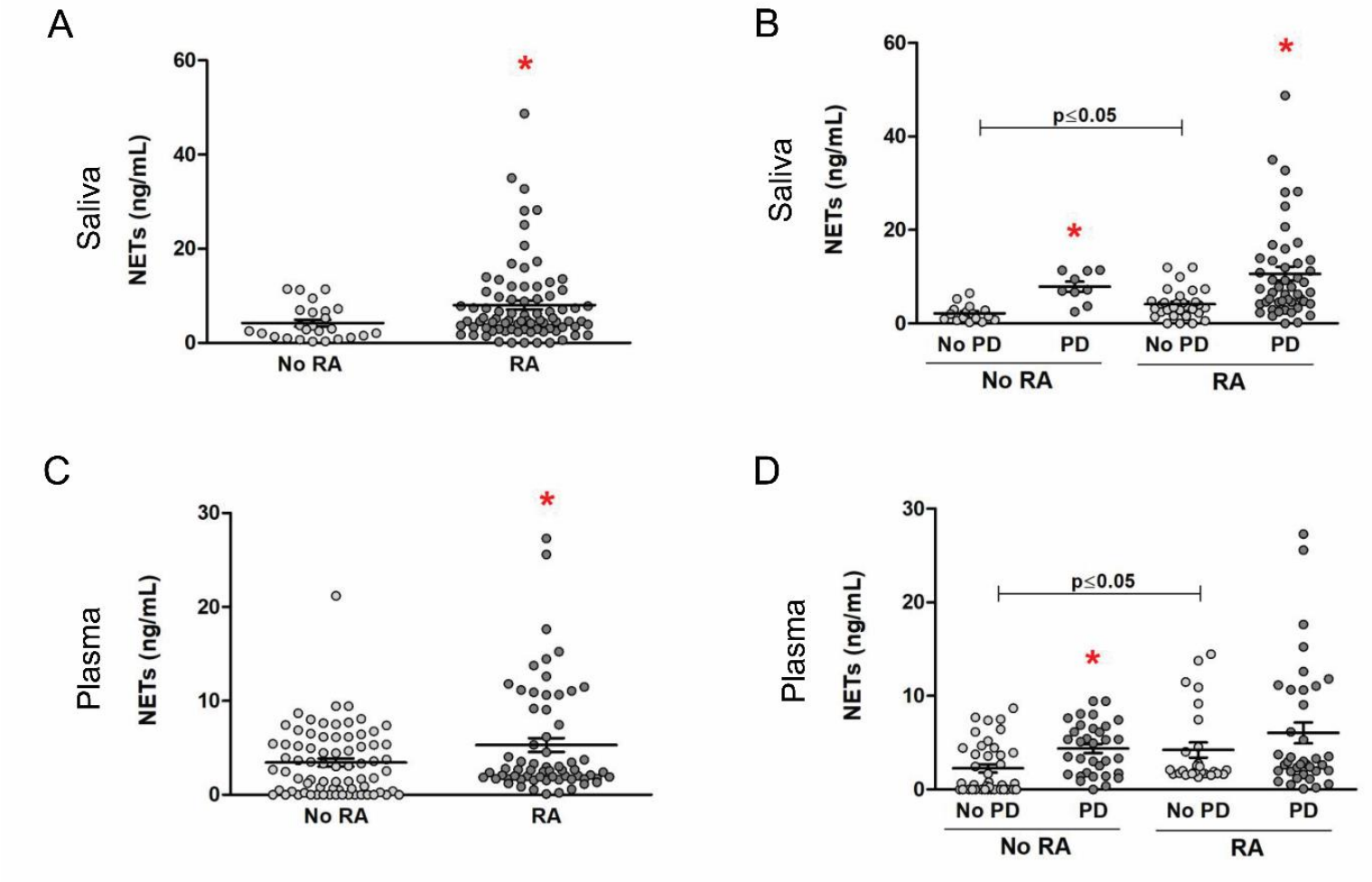
test and chi-square test were used to assess the differences between groups regarding the quantitative and qualitative variables. Bivariate analysis (Student *t*-test) was used to determine the association of the variables (health status, periodontitis, and clinical periodontal parameters) with the variable (NET concentration). The *t*-test was employed to assess differences between groups before and after periodontal therapy. Pearson's chi-square test was used to evaluate the association between concentration of cytokines dichotomized by the median and presence or absence of periodontitis. Independent variables were incorporated into linear regression models to determine their association with NET concentration. For all analyses, the level of significance was ≤0.05.

Supplementary Table 1. Regression analysis for the assessment of the association of neutrophil extracellular traps (NETs) in saliva and plasma with health status (no rheumatoid arthritis [RA]/early RA and no RA/established RA), periodontitis, tobacco smoking, and age

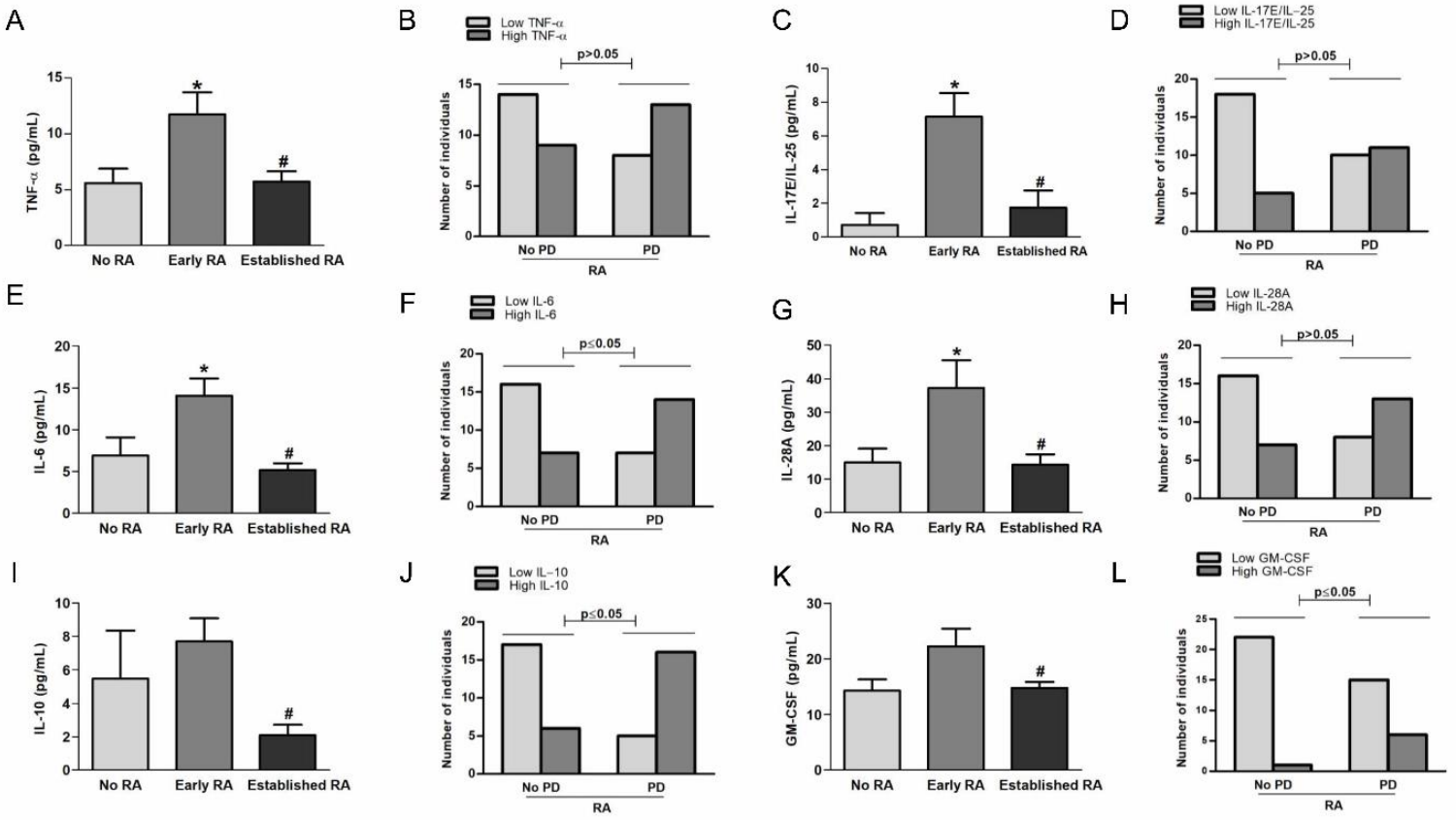
Variables	NETs in saliva (ng/mL)			NETs in plasma (ng/mL)		
	Coefficient	t	*p* value*	Coefficient	t	*p* value*
Health status						
No RA (Ref)	0.345	2.006	0.052	0.660	6.788	<0.001*
Early RA						
Periodontitis						
No (Ref)	0.439	3.128	0.003*	0.172	2.118	0.037*
Yes						
Tobacco smoking						
Never (Ref)	-0.043	-0.274	0.785	-0.022	-0.238	0.812
Still/stopped						
Age	-0.076	-0.551	0.585	-0.021	-0.244	0.808
Health status						
No RA (Ref)	0.089	0.814	0.418	0.014	0.141	0.888
Established RA						
Periodontitis						
No (Ref)	0.381	3.636	<0.001*	0.123	1.348	0.180
Yes						
Tobacco smoking						
Never (Ref)	-0.078	-0.733	0.466	-0.045	-0.464	0.643
Still/stopped						
Age	0.096	0.862	0.391	-0.104	-1.056	0.293

Ref, reference category.
*Statistically significant at $p \leq 0.05$.

Supplementary Figure 1. Concentration of neutrophil extracellular traps (NETs) in saliva according to **(A)** health status of individuals categorized as no rheumatoid arthritis (RA) and RA (early + established), **(B)** periodontal status (no PD/PD) of individuals without RA and with RA (early + established). Concentration of NETs in plasma according to **(C)** health status of individuals categorized as no RA and RA (early + established), **(D)** periodontal status (no PD/PD) of individuals without RA and with RA (early + established). Note: PD, periodontitis; (*) denotes a difference between individuals without RA and/or without PD ($p \leq 0.05$). The *t*-test was employed to determine whether there was a difference between groups.



Supplementary Figure 2. Assessment of the concentrations of inflammatory mediators in saliva among individuals without rheumatoid arthritis (RA), with early RA and with established RA, and association between periodontitis and the salivary concentration of the following cytokines dichotomized by the median into low and high concentration, in individuals with RA (early and established clustered together): **(A)** TNF-α, **(B)** TNF-α (low ≤5.97 pg/mL; high >5.97 pg/mL), **(C)** IL-17E/IL-25, **(D)** IL-17E/IL-25 (low ≤10.00 pg/mL; high >10.00 pg/mL), **(E)** IL-6, **(F)** IL-6 (low ≤6.05 pg/mL; high >6.05 pg/mL), **(G)** IL-28A, **(H)** IL-28A (low ≤10.00 pg/mL; high >10.00 pg/mL), **(I)** IL-10, **(J)** IL-10 (low ≤2.51 pg/mL; high >2.51 pg/mL), **(K)** GM-CSF, **(L)** GM-CSF (low ≤20.00 pg/mL; high >20.00 pg/mL). Note: (*) denotes a difference between the group of individuals with early RA and the group of individuals with no RA ($p \leq 0.05$); ($^{\#}$) denotes a difference between the group of individuals with established RA and the group of individuals with early RA ($p \leq 0.05$); one-way ANOVA followed by the Tukey post hoc test was employed to determine whether there was a difference between groups and the chi-square test was used to determine whether the difference was statistically significant. Statistically significance was set at $p \leq 0.05$.



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4.2 Artigo 2

Os resultados relacionados aos objetivos específicos de investigar a produção das NETs na cultura de neutrófilos isolados do sangue periférico de indivíduos controles saudáveis e indivíduos com AR e determinar se a presença do LPS de *Porphyromonas gingivalis* influencia na produção das NETs; investigar a associação entre a produção das NETs na cultura de neutrófilos de indivíduos com AR, a presença da periodontite e a atividade da doença; caracterizar a presença das NETs em tecido gengival parafinizado através da marcação por imunofluorescência de células MPO/histonas citrulinas H3 positivas e comparar o número de células positivas de acordo com o status periodontal; investigar a relação entre SNPs no gene *PADI4* e o risco de AR; e, por fim, investigar a associação entre a presença de SNPs no gene *PADI4,* a produção de NETs na cultura de neutrófilos e os parâmetros clínicos periodontais de indivíduos com AR; são apresentados no manuscrito intitulado *"Neutrophil extracellular traps in rheumatoid arthritis and periodontitis: contribution of PADI4 gene polymorphisms"* que será submetido para publicação no periódico *Oral diseases* (Fator de impacto: 4.0).

Neutrophil extracellular traps in rheumatoid arthritis and periodontitis: contribution of *PADI4* gene polymorphisms

Abstract

Rheumatoid arthritis (RA) is a multifactorial disease. Individuals with RA have an increased incidence of periodontitis (PD) and neutrophil extracellular traps (NETs) may be the link between both diseases. **Objectives:** Herein, we investigated NETs production in neutrophils from individuals with RA and controls. We also compared the presence of NETs in gingival tissues of individuals with RA. Furthermore, we investigated the association between single nucleotide polymorphisms (SNPs) of peptidyl arginine deaminase type 4 *(PADI4)* gene, and the GTG haplotype with RA, PD, and NETs *in vitro*. **Methods:** Peripheral blood neutrophils were isolated by density gradient. The concentration of NETs was determined using the PicoGreen® kit. Immunofluorescence was performed to identify NETs by colocalization of myeloperoxidase (MPO) and citrullinated histone H3. Genotyping was performed for the SNPs (*PADI4*_89; *PADI4*_90; *PADI4*_92; *PADI4*_104). **Results:** The production of NETs by neutrophils in culture was higher in individuals with RA and PD. Gingival tissues from individuals with RA and PD showed a higher concentration of positive MPO/H3 cells. Individuals with the GTG haplotype had a higher production of NETs *in vitro* and worse periodontal clinical parameters. **Conclusion**: The production of NETs in culture is associated with RA and PD and is influenced by the presence of the GTG haplotype.

Key words: Rheumatoid arthritis; periodontitis; polymorphisms; neutrophil extracellular traps.

1 Introduction

Rheumatoid arthritis (RA) is an autoimmune disease linked to genetic, environmental, and behavioral factors (Deane et al., 2017). Individuals with RA are greatly affected by periodontitis (PD) (Qiao et al., 2020; Bolstad, Fevang, & Lie, 2023). PD is a chronic infectious disease of tooth supporting structures that might lead to tooth loss and systemic repercussions (Potempa, Mydel, & Koziel, 2017; Kapila, 2021).

Despite the differences in the etiologies of RA and PD, diseases cross talk occurs by means of inflammatory mediators and anti-citrullinated protein antibodies (ACPAs) (Engström et al., 2018; Vitkov, Hannig, Minnich, & Herrmann, 2018). Citrullination is a post-translational modification that might be caused by periodontopathogenic bacteria, such as *Porphyromonas gingivalis* (Shimada et al., 2016). Accordingly, the presence of this bacterium at periodontal pockets has been linked to increased production of ACPAs (Mikuls et al., 2014; Bello-Gualtero et al., 2016). Moreover, the enzyme peptidyl arginine deaminase type 4 (PAD4), which is expressed primarily by neutrophils, eosinophils, and monocytes (Nakashima, Hagiwara, & Yamada, 2002; Vossenaar et al., 2004), catalyzes the conversion of arginine to citrulline residues (Mondal & Thompson, 2019). Studies have shown that an increase of PAD4 activity has been associated with the onset and progression of RA (Reyes-Castillo, Muñoz-Valle, & Llamas-Covarrubias, 2018) and that single nucleotide polymorphisms (SNPs) in the gene encoding PAD4 enzyme confers greater susceptibility to RA (Suzuki et al., 2003; Lee & Bae, 2015; Matuz-Flores et al., 2022).

PAD4 also participates in the formation of neutrophil extracellular traps (NETs), which are considered a source of citrullinated antigens (Tatsiy & McDonald, 2018; Corsiero et al., 2016). While playing an essential role in the innate immune system, the excessive production of NETs exacerbates tissue damage in RA (Lee et al. al., 2017; Papayannopoulos, 2018) and PD (Magán-Fernández et al., 2020; Wang et al., 2021). Both RA and PD contribute to an elevated concentration of NETs in saliva and plasma (Kaneko et al., 2018; Oliveira et al., 2020). However, the NETs production by circulating neutrophils along with *PADI4* gene SNPs was not demonstrated in individuals with RA and PD. Our hypothesis is that peripheral blood neutrophils from individuals with RA and PD show a higher production of NETs in the culture, and result in a significant positivity of NETs in the gingival tissues. Furthermore, the presence of *PADI4* SNPs might account for elevated NETs production worsening the course of PD and RA.

2 Methods

2.1 Study design, sampling, setting, and ethical issues

The cross-sectional study was conducted with adult individuals with a diagnosis of RA (Aletaha et al., 2010) who were attending at the University Hospital, Federal University of Minas Gerais, Belo Horizonte, Brazil and the Rheumatology Outpatient Clinic of the Ribeirão Preto Medical School Hospital, University of São Paulo, Ribeirão Preto, Brazil. Control volunteers, older than 18 years and without systemic alterations were also included in the study. Informed consent was obtained from all participants. The exclusion criteria were individuals who had been diagnosed with another autoimmune disease, diabetes, malignant neoplasms, individuals wearing orthodontic appliances, those who had taken antibiotics within the last three months, individuals who had undergone treatment for periodontal disease within the last six months, women lactating or pregnant women, and individuals who had less than eight teeth.

The reporting of the study was based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (Knottnerus & Tugwell, 2008). The study was approved by the Ethics Committee of both universities (#05934818.3.0000.5440; #03128012.0.0000.5149).

2.2 Data assessment and collection

Data on sex, age, and smoking status were collected both from the group of individuals with RA and from control volunteers. In addition, periodontal status was assessed by two calibrated examiners using a periodontal probe in individuals with RA (modelo PCP15, Hu-Friedy®; Chicago, IL, EUA). Participants were classified according to the 2018 classification for PD (Tonetti, Greenwell, H., & Kornman, 2018) and assigned to two groups: individuals without PD [periodontal health and stage I (borderline between gingivitis and PD)] and individuals with PD (stages II, III, or IV). The following data were collected from the medical records of individuals with RA: DAS 28, concentration of ACPA, stage of disease activity (Prevoo et al., 1995) and time of symptoms.

2.3 Peripheral blood collection

Peripheral blood samples from RA patients and control volunteers were collected into tubes containing K_2EDTA (BD Vacutainer™, Franklin Lakes, NJ, USA).

An aliquot of whole blood was stored at -70° C for genomic DNA extraction and subsequent genotyping. The remaining collected sample was used to perform the isolation of neutrophils from peripheral blood.

2.4 NETs production by isolated human blood neutrophils in vitro

Neutrophils were isolated from peripheral blood by means of Percoll gradients (72, 63, 54, and 45%). After centrifugation, polymorphonuclear leukocytes accumulated as a band between 72 and 63% Percoll. Total cell numbers were counted using light microscopy. The percentage of neutrophils was determined microscopically through blue turky staining. One million neutrophils were incubated at 25°C in Hanks' buffered saline solution (HBSS) medium (Corning, Manassas, VA, USA) for 4 hours. To analyze the stimulated production of NETs, cells were incubated with 1 µg/µL of lipopolysaccharide (LPS) from heat Killed *Porphyromonas gingivalis* (Invivo Gen, San Diego, CA, USA) (Chen et al., 2022). The supernatants were used to determine NETs concentration. The Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) was used to quantify NETs levels by identifying the myeloperoxidase (MPO)-DNA complex in saliva, serum, and in supernatants of the neutrophils' culture (Schneider et al., 2020; Oliveira et al., 2021). Briefly, 50 µL of each sample were placed in a 96-well clear-bottom black plate covered with anti-MPO antibody (PA5-16672, dilution 1:500, Invitrogen, Carlsbad, CA, USA). The amount of DNA bound to MPO was quantified using the Quant-iT™ PicoGreen® kit, according to the manufacturer's instructions. Fluorescence intensity (excitation at 488 nm and emission at 525 nm wavelength) was determined with a FlexStation 3 Microplate Reader (Molecular Devices, San Jose, CA, USA).

2.5 NETs in gingival tissues

Gingival tissue samples were obtained from individuals with RA who need underwent periodontal surgery. The samples were fixed with 10% buffered paraformaldehyde for 48 hours, processed and embedded in paraffin. Paraffin blocks were serially sectioned using a microtome at 4 µm sections. The slides were dewaxed in xylene and rehydrated. For antigen retrieval, the slides were immersed in Citrate solution, pH 6.0, at 95 °C for 20 minutes. Protein blocking was performed with incubation in 2% bovine serum albumin (BSA) and Triton® X-100 (Sigma-Aldrich®, Saint Louis, MO, EUA) for 2 hours.

Subsequently, the slides were incubated overnight with primary antibodies anti-citrullinated histones H3 (1:100- isotype rabbit) and anti-MPO (1:50-isotype mouse) (Thermo Fisher Scientific®, Waltham, MA, USA). Detection was performed using secondary antibodies anti-rabbit (AlexaFluor 488) (1:2000) and an anti-mouse (AlexaFluor 594) (1:1250) (Thermo Fisher Scientific®, Waltham, MA, USA). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Abcam®, Cambridge, UK) and the sections were analyzed under a ZEISS Axioscope 5 fluorescence microscope (ZEISS®, Oberkochen, Germany). Each field was imaged with a laser at 405 nm (DAPI—blue), 488 nm (Fitc—green), and 594 nm (Rhod—red), and the images were processed and combined into a merged image. The presence of NETs in the sections was confirmed through analysis among DAPI, MPO, and citrullinated histones H3. Labeled cells were counted in 10 consecutive fields at 400× magnification.

2.6 Genotyping of PADI4 polymorphisms

Genomic DNA was extracted from peripheral blood mononuclear cells using the QIAamp® DSP DNA Blood Mini Kit (QIAGEN®, Hilden, Germany). The quality and concentration of the extracted DNA was determined using a Nano Drop spectrophotometer (Nano Drop 2000, Thermo Scientific, USA).

All participants with RA and control volunteers were genotyped for 4 SNPs: *PADI4*_89 (rs11203366), *PADI4*_90 (rs11203367), *PADI4*_92 (rs874881), and *PADI4*_104 (rs1748033) using TaqMan™ SNP Genotyping Assay (Thermo Fisher Scientific®, Waltham, MA, EUA) by StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, USA). For performing PCR amplification, each reaction mixture contained 1 µl of extracted DNA (with 20 ng/µl concentration), 10 µl of TaqMan Master Mix (containing Taq DNA polymerase and dNTPs), 0.5 µl TaqMan Genotyping Assay mix (containing pre-designed primers and FAM or VIC labeled probes; Applied Biosystems, Foster City, USA), and distilled water to the ultimate volume of 20 µl. The thermocycling conditions for the PCR amplification were initial denaturation at 94 ◦C for 3 min followed by 35 cycles of 30 s at 94 ◦C, 30 s at 56 ◦C, and 30 s at 72 ◦C; then, a final extension of 1 minute at 72 ◦C. Determination of alleles in each sample was conducted via analysis of the allelic discrimination plots through TaqMan® Genotyper Software (Thermo Fisher Scientific®, Waltham, MA, EUA).

We performed a haplotype analysis of the *PADI4* gene for the three non-synonymous SNPs investigated (Bang et al., 2010). Individuals were classified as GTG

+ haplotype when they presented homozygous genotypes for the polymorphism in the three SNPs: *PADI4*_89, *PADI4*_90 and *PADI4*_92, respectively. The other individuals were classified as GTG -.

2.7 Data analysis

The Statistical Package for the Social Sciences (SPSS) (version 25.0, Armonk, USA) and the GraphPad Prism (version 7.00, La Jolla, USA) were used. Descriptive analysis was applied to clinicodemographic data. Results of analyses of continuous variables were reported as mean (± standard deviation). ANOVA test was used to assess the differences between groups regarding the quantitative variables. Student *t*-test and Paired *t*-test were also employed. Pearson's chi-square test was used to assess the association between qualitative variables. Logistic regression was adopted to calculate the odds ratio that compared the observed genotypes between the control volunteers and RA groups. For all analyses, the level of significance was set at <0.05.

3 Results

Clinical and demographic characteristics

A total of 198 individuals were included in this study, among whom 111 were control volunteers and 87 individuals had been diagnosed with RA. Among the control volunteers, 83 (74.8%) were female individuals and 28 (25.2%) were male individuals. The mean age observed in the control group was 46.4 years (±12.62). Still among the 111 control individuals included, 72 (64.9%) had never smoked, 24 (21.6%) were smokers, and 14 (12.6%) had stopped smoking. One individual did not report his status in relation to smoking (0.9%). Of 87 individuals with RA, 77 (88.5%) were females and 10 (11.5%) were males, with a mean age of 55.2 years (±12.31). Of these, 55 (63.2%) had never smoked, seven (8.0%) were still smokers, 19 (21.8%) had stopped smoking, and six (6.9%) did not report their smoking status. Regarding periodontal status, 67.2% had PD and 32.8% had no PD. The mean of DAS28 observed was 3.53 (± 1.50). The mean serum concentration of ACPA observed in the RA subjects was 246.21 U/mL (±148.74). About stage of disease activity, 87.1% of individuals were in a moderate or high disease activity stage and 12.9% were in remission or low disease activity. The mean duration of RA symptoms was 133.49 months (min: 2 - max: 540) (±147.80).

Production of NETs in culture was increased in RA patients and after *Porphyromonas gingivalis* stimuli

We observed that the production of NETs by neutrophils isolated from peripheral blood of individuals with RA was significantly higher than control volunteers (p=0.019). Neutrophils stimulated with *Porphyromonas gingivalis* LPS had pronounced NETs production in both RA and controls groups p=0.014 and p=0.002, respectively. Additionally, neutrophils from individuals with RA showed significantly greater production of NETs when stimulated by *Porphyromonas gingivalis* when compared to stimulated neutrophils from control volunteers (p=0.038) (Figure 1A).

The production of NETs was associated with the RA activity and PD

We verified that individuals in moderate and high disease activity (>3.2) had a significantly higher concentration of NETs compared to individuals in remission and low disease activity (≤3.2) (p=0.030) (Figure 1B).

Considering the exacerbation of production of NETs by the periodontopathogen *Porphyromonas gingivalis*, the periodontal status was included in the analysis. Individuals with RA and PD had a significantly higher production of NETs when compared to individuals without PD (p=0.032) (Figure 1C).

Identification of NETs in the gingival tissue of individuals with RA

Based on the significant production of NETs by peripheral neutrophils in individuals with RA and PD, we next evaluated the NETs-producing neutrophils in the gingival tissues of RA individuals without PD (Figure 2A), and of RA individuals with PD (Figure 2B). RA individuals with PD had a significantly higher number of MPO-H3 positive cells compared to RA individuals without PD (p<0.001) (Figure 2C).

Frequency of genotypes associated with PADI4 polymorphisms

Considering the relevance of enzyme PAD4 to NETs generation and its participation in the pathophysiology of RA and PD (Reyes-Castillo et al., 2018; Magán-Fernández et al., 2020), the polymorphisms in the *PADI4* gene was investigated through the genotyping of 4 SNPs: *PADI4*_89, *PADI4*_90, *PADI4*_92 and, *PADI4*_104. The genotype distributions of control and RA groups were found to be in Hardy-Weinberg equilibrium. The frequency of the observed genotypes is detailed in Table 1.

In *PADI4*_89, in the control volunteers and in the RA individuals, the following frequency of genotypes was observed, respectively: AA: 31.5% and 26.7%, AG: 52.3% and 53.5%, GG: 16.2% and 19.8%. The frequency of allele A observed in control and RA individuals was 57.7% and 53.5%, respectively. The frequency of allele G in control and individuals with RA was 42.3% and 46.5%, respectively. As for the *PADI4*_90, the genotypes observed within the control volunteers was: 30.3% of CC, 54.1% of CT, and 15.6% of TT. The frequency of allele C observed was 57.3 and of allele T was 42.7% T. In the RA group, the observed frequency was 28.7% of CC, 52.9% of CT, and 18.4% of TT. The frequency of allele C observed was 55.2% and of allele T was 44.8%. When analyzing the *PADI4*_92, the frequency observed in the control and RA individuals was respectively, CC: 30.6% and 22.7.5%; CG: 52.8% and 56.0%; GG:16.6% and 21.3%. The frequency of allele C observed in control and RA individuals was 56.9% and 50.6%, respectively. The frequency of allele G in controls and individuals with RA was 43.1% and 49.4%, respectively. Finally, the observed frequency of the *PADI4*_104 was CC: 37.8% and 37.2%; CT: 50.0% and 51.2%; TT: 12.2% and 11.6% for control and RA group, respectively. The frequency of allele C was 62.8% and 62.8% and the frequency of allele T was 37.2% and 37.2% in control and RA individuals, respectively. No statistically significant differences were observed between the distribution of genotypes and GTG+ haplotypes in the control and RA groups ($p>0.05$). No significant differences were observed between the polymorphic genotypes and GTG+ haplotypes and the chance of developing RA ($p>0.05$) (Table 1).

Polymorphisms and the production of NETs and ACPAs

Considering the role of the PAD4 in the proteins citrullination, we investigated whether of *PADI4* SNPs are associated with the concentration of ACPAs. We observed that, the neutrophils of individuals with homozygous genotypes for the polymorphism GG (*PADI4*_89), TT (*PADI4*_90) and GG (*PADI4*_92) showed similar concentration of ACPAs, ($p>0.05$) (Supplementary figure 1). However, the individuals carrying GTG+ haplotype had higher concentrations of ACPAs when compared to GTG- individuals ($p=0.010$) (Figure 3A).

As the *in vitro* production of NETs by neutrophils from individuals with RA, we investigated whether the production of NETs in the culture was associated with *PADI4* SNPs. Neutrophils from individuals with homozygous genotypes for the polymorphism GG (*PADI*4_89), TT (*PADI*4_90) and GG (*PADI*4_92) showed a similar

production of NETs (p>0.05). Interestingly, *PADI*4_104 individuals and that with genotype CC and TT showed a significantly higher production of NETs compared to CT genotype (p=0.017) (Supplementary figure 2). Moreover, the GTG+ haplotype individuals were high producers of NETs (p=0.035) (Figure 3B).

The GTG haplotype and PD

After observed a greater production of NETs in the culture of neutrophils from individuals with the GTG+ haplotype, we investigated the association between this haplotype and PD. We observed that individuals with the GTG+ haplotype had worse periodontal clinical parameters such as probing depth, clinical attachment loss and bleeding on probing (Figure 4). Furthermore, the presence of the GTG+ haplotype was significantly associated with the presence of PD, as 100% of RA individuals with PD had the GTG+ haplotype. Individuals with RA and with GTG+ haplotype are 1.75 times more chance of developing PD (p=0.006) (Table 2).

Discussion

In this study, we found an association between the production of NETs by neutrophils *in vitro* with RA and PD. We also observed the influence of LPS from *Porphyromonas gingivalis* on the production of NETs and identified, for the first time, through immunofluorescence, a higher concentration of positive MPO/H3 fields in gingival tissues of individuals with RA and PD. Furthermore, the contribution of homozygous haplotype for the polymorphism (GTG) in the *in vitro* production of NETs, concentration of circulating ACPAs, and worse periodontal clinical parameters was revealed.

In our study, we observed a greater production of NETs *in vitro* by neutrophils from individuals with RA and the association of this NETs production with more severe disease activity. NETs externalize immunostimulatory molecules and citrullinated autoantigens that, in predisposed individuals, can perpetuate a vicious cycle leading to the generation of specific autoantibodies, exacerbation of immune responses, and subsequent tissue damage (Khandpur et al., 2013; Pratesi et al., 2014; Potempa et al., 2017). Previous studies have also demonstrated that neutrophils isolated from individuals with RA are more reactive and exhibit greater production of NETs *in vitro* without stimulation of a microbial challenge when compared to neutrophils

from healthy controls (Khandpur et al., 2013; Sur Chowdhury et al., 2014; Schneider et al., 2020).

LPS is one of the most important virulence factors of *Porphyromonas gingivalis*, that plays a critical role in mediating inflammation and inducing various immune cells to secrete pro-inflammatory cytokines (Zhu et al., 2016). Herein, we observed an increase in the production of NETs when the culture was stimulated by LPS from *Porphyromonas gingivalis*, both in neutrophils from individuals with RA and in neutrophils from controls. Similar results were observed by Chen and collaborators, that observed an increase in the production of NETs by neutrophils stimulated by *Porphyromonas gingivalis*. In addition, these authors also detected increased levels of intracellular $Ca2^{+}$ and PAD4 in stimulated neutrophils (Chen et al., 2022). Other authors also demonstrated that exposure of neutrophils to LPS from *Porphyromonas gingivalis* significantly increased the production of NETs *in vitro* (Alizadehgharib, Östberg, Dahlstrand, Dahlgren, & Christenson, 2021; Tong, Xin, Fu, Shi, & Sun, 2023).

This study with a Brazilian population evaluated, for the first time, the genotypic and GTG haplotype frequencies of *PADI4* gene SNPs and no association with the risk of developing RA was observed. Studies conducted mainly with Asian populations observed an association between the presence of polymorphisms and susceptibility to RA (Suzuki et al., 2003; Kang et al., 2006; Bang et al., 2010), as did a recent study carried out in Mexico (Matuz-Flores et al., 2022). On the other hand, in a large study conducted in a Caucasian population of European descendants, *PADI4* polymorphisms were not considered a significant risk factor for RA (Burr et al., 2011). Other studies conducted in Caucasian populations also found no association between *PADI4* and risk for RA (Caponi et al., 2004; Barton et al., 2004; Julià et al., 2008). Systematic reviews have shown that *PADI4*-associated polymorphisms may contribute differently depending on ethnicity, being mainly important in Asian populations, but not in the same way in Caucasian populations (Hou et al., 2013; Lee & Bae, 2016; Lu et al., 2018). An important issue that should be taken into account; however, is miscegenation worldwide. Brazilians, for instance, are characterized by their heterogeneity as a result of more than 500 years of miscegenation involving Europeans, Amerindians, Africans, and East Asians (Moura et al., 2015). Thus, further studies involving a larger number of individuals are needed to verify whether the presence of SNPs in the *PADI4* gene confer a risk for RA in other miscegenetic ethnic groups, such as the Brazilian population.

Here, we observed an association between the presence of the GTG haplotype and higher levels of circulating ACPAs. Other studies have also observed an association between ACPAs and the presence of *PADI4* polymorphisms (Gandjbakhch et al., 2009; Gzmán-Guzmán et al., 2015). Recent studies have shown increased *PADI4* gene expression in individuals with RA carrying the GTG haplotype (Matuz-Flores et al., 2022). Corroborating these findings, previous studies have shown an increase in mRNA and higher levels of PAD4 transcripts in individuals carrying the GTG haplotype, which may lead to an increase in the production of citrullinated peptides that act as autoantigens (Hill et al., 2003; Suzuki et al., 2003; Guzmán-Guzmán et al., 2015; Matuz-Flores et al., 2022).

We also observed an association between a greater production of NETs in the culture of neutrophils from individuals with RA and PD and also from individuals who had a positive GTG haplotype. Likewise, we observed that GTG-positive individuals had worse periodontal clinical parameters. Previous *in vitro* studies using leukocytes from individuals with RA have been observed greater activity of the PAD4 enzymatic and greater affinity for calcium ions in carriers of the positive GTG haplotype (Hung et al., 2007; Matuz-Flores et al., 2022). One should recognize, the role of the PAD4 enzyme in the production of NETs through intra and extracellular calcium pools (Gupta, Giaglis, Hasler, & Hahn, 2014; White, Chicca, Cooper, Milward, & Chapple, 2016), and the fact that previous studies had already demonstrated the association between the concentration of circulating NETs and worse periodontal parameters in individuals with RA (Kaneko et al., 2018; Oliveira et al., 2020); Herein, we hypothesize that the increased production of NETs in individuals with the GTG haplotype may be caused by an increase in the activity of the PAD4 enzyme and that this increase directly influences the periodontal condition and also the disease activity of these individuals. However, further studies are needed to better clarify this mechanism.

Based on our observations, we concluded that the production of NETs in the culture of individuals with RA is associated with the severity of the disease and the presence of PD. Furthermore, we observed, for the first time, the association between the production of NETs in the culture and worse periodontal clinical parameters with the presence of the GTG susceptibility haplotype.

Table 1: Frequency of distribution of genotypes observed in control volunteers and in individuals with RA

SNPs	*Control n (%)*	*RA n (%)*	*OR (CI 95%)*	*p*
PADI4_89				
AA[∞]	35 (31.5)	23 (26.7)	1	
AG	58 (52.3)	46 (53.5)	1.20 (0.62-2.31)	0.573
GG	18 (16.2)	17 (19.8)	1.43 (0.61-3.35)	0.401
PADI4_90				
CC[∞]	33 (30.3)	25 (28.7)	1	
CT	59 (54.1)	46 (52.9)	1.02 (0.53-1.96)	0.931
TT	17 (15.6)	16 (18.4)	1.24 (0.52-2.93)	0.620
PADI4_92				
CC[∞]	33 (30.6)	17 (22.7)	1	
CG	57 (52.8)	42 (56.0)	1.43 (0.70-2.90)	0.322
GG	18 (16.6)	16 (21.3)	1.72 (0.70-4.21)	0.231
PADI4_104				
CC[∞]	34 (37.8)	16 (37.2)	1	
CT	45 (50.0)	22 (51.2)	1.03 (0.47-2.27)	0.924
TT	11 (12.2)	5 (11.6)	0.96 (0.28-3.24)	0.955
Haplotypes[†]				
GTG negative[∞]	89 (84.0)	58 (78.4)	1	
GTG positive	17 (16.0)	16 (21.6)	1.44 (0.676-3.084)	0.342

[∞]1 = Reference category; OR = odds ratio; 95% CI = confidence interval.
[†]The letters in *PADI4* haplotype represent nucleotides in *PADI*4_89, *PADI*4_90, and *PADI*_92 SNPs, respectively.
p-values were calculated using logistic regression.

Table 2: Frequency of distribution of haplotypes observed in individuals with according to periodontal status

Haplotypes	*No periodontitis n (%)*	*Periodontitis n (%)*	*OR (CI 95%)*	*p*
GTG negative	18 (42.9)	24 (57.1)	1	
GTG positive	0 (0)	14 (100)	1.75 (0.44-0.74)	0.006

Pearson's chi square test. OR = odds ratio; 95% CI = confidence interval.
[∞] = Reference category
[1]The letters in *PADI4* haplotype represent nucleotides in *PADI*4_89, *PADI*4_90, and *PADI*_92 SNPs, respectively.

Figure 1. (A) Concentration of neutrophil extracellular traps (NETs) in unstimulated and *Porphyromonas gingivalis (Pg)* stimulated human neutrophils culture supernatants of individuals with rheumatoid arthritis (RA) and control volunteers. * denotes significant difference between the culture stimulated with *Pg* and the culture not stimulated. # denotes significant difference between the culture of the RA individuals and of the control volunteers (p<0.05) (Paired t Test was used to compare stimulated and unstimulated NETs concentration within the same group. Unpaired t test was used in the comparison between the concentration of NETs of individuals with RA and control volunteers). (B) Concentration of NETs in human neutrophils culture supernatants of individuals with RA, according to the disease activity score (DAS) 28. (C) Concentration of NETs in human neutrophils culture supernatants of individuals with RA, according to the periodontal status (p<0.05) (Unpaired t Test).

Figure 1

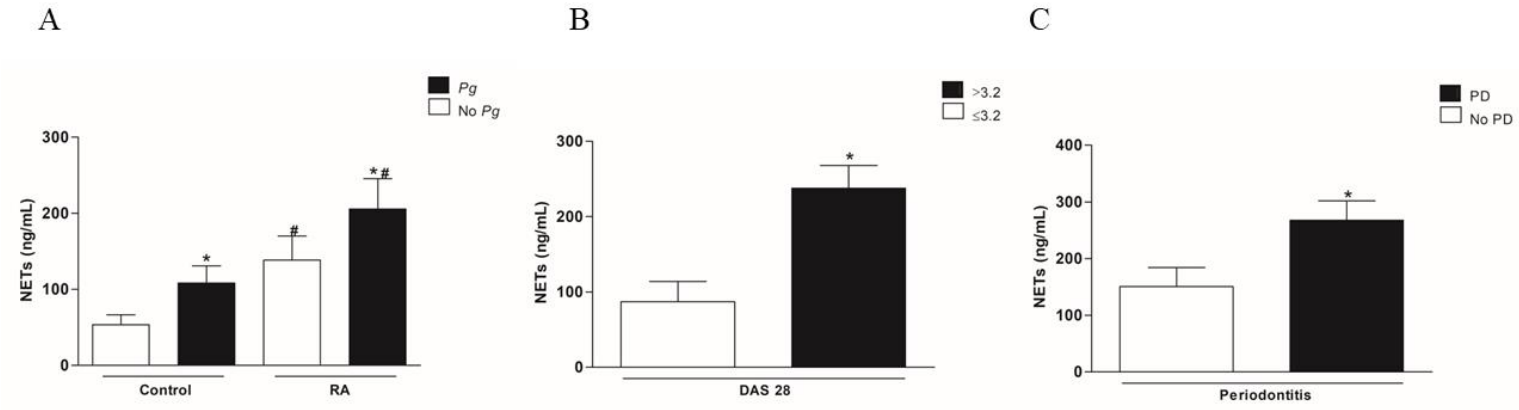


Figure 2. Representative images of immunofluorescence of gingival tissue from (A) individuals with rheumatoid arthritis (RA) without periodontitis (PD); and in (B) individuals with RA and with PD. 4′,6-diamidino-2-phenylindole (DAPI-Blue); citrullinated histones H3 (H3-green); myeloperoxidase (MPO-red) and merged immunofluorescence image. (C) Mean of MPO/H3 positive cells per field at 400x magnification (p<0.05) (Unpaired t Test) (n=5).

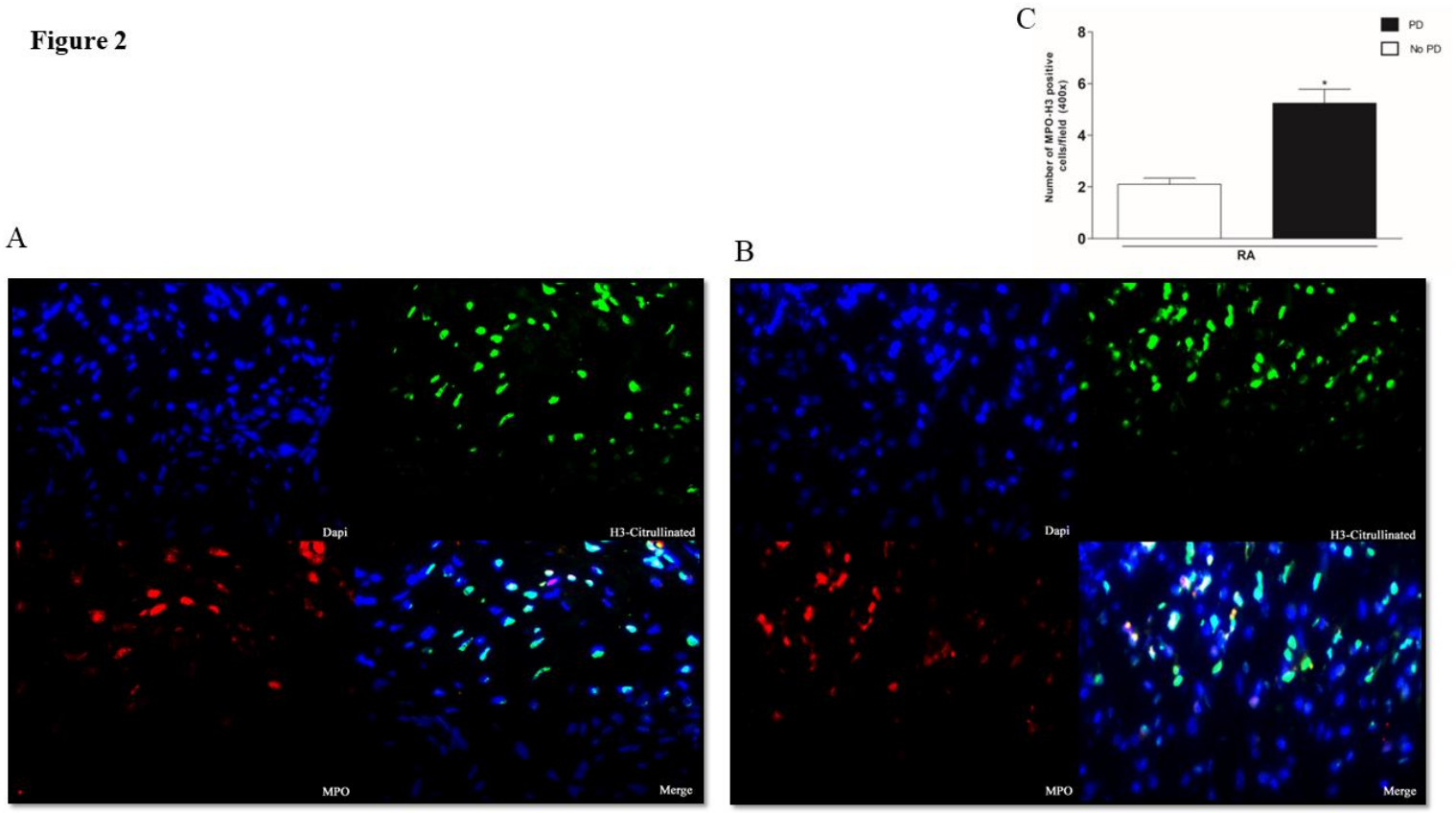


Figure 3. Association of haplotype GTG+/GTG- and (A) concentration of anti-citrullinated protein antibodies (ACPAs) in serum from individuals with rheumatoid arthritis (RA); and (B) production of neutrophil extracellular traps (NETs) in culture by neutrophils from individuals with RA (p<0.05) (Unpaired *t* Test). The letters in peptidyl arginine deaminase type 4 *(PADI4)* haplotype represent nucleotides in *PADI*4_89, *PADI*4_90, and *PADI*_92 single nucleotide polymorphisms (SNPs), respectively.

Figure 3

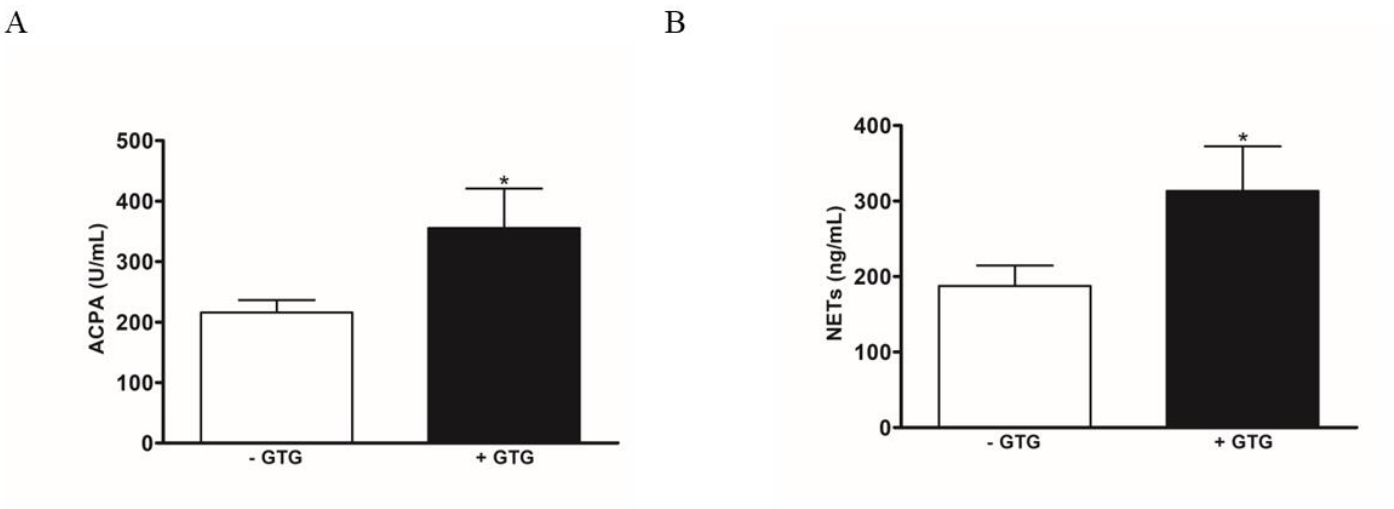
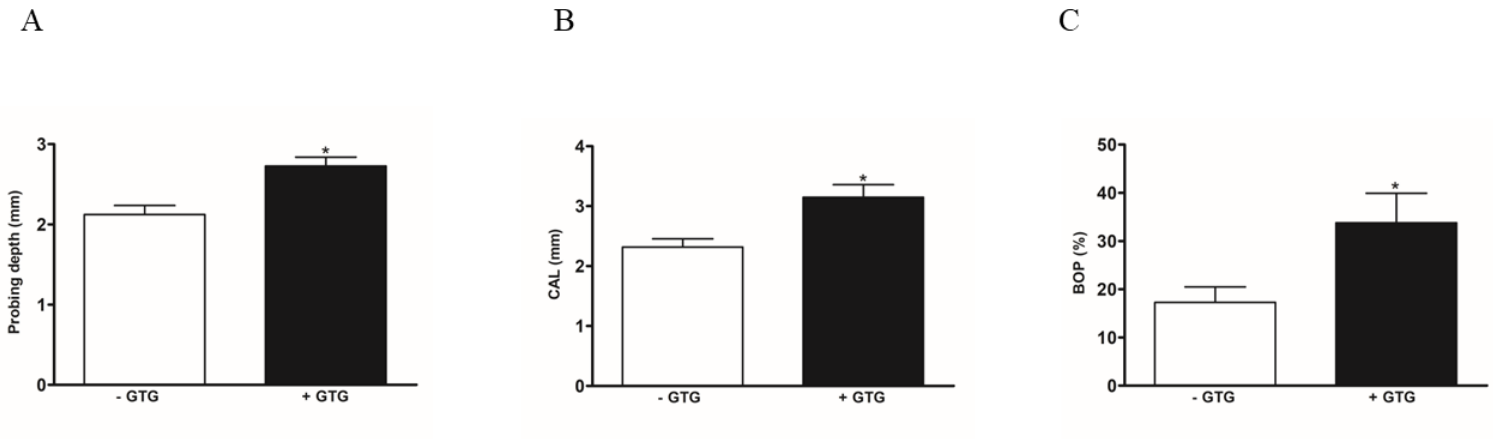


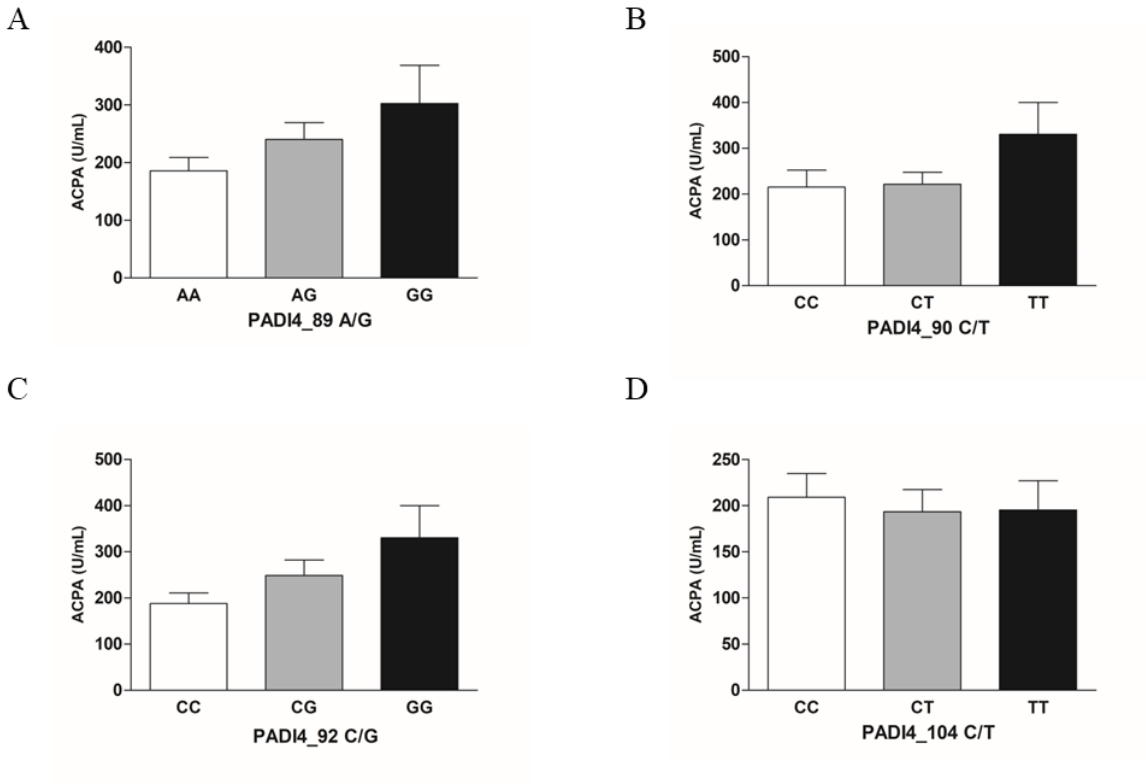
Figure 4. Association of haplotype GTG+/GTG- and periodontal clinical parameters (A) Probing deep; (B) Clinical attachment level (CAL) and (C) Bleeding on probing (BOP) in individuals with rheumatoid arthritis (RA) (p<0.05) (Unpaired t Test). The letters in peptidyl arginine deaminase type 4 *(PADI4)* haplotype represent nucleotides in *PADI*4_89, *PADI*4_90, and *PADI*_92 single nucleotide polymorphisms (SNPs), respectively.

Figure 4



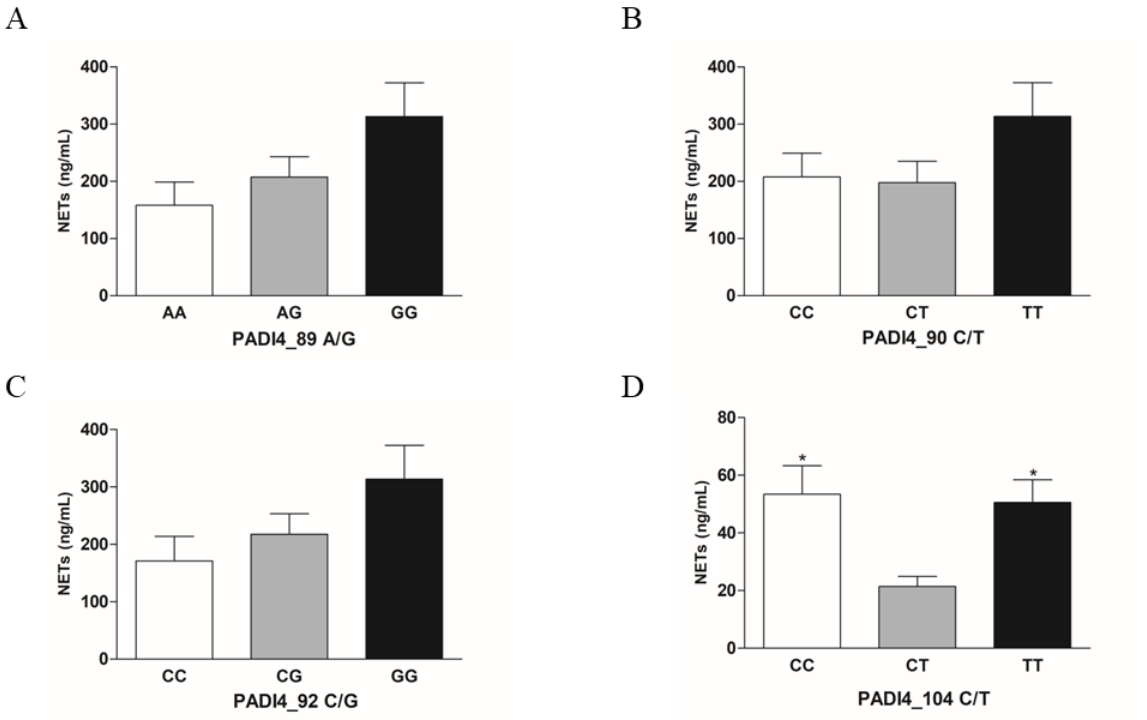
Supplementary figure 1. Association of genotypes observed in single nucleotide polymorphisms (SNPs) (A) *PADI4*_90, (B) *PADI4*_92, (C) *PADI4*_89, (D) *PADI4*_104 and the concentration of anti-citrullinated protein antibodies (ACPAs) in serum from individuals with rheumatoid arthritis (RA) (p<0.05) (ANOVA).

Supplementary figure 1



Supplementary figure 2. Association of genotypes observed in single nucleotide polymorphisms (SNPs) (A) P*ADI4*_90, (B) *PADI4*_92, (C) *PADI4*_89, (D) *PADI4*_104 and the production of neutrophil extracellular traps (NETs) in culture by neutrophils from individuals with rheumatoid arthritis (RA) (p<0.05) (ANOVA).

Supplementary figure 2



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4.3 Artigo 3

Os resultados relacionados ao objetivo específico de realizar uma avaliação geral das revisões sistemáticas disponíveis na literatura com o objetivo de identificar as evidências sobre o efeito do tratamento periodontal não cirúrgico nos parâmetros clínicos e laboratoriais da AR, principalmente sobre a atividade da doença; são apresentados no manuscrito intitulado *"Does non-surgical periodontal treatment contribute to rheumatoid arthritis amelioration? Evidence based on a systematic overview and meta-analysis?"* que será submetido para publicação no periódico *Rheumatology* (Fator de impacto: 5.5).

Does non-surgical periodontal treatment contribute to rheumatoid arthritis amelioration? Evidence based on a systematic overview and meta-analysis

Abstract

Study design: Overview of systematic reviews (SRs) and meta-analysis.

Objective: To perform an overview by analyzing clinical outcomes in individuals with rheumatoid arthritis (RA) who underwent non-surgical periodontal treatment (NSPT).

Methods: Electronic searches were conducted across four databases and gray literature with no restriction on language or publication date. The study followed the 2020 PRISMA statement. A meta-analysis was performed comprising 18 primary studies from SR to examine the effects of NSPT (n=201 subjects in the non-NSPT group vs. n=199 in the NSPT) on 28-joint Disease Activity Score (DAS28).

Results: Nine SRs were analyzed; of these, six with meta-analyses and three without meta-analyses. The main outcomes evaluated were DAS28, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR). NSPT resulted in a significant decrease in CRP, ESR and DAS28, both in studies that included a control group (without NSPT) or in those that compared individuals before and after periodontal therapy. Follow-up period after NSPT ranged from 6 to 24 weeks.

Conclusion: Despite the heterogeneity of data related to RA and periodontitis status and antirheumatic therapy, NSPT decreases the levels of systemic inflammatory markers and RA activity.

Keywords: Dental scaling; Disease activity; Periodontitis; Rheumatoid arthritis; Root planning.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial inflammation, progressive destruction of cartilage and bone tissue resulting in structural damage, disability, and loss of function [1]. Individuals with RA are affected by multiple comorbidities and also have a higher risk of developing periodontitis and tooth loss [2,3]. Among multiple pathophysiological factors implicated in RA-related periodontitis, a major role is played by oral microbiota imbalance characterized by a higher abundance of pathogenic species [4-6].

Periodontitis is a multifactorial inflammatory disease, and one of the most prevalent oral conditions in the general adult population [7,8]. Periodontitis is initiated by dysbiotic microbial communities which lead to tissue-destructive and systemic inflammation [4]. Several studies have implicated key periodontopathogenic bacteria, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, in the generation of citrullinated antigens, which drives the production of anti-citrullinated proteins autoantibodies, a pathological marker of RA [9-11]. Furthermore, high concentrations of circulating systemic inflammatory markers, such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are detected in individuals with RA with worse periodontal condition [12-16]. Therefore, the pathogenic mechanisms of periodontitis mirror those of RA and these conditions can “feed” each other.

Management of periodontitis aims to dismantle the related bacterial biofilm and involves non-surgical periodontal treatment (NSPT) with scaling and root planning, along with oral hygiene education [17,18]. Although previous Systematic Reviews (SRs) revealed that RA outcomes, particularly disease activity, can improve with NSPT [19-22], the results of some SRs [23-25] and recent individual study [26] failed to confirm that. Given this controversial scenario and to build more robust evidence on the effect of NSPT in RA outcomes, we performed an overview and a meta-analysis pooling data from all primary studies identified through the included SRs. This knowledge might support decision-making processes in clinical practices of dentists and rheumatologists.

2. Material and Methods

2.1 Study design and eligibility criteria

Inclusion criteria were SRs assessing the effect of NSPT on RA disease activity as well as clinical and biochemical parameters of RA. A standard definition of SRs was used [27]. The PICOS question was:

P (Population): Individuals with RA;

I (Intervention): NSPT;

C (Comparison): a) No NSPT; b) assessment before (baseline) and after NSPT;

O (Outcome): disease activity (28-joint Disease Activity Score - DAS28), tender joint count (TJC), swollen joint count (SJC), visual analogue scale (VAS), morning stiffness (MS), ESR, and CRP;

S (Studies): SRs with or without meta-analyses.

Exclusion criteria were animal and *in vitro* studies, critical and narrative reviews, letters to the editor, expert opinions, and conference/meeting abstracts.

2.2 Sources of information

Electronic searches were conducted in April 2023 and updated in July 2023 in the following databases: PubMed (National Library of Medicine), Scopus (Elsevier), Embase (Elsevier), and Web of Science (Thomson Reuters). The grey literature (Open Grey) and Google Scholar were also examined, with searches limited to the first 200 results [28]. Searches in the reference list of the included articles were carried out as well. Duplicate references in different databases were encountered and removed using the EndNote program (EndNote®, Clarivate Analytics, Toronto, Canada). No restrictions on the language, date of publication or geographic region were imposed.

2.3 Search strategy

The search strategies used in the databases with Boolean operators linking terms and keywords are depicted in detail in **Supplementary file 1**. In PubMed, the following search strategy was used:

Periodontitis OR Periodontal disease OR Gingivitis OR Periodontal infection OR Gum disease OR Periodontal inflammation OR Gingival inflammation OR Periodontal condition OR Probing depth OR Periodontal probing OR Bleeding on probing OR Clinical attachment loss OR Clinical attachment level OR Plaque AND Periodontal therapy OR Periodontal treatment OR Scaling OR Root planning OR Non-surgical periodontal treatment OR Non-surgical periodontal therapy OR Full-mouth

treatment OR Full-mouth scaling OR Full-mouth disinfection AND Rheumatoid Arthritis OR Arthritis.

2.4 Study selection

The selection of the studies was performed in two phases. The titles/abstracts of all references retrieved during the search were read independently by two authors (S.R.O. and T.A.S.). References whose titles/abstracts met the eligibility criteria were included. When the title/abstract was unavailable or did not provide sufficient information for a decision, the full text was read and evaluated. Articles that met inclusion criteria were also included. Disagreements between the two authors were resolved with a third author (L.G.A.) until reaching a consensus

2.5 Data extraction

The following information was extracted from each included SR: first author's last name, year of publication, country where the study was conducted, databases in which the searches had been carried out, period in which the search was carried out, language restriction applied on search, inclusion and exclusion criteria applied, performance of meta-analysis, performance of risk of bias analysis, number of articles included, number of individuals included, follow up time, main findings after NSPT. Data extraction was performed by two authors independently (S.R.O. and T.A.S.) and disagreements between authors were solved by discussion.

2.6 Synthesis of results (quantitative analysis)

Data extracted from SRs were tabulated with Microsoft Office Excel 2019 (software Microsoft®, Redmond, WA, EUA) and analyzed descriptively.

Meta-analyses of the primary studies included in the SRs were performed using the Review Manager 5.3 (The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). The aim was to pool data of primary studies incorporated into previous meta-analyses and data from primary studies that had not been previously meta-analysed [29].

Four meta-analyses were carried out: 1) a meta-analysis comparing the DAS28 before and ≤3 months after NSPT; 2) a meta-analysis comparing the DAS28 before and 6 months after NSPT; 3) a meta-analysis comparing the change in DAS28 after ≤3 months between individuals who had not undergone any NSPT and individuals

who had undergone NSPT and; 4) a meta-analysis comparing the change in DAS28 after 6 months between individuals who had not undergone any NSPT and individuals who had undergone NSPT.

In the four meta-analyses, data on DAS28 mean and standard deviation as well as sample size were used. When DAS28 data provided by studies were expressed as median and interquartile range, or median and minimum and maximum, equations for calculating mean and standard deviation were adopted [30] (**Supplementary file 2A and 2B**).

In the two meta-analyses comparing individuals who had undergone NSPT and individuals who had not undergone NSPT, the mean difference and the pooled standard deviation of the DAS28 between the follow-up (≤3 months or 6 months) and the baseline were also calculated based on equations established [31] (**Supplementary file 2C**).

In all analyses, the random-effects model was used [32]. The results of the meta-analyses were reported in mean difference (MD) and 95% confidence interval (CI).

2.7 Critical appraisal of the studies

The quality assessment was performed independently by two authors (S.R.O. and T.A.S.), and disagreements were resolved by discussion. The methodology of included SRs was examined using the A MeaSurement Tool to Assess SRs (AMSTAR-2) checklist [33].

2.8 Protocol and registration

The reporting of this overview of SRs complies with the Preferred Reporting Items for SRs and Meta-Analysis (PRISMA) [34]. We followed the extensive new material on methods of SRs available in the fully updated edition of the Cochrane Handbook [29]. A protocol was registered in the International Prospective Register of SRs (PROSPERO) under registration number (CRD42023414714).

3. Results

3.1 Study selection

Across the electronic databases, 2,172 references were identified; of these, 966 were retrieved from PubMed, 897 from Web of Science, 156 from Scopus, and 153 from Embase. After removal of 434 duplicates, 1,738 titles/abstracts were evaluated. Of these, six articles [19, 20, 22, 23, 24, 35] had titles/abstracts that satisfied the eligibility criteria and were included. The title/abstracts of 10 articles contained insufficient information for a decision and full texts were evaluated considering the eligibility criteria; seven articles were excluded for different reasons **(Supplementary file 3)** and three [21, 25, 36] were included. No references that met the eligibility criteria were found in searches in the reference list of included articles or in the searches in grey literature. Finally, nine SRs [19-25, 35, 36] were included in this study. The flowchart of the study is displayed in **Figure 1**.

3.2 *Characteristics of the SRs*

Three studies were SRs without meta-analysis [23, 35, 36] and six were SRs with meta-analyses [19-22, 24, 25]. The studies were developed in Brazil [21, 22], China [20, 25], Australia [24, 36], United Kingdom [35], Portugal [19], and Spain [23] and were published between 2011 and 2022. Most of the included studies were published in English [19-21, 23, 24, 35, 36], but there were also publications in Portuguese [22] and Chinese [25].

The number of articles included in each SR ranged from 3 to 21 studies and the post-NSPT follow-up ranged from 45 days to 24 weeks (**Table 1** and **Table 2**). The inclusion and exclusion criteria adopted in each SR are described in **Supplementary file 4**.

Regarding quality analysis/risk of bias assessment**,** one study [23] did not perform any evaluation. The other studies used different tools, such as Cochrane [19-21, 25, 35], Risk of Bias In Non-randomized Studies of Interventions (ROBINS-I) [35], the Newcastle-Ottawa scale (NOS) [21], PEDro [22], and Joanna Briggs Institute Meta-Analysis of Statistics Assessment and Review Instrument (JBI-MAStARI) [24, 36].

3.3 NSPT adopted in the SRs

In most studies, periodontal treatment [20-25, 36] consisted of full-mouth scaling and root planning using manual or ultrasonic instruments along with oral hygiene instruction. The SRs conducted by Mustufvi et al., 2022 [35] and Silva et al.,

2021 [19] also included intervention studies that, in addition to the NSPT, used local or systemic antibiotics as adjuvants.

3.4 NSPT and the rheumatologic outcomes

The main outcomes related to NSPT effects on clinical and laboratory parameters are described in **Table 1** and **Table 2**.

3.4.1 NSPT and RA clinical parameters

Of the nine studies included, three evaluated the effects of NSPT on clinical parameters of RA such as TJC, SJC and MS, in addition to patient's perception of pain through the VAS scale. The meta-analysis conducted by Sun et al., 2021 [20] showed that NSPT resulted in a significant reduction of DAS28 components TJC, SJC, and VAS. In contrast, MS was not modified by NSPT [20]. In the review carried out by Mustufvi et al., 2022 [35], a total of four studies included data on the effect of NSPT on TJC and SJC. Two of them reported that there was a significant improvement in TJC and SJC after NSPT and no studies showed decrease in MS. In the SR conducted by Calderaro et al., 2017 [22], no significant reduction in TJC, SJC and VAS after NSPT was observed.

3.4.2 NSPT and disease activity score

The DAS28 is widely used and a validated instrument that combine several parameters such as TJC, SJC, ESR or CRP and patient global assessment using a visual analog scale (VAS) [37]. DAS28 is used routinely in guiding individual treatment and in assessing the efficacy of therapies.

The meta-analysis performed by Calderaro et al., 2017 [22] showed that NSPT resulted in significant reduction of DAS28. In this SR, studies that used CRP to calculate the DAS28, and that did not present DAS28 data at baseline and follow-up, were excluded; therefore, the meta-analysis was performed with two articles only. Silva et al., 2022 [19], performed a meta-analysis with seven of the 14 studies included in the review. A significant reduction in the disease activity was observed when comparing the group that had undergone NSPT with the group that had not undergone NSPT. The studies included both DAS28 and the SDAI (Simplified Disease Activity Index), another validated instrument to measure RA activity. The meta-analysis conducted by Sun et al., 2021 [20] included seven articles that used the DAS28 to

assess disease activity and observed a significant reduction in the DAS28 after NSPT. Rosa et al., (2021) [21] performed an intragroup meta-analysis, comparing the DAS28 observed in the follow-up after the NSPT with the DAS28 measured at baseline within individuals the who had undergone the NSPT. The authors included in the meta-analysis five of the seven articles included in the SR. The meta-analysis also showed a significant reduction in the DAS28 after the NSPT.

In the SR performed by Mustufvi et al., (2022) [35], of the 17 studies included, nine showed a significant improvement in DAS28 after NSPT compared to baseline. In addition, six out of 10 articles showed a significant difference of DAS28 between the group who had undergone NSPT and the group who had not undergone NSPT. In this SR, the DAS28 was calculated based on the ESR in eight included studies, while two studies used CRP to calculate the DAS28. Seven studies did not report the basis for DAS28 calculation, and one study reported both formats [35]. Other studies did not observe significant differences in DAS28 after NSPT [23-25].

3.4.3 NSPT on laboratory parameters

ESR and CRP are acute phase markers elevated in RA and useful to evaluate the disease activity [38]. The study of Kaur et al., 2013 [36] included three articles demonstrating significant reduction in ESR after NSPT. A meta-analysis confirmed the significant decrease in ESR in individuals submitted to NSPT, but no statistically significant decrease in CRP levels after NSPT was observed [24]. Similarly, the meta-analysis performed by Rosa et al., 2021 [21], showed a significant reduction of ESR but not of CRP after the NSPT. The studies included in the SR carried out by Silvestre et al., 2016 [23], also showed a significant decrease in ESR levels, suggesting a reduction in systemic inflammation following NSPT; however, there was no statistically significant decrease in CRP levels.

In the SR conducted by Mustufvi et al., 2022 [35], of the seven studies that analyzed CRP and the 10 studies that analyzed ESR, four and six studies, respectively, showed a decrease in these markers after NSPT. Likewise, the meta-analysis by Silva et al., 2022 [19] also showed ESR and CRP reduction after the NSPT.

On the other hand, the meta-analysis performed by Sun et al., 2021 [20], which included five intervention studies, reported a significant decrease of CRP but not of ESR in the group that had undergone NSPT. Other studies also observed a

decrease, albeit not significant, in ESR and CRP levels in individuals with RA after performing the NSPT [22].

3.5 Meta-analyses of the effect of NSPT on DAS28

To synthesize the results of multiple SRs comparing NSPT impact on DAS28, we pooled data from all primary studies identified in the SRs. Considering the type of comparison (before and after NSPT or NSPT versus non-NSPT) and follow up period of NSPT, four meta-analysis were conducted.

In the meta-analysis comparing the DAS28 before NSPT and ≤3 months after NSPT, data from 17 studies were pooled [15, 39-54]. Data from 436 patients, demonstrated that DAS28 after NSPT (≤3 months) was significantly lower than DAS28 before NSPT (baseline) (MD = -0.75; 95% CI = -1.03 - -0.48) (Figure 2A).

In the meta-analysis comparing the DAS28 at baseline and 6 months after NSPT, data of five studies [41, 42, 50, 54] comprising 101 individuals were merged. The result showed that DAS286 months after NSPT was significantly lower than DAS28 before NSPT (baseline) (MD = -1.08; 95% CI = -1.68 - -0.49) (Figure 2B).

In the meta-analysis comparing the change in DAS28 after follow-up (≤3 months) between individuals who had undergone NSPT and individuals who had not undergone any NSPT data from eight studies comprising 199 and 201individuals respectively [39, 45, 48, 50-52, 54, 55] were pooled. Compared to the baseline, the reduction of DAS28 among individuals who had undergone NSPT was significantly higher than among individuals who had not undergone NSPT (follow-up ≤3 months) (MD = -0.55; 95% CI = -0.83 - -0.27) (Figure 3A).

In the meta-analysis comparing the change in DAS28 after 6 months between individuals who had undergone NSPT and individuals who had not undergone NSPT, data of two studies comprising 61 and 64 individuals respectively [50, 54] were used. The reduction of DAS28 after 6 months was higher among individuals who had undergone NSPT than among individuals who had not undergone NSPT, but not reaching statistical significance (MD = -1.36; 95% CI = -3.61 - 0.89) (Figure 3B).

3.6 Critical appraisal of SRs

The quality of the included SRs was reasonable. The results of the quality analysis of the SRs included are described in **Supplementary file 5**.

4. Discussion

This overview identified nine SRs addressing the influence of NSPT on key clinical and laboratory outcomes of RA. According to our quantitative synthesis, NSPT significantly decreased DAS28 at the three and six months of follow-up. In addition, the group of patients with RA submitted to NSPT showed a significant reduction in DAS28 than the group not submitted to NSPT at the three months follow-up. Of relevance, parameters, such as TJC, SJC, ESR, and CRP, of individuals with RA also decreased after NSPT, suggesting a potential contribution of periodontal therapy on control of RA.

NSPT can effectively decrease bacteria, bacterial antigens, and pro-inflammatory mediators that invade the circulatory system [15]. The reduction of systemic inflammation observed after NSPT seems to contribute to a better clinical outcome of RA [43]. Herein, we observed that most previous studies showed a significant decrease in the DAS28 after NSPT [19, 20, 21, 22] which was confirmed by our meta-analysis. Conversely, the meta-analysis performed by Kaur et al., 2014 [24] and Lu et al., 2011 [25] and the SR of Silvestre et al., 2016 [23], did not observe significant reductions in DAS28 after NSPT. Conflicting results reported by previous studies [23-25] can be explained by factors such as small sample size, different stages of periodontitis and RA at the baseline, and follow up period and antirheumatic treatment. In order to homogenize the sample, some authors allocated participants according to RA disease activity [44], the use of DMARDs [52], or by periodontal status [15, 45].

Regarding the NSPT follow-up, most studies included in the SRs had a primary endpoint of 3 months or less. In our meta-analysis, we observed a significant decrease in DAS28 both at three and six months of follow-up. However, a slight decrease in DAS28 after NSPT may not directly be translated into an improvement in the stage of RA disease activity (e.g., moderate to low), or in the clinical signs and symptoms of the disease; some patients may have tender joints caused by concomitant osteoarthritis, and in others, acute phase reactants may be elevated due to comorbid conditions [56].

This study also revealed positive effects of NSPT on individual parameters included in the DAS28 score, such as TJC, SJC and on laboratory parameters (CRP and ESR), as also demonstrated elsewhere [57]. Periodontitis-related cytokines trigger increased hepatic synthesis and rapid secretion of plasma proteins, including CRP and

fibrinogen [58], consequently increasing ESR [59]. While former studies [21, 23, 24, 36] observed a significant reduction in ESR levels after NSPT, Sun et al., 2021 [20] demonstrated a significant decrease in circulating serum CRP levels. Accordingly, recent clinical trials have shown a significant decrease in ESR and serum CRP [43,50,60].

Due to the cross-talk between periodontitis and RA, it is possible that antirheumatic treatment have an impact on the periodontal condition. An improvement of periodontal health in individuals using biological DMARDs, such as anti-TNF-α [61-63] and anti-IL-6 agents [64] has been shown. Similar results were observed in individuals using non-biological DMARDs (i.e., MTX) [65]. Recent SRs have indicated anti-rheumatic agents may influence the periodontal condition in individuals with RA and periodontitis [66, 67]. In this sense, the influence of antirheumatic therapy on periodontal health should also be considered.

Regarding potential limitations of the studies included, it is worth mentioning the small number of individuals recruited per group, lack of standardization in relation to the criteria for defining periodontitis, different stages of disease severity and duration of periodontitis and RA, relatively short follow-up, lack of standardization of the NSPT (e.g., instruments used, operator skills, number of sessions, and adjuvant use of antibiotics) as well as the different antirheumatic and corticosteroids therapies. It is also important to point out that the included studies adopted different laboratory tests for the calculation of the DAS28 (i.e., ESR vs. CRP). Another important point that should be considered is the lack of appropriate control of confounding factors such as comorbidities that can influence both periodontitis and RA, including smoking. Multicenter double-blind randomized controlled clinical studies that adopt the mentioned standards and with longer follow-up times are highly needed.

In light of the findings observed in this study and in view of the evidence on the role of periodontal inflammation and the participation of periodontopathogens in the onset of RA and in the increase of disease activity [10, 68, 69] both rheumatologists and dentists should be aware of the importance of periodontal health in individuals with RA, adopting preventive and therapeutic measures [66, 70, 71].

Conclusion

NSPT contributed to the reduction of disease activity in individuals with RA. Future studies with a longer follow-up period, a larger number of participants and

control for confounding factors are needed to specifically weight the contribution of antirheumatic medication and other likely variables.

Table 1. Characteristics of the SRs with meta-analyses included in the overview (n=6)

Reference	Country	Data bases	Search period	Language restriction	Quality analysis/ Risk of bias	Number of articles in SR	Number of articles in meta-analyses	Number of individuals included in meta-analyses	Follow-up time NSPT	Outcomes of meta-analyses	Main findings
Silva et al., 2022 [19]	PRT	PubMed/ Medline; Cochrane library; Embase; ClinicalTrials.gov; WHO – ICTRP	No restriction of data	No restriction	Cochrane tool	14[a]	DAS28/SDAI: n=7 ESR/CRP: n= 7	DAS28/SDAI: Control group = 147; NSPT group= 149 ESR/CRP: Control group = 134; NSPT group= 136	6 to 24 weeks	DAS28 /SDAI ESR/CRP	Decrease in disease activity and systemic inflammatory markers (ESR/CRP)
Sun et al., 2021 [20]	CHN	PubMed; Embase; Cochrane library	Until october 2020	No restriction	Cochrane tool	9	DAS28: n=7 ESR: n=6 CRP: n=5 TJC: n=5 SJC: n=5 VAS: n=5 MS: n=1	DAS28: Control group = 147; NSPT group= 149 ESR: Control group = 105; NSPT group= 110 CRP: Control group = 102; NSPT group= 99 TJC: Control group = 111; NSPT group= 113 SJC: Control group = 111; NSPT group= 113 VAS: Control group = 111; NSPT group= 113 MS: Control group = 12; NSPT group= 17	6 to 24 weeks	DAS28 ESR CRP TJC SJC VAS MS	Decrease in: DAS28¸ TJC, SJC, VAS, CRP
Rosa et al., 2021 [21]	BRA	PubMed/ Medline; Scopus; Cochrane library	Until April 2019	No restriction	Cochrane tool; NOS	7	DAS28: n=5 ESR: n=4 CRP: n=2	DAS28: Control group = 130; NSPT group= 135 ESR: Control group = 120; NSPT group= 125	4 to 24 weeks	DAS28 ESR CRP	Decrease in DAS28; decrease in ESR

								CRP: Control group = 59; NSPT group= 56			
Calderaro et al., 2017 [22]	BRA	Pubmed/ Medline; The Cochrane Library; Clinical Trials; SciELO; Lilacs	Until December 2014	No restriction	PEDro scale	4	DAS28: n=2 ESR: n=2 CRP: n=2 TJC: n=2 SJC: n=2 VAS: n=2	DAS28: Control group = 22; NSPT group= 27 ESR: Control group = 27; NSPT group= 32 CRP: Control group = 44; NSPT group= 41 TJC: Control group = 41; NSPT group= 43 SJC: Control group = 41; NSPT group= 43 VAS: Control group = 41; NSPT group= 43	6 to 24 weeks	DAS28 ESR CRP TJC SJC VAS	Decrease in DAS28
Kaur et al., 2014 [24]	AUS	PubMed/ Medline; CINAHL; DOSS; Embase; Scopus; Web of Knowledge; MedNar; Lilacs; ProQuest Theses and Dissertations	Until September 2013	English	JBI-MAStARI	5	DAS28: n=3 ESR: n=3 CRP: n=2	DAS28: Control group = 51; NSPT group= 53 ESR: Control group = 37; NSPT group= 42 CRP: Control group = 44; NSPT group= 41	6 to 24 weeks	DAS28 ESR CRP	Decrease in ESR
Lu et al., 2011 [25]	CHN	Medline; Embase; China Biomedical Literature Database; Cochrane library	1950-2010	No restriction	Cochrane tool	4	DAS28: n=2	DAS28: Control group = 22; NSPT group= 27	6 to 24 weeks	DAS28	No decrease in DAS28

Note: AUS: Australia; BRA: Brazil; CHN: China; CRP: C-reactive protein; DAS28: 28-joint Disease Activity Score; ESR: Erythrocyte sedimentation rate; JBI-MAStARI: Joanna Briggs Institute Meta Analysis of Statistics Assessment and Review Instrument; MS: Morning stiffness; NOS: Newcastle-Ottawa scale; NSPT: Non-surgical periodontal treatment; PRT: Portugal; SDAI: Simplified Disease Activity Index; SJC: Swollen join counts; SR: Systematic review; TJC: Tender joint counts; VAS: Visual analogical scale; WHO-ICTRP: World Health Organization International Clinical Trial Registry Platform portal.

ƨ Of these, four are ongoing studies and were not considered in the follow-up time and number of individuals included.

Table 2. Characteristics of the SRs without meta-analyses included in the overview (n=3)

Reference	Country	Data bases	Search period	Language restriction	Quality analysis/ Risk of bias	Number of articles in SR	Total number of individuals included in SR	Follow-up time NSPT	Main findings
Silvestre et al., 2016 [23]	ESP	PubMed/ Medline; Cochrane library; Embase; Scopus	Until February 2015	English	No	8	291	6 to 24 weeks	Decrease in ESR*****; tendency of decrease in DAS28∞ and CRP∞
Mustufvi et al., 2022 [35]	GBR	PubMed/ Medline; OVID Embase; Cochrane library	1944 to January 2022	English	ROBINS-I; Cochrane tool	21	805	45 days to 24 weeks	9 out of 17 studies showed a decrease in DAS28*****; 4 out of 7 studies showed a decrease in CRP*****; 6 out of 10 studies showed a decrease in ESR*****; 2 out of 4 studies showed an improvement in TJC* and SJC*****; no decrease in MS
Kaur et al., 2013 [36]	AUS	PubMed/ Medline; CINAHL; DOSS; Embase; Scopus; Web of Knowledge; MedNar; ProQuest Theses and Dissertations	Until June 2012	English	JBI-MAStARI	3	101	8 to 24 weeks	Decrease in ESR*****

Note: AUS: Australia; CRP: C-reactive protein; DAS28: 28-joint Disease Activity Score; ESP: Spain; ESR: Erythrocyte sedimentation rate; GBR: United Kingdom; JBI-MAStARI: Joanna Briggs Institute Meta Analysis of Statistics Assessment and Review Instrument; MS: Morning stiffness; NSPT: Non-surgical periodontal treatment; ROBINS-I: Risk Of Bias In Non-randomized Studies of Interventions; SJC: Swollen join counts; SR: Systematic review; TJC: Tender joint counts.
* Statistically significant result; ∞ Result not statistically significant.

Figure 1. Flow diagram of the literature search and selection of studies based on the Preferred Reporting Items for SRs and Meta-analyses (PRISMA).

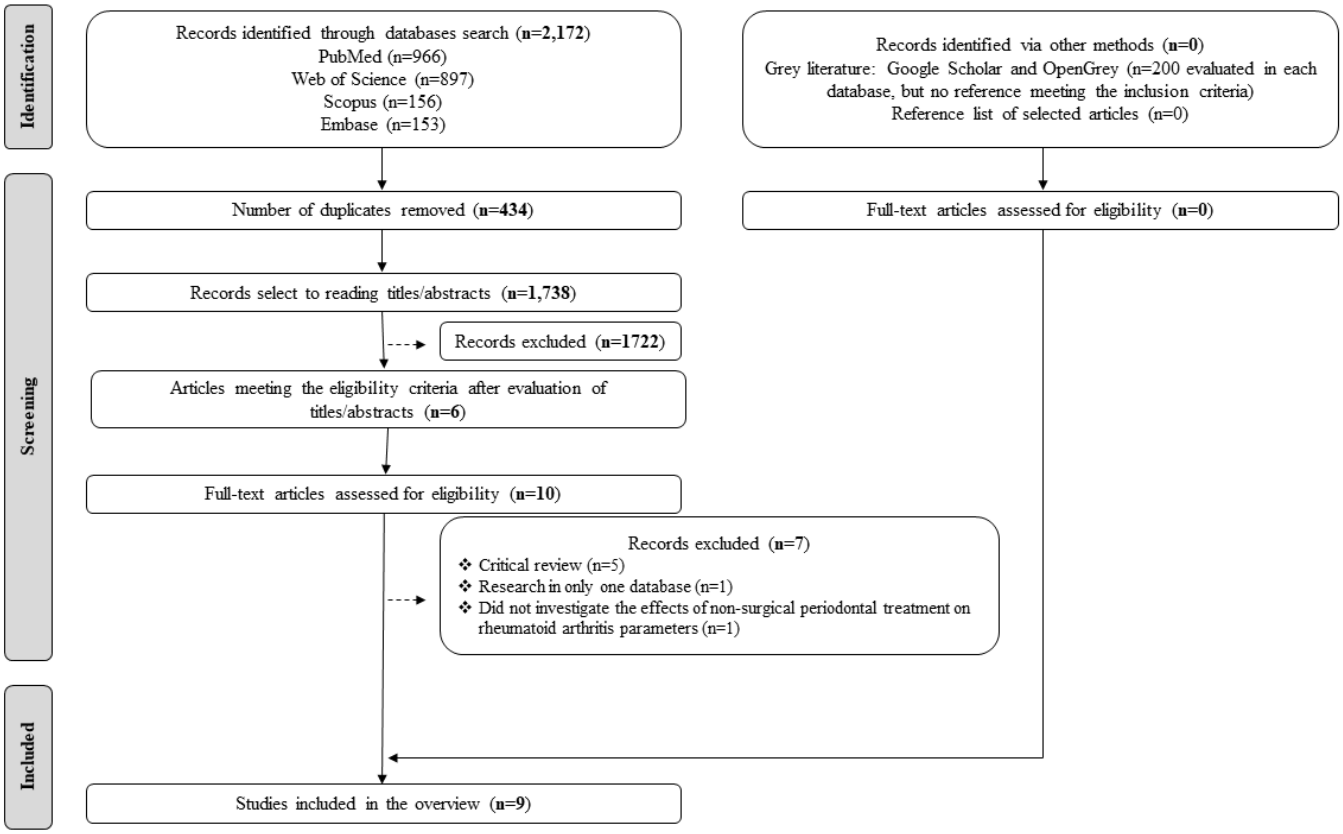
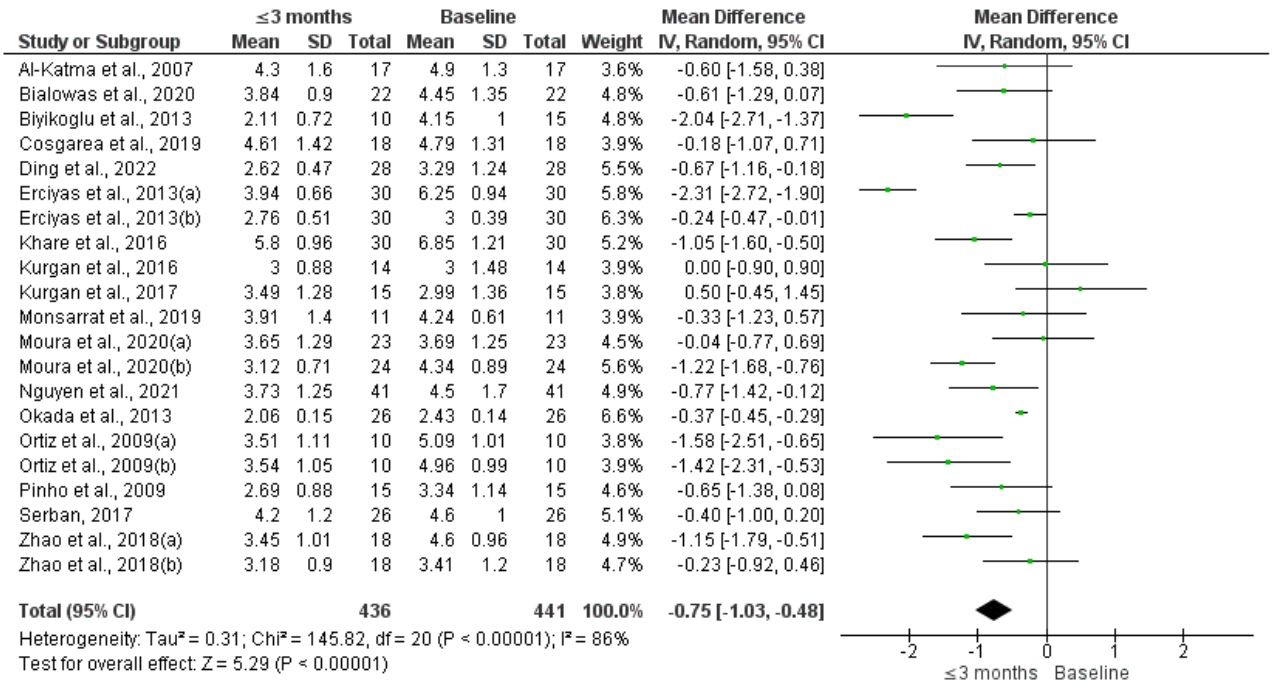


Figure 2. (A) Meta-analysis of the effect of non-surgical periodontal treatment (NSPT) on disease activity score 28 (DAS28) comparing baseline and follow-up for ≤ 3 months in the rheumatoid arthritis (RA) group who had undergone NSPT. (B) Meta-analysis of the effect of non-surgical periodontal treatment (NSPT) on disease activity score 28 (DAS28) comparing baseline and 6 months follow-up in the rheumatoid arthritis (RA) group who had undergone NSPT.

A



B

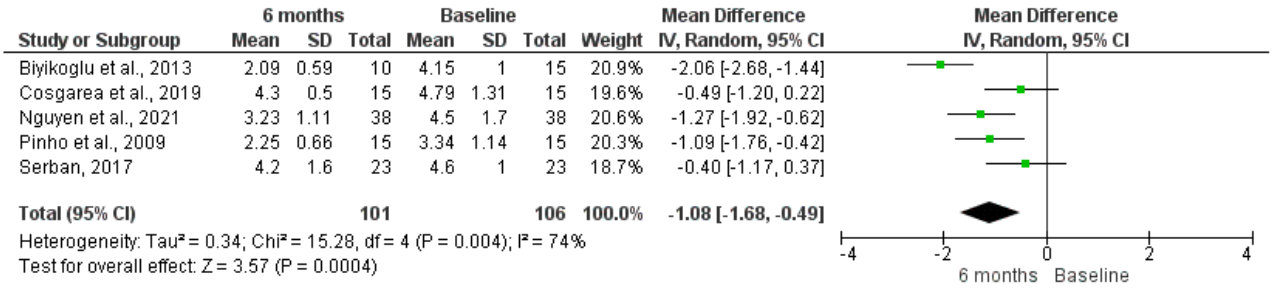
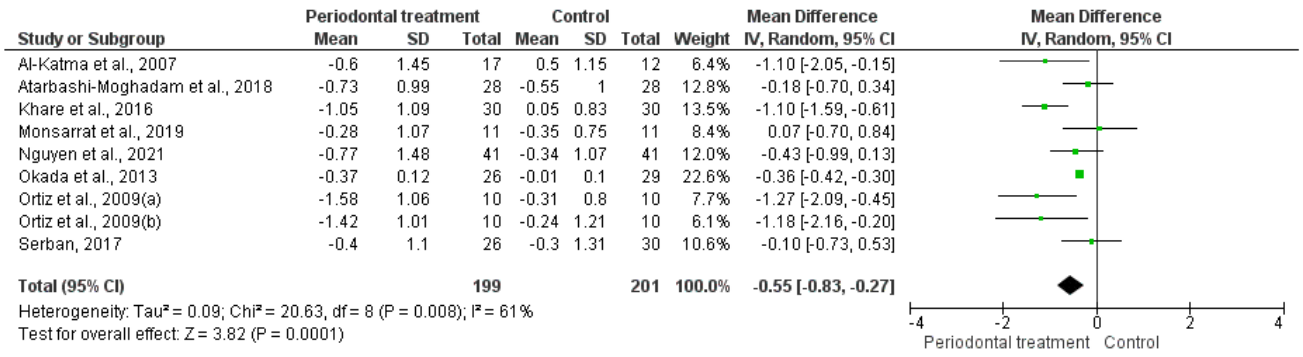
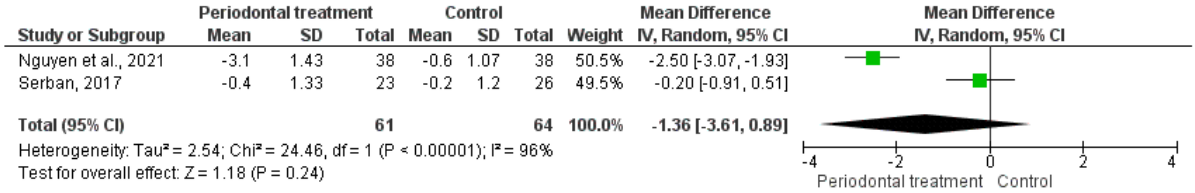


Figure 3. (A) Meta-analysis comparing the change (baseline and at follow-up ≤ 3 months) on disease activity score 28 (DAS28) between individuals with rheumatoid arthritis (RA) group who had undergone NSPT and individuals with rheumatoid arthritis (RA) group who had not undergone NSPT. (B) Meta-analysis comparing the change (baseline and at follow-up 6 months) on disease activity score 28 (DAS28) between individuals with rheumatoid arthritis (RA) group who had undergone NSPT and individuals with rheumatoid arthritis (RA) group who had not undergone NSPT.

A



B



Supplementary files

Supplementary file 1. Search strategies employed to identify articles in databases

Database	Search strategy
PubMed	Periodontitis OR Periodontal disease OR Gingivitis OR Periodontal infection OR Gum disease OR Periodontal inflammation OR Gingival inflammation OR Periodontal condition OR Probing depth OR Periodontal probing OR Bleeding on probing OR Clinical attachment loss OR Clinical attachment level OR Plaque AND Periodontal therapy OR Periodontal treatment OR Scaling OR Root planning OR Non-surgical periodontal treatment OR Non-surgical periodontal therapy OR Full-mouth treatment OR Full-mouth scaling OR Full-mouth disinfection AND Rheumatoid Arthritis OR Arthritis
Web of Science	Periodontitis OR Periodontal disease OR Gingivitis OR Periodontal infection OR Gum disease OR Periodontal inflammation OR Gingival inflammation OR Periodontal condition OR Probing depth OR Periodontal probing OR Bleeding on probing OR Clinical attachment loss OR Clinical attachment level OR Plaque AND Periodontal therapy OR Periodontal treatment OR Scaling OR Root planning OR Non-surgical periodontal treatment OR Non-surgical periodontal therapy OR Full-mouth treatment OR Full-mouth scaling OR Full-mouth disinfection AND Rheumatoid Arthritis OR Arthritis
Embase	Periodontitis OR “Periodontal disease” OR Gingivitis OR “Periodontal infection” OR “Gum disease” OR “Periodontal inflammation” OR “Gingival inflammation” OR “Periodontal condition” OR “Probing depth” OR “Periodontal probing” OR “Bleeding on probing” OR “Clinical attachment loss” OR “Clinical attachment level” OR Plaque AND “Periodontal therapy” OR “Periodontal treatment” OR Scaling OR “Root planning” OR “Non-surgical periodontal treatment” OR “Non-surgical

	periodontal therapy” OR “Full-mouth treatment” OR “Full-mouth scaling” OR “Full-mouth disinfection” AND “Rheumatoid Arthritis” OR Arthritis
Scopus	Periodontitis OR “Periodontal disease” OR Gingivitis OR “Periodontal infection” OR “Gum disease” OR “Periodontal inflammation” OR “Gingival inflammation” OR “Periodontal condition” OR “Probing depth” OR “Periodontal probing” OR “Bleeding on probing” OR “Clinical attachment loss” OR “Clinical attachment level” OR Plaque AND “Periodontal therapy” OR “Periodontal treatment” OR Scaling OR “Root planning” OR “Non-surgical periodontal treatment” OR “Non-surgical periodontal therapy” OR “Full-mouth treatment” OR “Full-mouth scaling” OR “Full-mouth disinfection” AND “Rheumatoid Arthritis” OR Arthritis
Google Scholar	(Periodontitis OR “Periodontal disease” OR Gingivitis OR “Periodontal infection” OR “Gum disease” OR “Periodontal inflammation” OR “Gingival inflammation” OR “Periodontal condition” OR “Probing depth” OR “Periodontal probing” OR “Bleeding on probing” OR “Clinical attachment loss” OR “Clinical attachment level” OR Plaque) AND (“Periodontal therapy” OR “Periodontal treatment” OR Scaling OR “Root planning” OR “Non-surgical periodontal treatment” OR “Non-surgical periodontal therapy” OR “Full-mouth treatment” OR “Full-mouth scaling” OR “Full-mouth disinfection”) AND (“Rheumatoid Arthritis” OR Arthritis)

Supplementary file 2: (A) Equations for calculating the mean and standard deviation from the median and interquartile range (Wan et al., 2014); (B) Equations for calculating the mean and standard deviation from median and minimum and maximum (Wan et al., 2014); (C) Equations for calculating the mean difference and the pooled standard deviation of the DAS28 between follow-up (≤3 months or 6 months) and the baseline, comparing individuals who had undergone NSPT and individuals who had not undergone NSPT (Cohen, 1988).

(A)

$$\bar{X} \approx \frac{q1 + m + q3}{3}$$

$$S \approx \frac{q3 - q1}{1.35}$$

$\bar{X}$= mean
q1 = the first quartile
m = the median
q3 = the third quartile
S= standard deviation

(B)

$$\bar{X} \approx \frac{a + 2m + b}{4}$$

$$S \approx \frac{b - a}{4}$$

$\bar{X}$= mean
a = the minimum value
m = median
b = the maximum value
S= standard deviation

(C)

$$\bar{X}_{difference} \approx \bar{X}_{follow-up} - \bar{X}_{baseline}$$

$$Pooled\ S = \sqrt{\frac{(S_{follow-up})^2 + (S_{baseline})^2}{2}}$$

$\bar{X}$= mean
S= standard deviation

References

Wan X, Wang W, Liu J, Tong T. Estimating the sample mean and standard deviation from the sample size, median, range and/or interquartile range. BMC Med Res Methodol. 2014;19(14):135. doi: 10.1186/1471-2288-14-135. PMID: 25524443; PMCID: PMC4383202.

Cohen, J (1988). Statistical Power Analysis for the Behavioral Sciences (2nd ed). Hillsdale, NJ: Lawrence Erlbaum Associates, Publishers.

Supplementary file 3. Articles excluded after full text reading with reasons for exclusion

References	Reasons for exclusion
1. Araújo VM, Melo IM, Lima V. Relationship between Periodontitis and Rheumatoid Arthritis: Review of the Literature. Mediators Inflamm. 2015;2015:259074.	This is a critical review
2. Hussain SB, Botelho J, Machado V, et al. Is there a bidirectional association between rheumatoid arthritis and periodontitis? A systematic review and meta-analysis. Semin Arthritis Rheum. 2020;50(3):414-422.	This study did not investigate the effects of non-surgical periodontal treatment on rheumatoid arthritis parameters
3. Wen S, Beltrán V, Chaparro A, Espinoza F, Riedemann JP. ¿La periodontitis crónica modifica la morbilidad de la artritis reumatoide?: Aspectos clínicos y moleculares. Una revisión sistemática [Association between chronic periodontitis and rheumatoid arthritis. A systematic review]. Rev Med Chil. 2019;147(6):762-775.	Research in only one database not characterizing a systematic review
4. Bartold PM, Marshall RI, Haynes DR. Periodontitis and rheumatoid arthritis: a review. J Periodontol. 2005;76(11):2066-2074.	This is a critical review

5. de Molon RS, Rossa C Jr, Thurlings RM, Cirelli JA, Koenders MI. Linkage of Periodontitis and Rheumatoid Arthritis: Current Evidence and Potential Biological Interactions. Int J Mol Sci. 2019;20(18):4541.	This is a critical review
6. Falcao A, Bullón P. A review of the influence of periodontal treatment in systemic diseases. Periodontol 2000. 2019;79(1):117-128.	This is a critical review
7. Rutger Persson G. Rheumatoid arthritis and periodontitis - inflammatory and infectious connections. Review of the literature. J Oral Microbiol. 2012;4:10.3402/jom.v4i0.11829.	This is a critical review

Supplementary file 4. Inclusion and exclusion criteria adopted in the SRs included in the overview

Reference	Inclusion criteria	Exclusion criteria
Mustufvi et al., 2022	RA was defined according to internationally accepted criteria; PD was defined according to internationally accepted criteria; study population had a clinically acceptable periodontal intervention as part of the trial; study population had a minimum follow-up period of 4 weeks; baseline and follow-up data included periodontal and RA parameters; relevant RA outcome measures were recorded, including DAS28, ACPA, ESR, CRP, RF, MS, HAQ and other ancillary biomarkers; studies had data that could be extracted.	Non-relevant study populations; non-intervention studies; studies with incomplete follow-up or missing data; studies not reporting on relevant RA outcome measure; studies including not clinically acceptable periodontal treatment; unclear methodology.
Silva et al., 2022	Randomised controlled trials; quasi-randomised studies trials; well-controlled cohort studies with individuals diagnosed RA and PD. Eligible studies compared any type of periodontal treatment with usual care and with a	Individuals with concomitant arthritic conditions or other rheumatic disease.

	sham or no-treatment comparator group in RA. Periodontal treatments included all surgical and mechanical NSPT and antimicrobial therapy (encompassing antiseptics and antibiotics), either locally applied or systemically administered.	
Sun et al., 2021	Studies that included individuals with RA and PD and that presented a test group composed of patients who received NSPT and a control group with patients who did not receive periodontal treatment; studies provided changes in clinical or biochemical parameters of RA after NSPT, such as DAS28, ESR, TJC, SJC, VAS; MS; RF; CRP; TNF-α; IL-6.	Used antibiotics in the last 3 months prior to NSPT, had systemic conditions likely to affect RA or PD, underwent periodontal treatment in the last 6 months and were using any antibiotic medication during periodontal treatment; review studies, abstracts from academic meetings, case reports, meta-analyses, studies in animal models and articles with no available date or full text.
Rosa et al., 2021	Randomized controlled trials; prospective studies with at least 10 participants diagnosed with RA and PD, who assessed RA activity after scaling and root planing using	Studies with patients under 18 years; systemic antibiotic therapy prescribed to patients 3 months before the study or during NSPT; studies with smoking or diabetic patients; as well as

	DAS28 and/or inflammatory measures such as ESR and CRP, and with at least four weeks of follow-up.	studies with patients who, during the follow-up period, changed their RA medication.
Calderaro et al., 2017	Intervention studies; Inclusion of adult patients diagnosed with PD and RA; NSPT; control group without NSPT; outcomes for RA that included at least one of the following characteristics: ESR, CRP, DAS28, SJC, TJC, VAS; follow-up of at least six weeks.	Case reports; review articles; editorials; comments; letters to the editor; experimental studies/basic sciences; articles on therapeutic interventions in RA and reports on patients with rheumatic diseases who not the RA.
Silvestre et al., 2016	Controlled clinical studies on the effect (intervention) of NSPT in patients with RA and PD; Studies with patients: diagnosed with RA and PD, aged over 30 years and without systemic inflammatory diseases capable of influencing RA or PD; the absence of antibacterial use in the three months prior to periodontal treatment; absence of periodontal treatment for at least the last 6 months; the presence of an age- and gender-matched control group without NSPT.	NR

Kaur et al., 2014	Quantitative studies that examined the effect of periodontal treatment of PD in patients diagnosed with both RA and PD; patients diagnosed with both RA and PD, aged 30 years or older; no antibiotic usage in the previous 3 months, no systemic conditions likely to affect either RA or PD and no periodontal treatment in the previous 6 months; periodontal intervention was to be non-surgical without the use of any adjunctive agents such as antibiotics or host modulating medications; control group of age and gender matched individual receiving no periodontal treatment for the duration of the study or baseline measure; outcome measures of RA activity to include CRP, ESR, ACPA, TNF-α, RF, IL-1β, IL-6 and DAS28; published in English.	NR

Kaur et al., 2013	Quantitative studies, mainly case-control studies and cohort prospective study designs; patients with diagnosed RA, with or without a clinical diagnosis of chronic PD; at least 30 years of age and of both genders.	Studies specifically focusing on aggressive forms of PD; studies where the focus was on patients with co-morbidities that may have an impact on PD and/or RA.
Lu et al., 2011	Studies that included patients: ≥ 30 years and diagnosed with RA and PD, more than 20 remaining teeth and diagnosis of moderate and severe PD; Studies that presented an experimental group that received periodontal treatment, and a control group that received periodontal treatment different from the experimental group or no periodontal treatment.	NR

Note: ACPA: Anti-citrullinated peptide antibodies; CRP: C-reactive protein; DAS28: 28-joint Disease Activity Score; ESR: Erythrocyte sedimentation rate; HAQ: Health assessment questionnaire; IL-6: Interleukin-6; IL-1β: Interleukin-1β; MS: Morning stiffness; NR: Not related; NSPT: Non-surgical periodontal treatment; PD: Periodontitis; RA: Rheumatoid arthritis; RF: Rheumatoid factor; SJC: Swollen joint counts; SR: Systematic review; TJC: Tender joint counts; TNF-α: Tumor necrosis factor-α; VAS: Visual analogical scale.

Supplementary file 5. Risk of bias assessed by A Measurement Tool to Assess the Methodological Quality of Systematic Reviews (AMSTAR) critical appraisal tools

Author, year of publication/reference	Mustufvi et al., 2022	Silva et al., 2022	Sun et al., 2021	Rosa et al., 2021	Calderaro et al., 2017	Silvestre et al., 2016	Kaur et al., 2014	Kaur et al., 2013	Lu et al., 2011
Did the research questions and inclusion criteria for the review include the components of PICO?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Did the report of the review contain an explicit statement that the review methods were established prior to the conduct of the review and did the report justify any significant deviations from the protocol?	No	Yes	No	Yes	No	No	No	No	No
Did the review authors explain their selection of the study designs for inclusion in the review?	No	No	No	No	No	No	No	No	No
Did the review authors use a comprehensive literature search strategy?	Yes	Yes	Partial Yes	Partial yes	Partial yes	Partial yes	Partial yes	Partial yes	Partial yes
Did the review authors perform study selection in duplicate?	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Did the review authors perform data extraction in duplicate?	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
Did the review authors provide a list of excluded studies and justify the exclusions?	Yes	No	Yes	Yes	No	No	Yes	No	No
Did the review authors describe the included studies in adequate detail?	Yes	Yes	Yes	Yes	Yes	Partial yes	Yes	No	Partial Yes
Did the review authors use a satisfactory technique for assessing the risk of bias (RoB) in individual studies that were included in the review?	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes
Did the review authors report on the sources of funding for the studies included in the review?	No	No	No	No	No	No	No	No	Yes
If meta-analysis was performed, did the review authors use appropriate methods for statistical combination of results?	No meta-analysis	No	Yes	No	Yes	No meta-analysis	Yes	No meta-analysis	Yes
If meta-analysis was performed, did the review authors assess the potential impact of RoB in	No meta-analysis	No	No	No	Yes	No meta-analysis	No	No meta-analysis	No

individual studies on the results of the meta-analysis or other evidence synthesis?									
Did the review authors account for RoB in primary studies when interpreting/discussing the results of the review?	No	Yes	No	No	Yes	No	No	No	No
Did the review authors provide a satisfactory explanation for, and discussion of, any heterogeneity observed in the results of the review?	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No
If they performed quantitative synthesis did the review authors carry out an adequate investigation of publication bias (small study bias) and discuss its likely impact on the results of the review?	No meta-analysis	No	Yes	No	No	No meta-analysis	No	No meta-analysis	No
Did the review authors report any potential sources of conflict of interest, including any funding they received for conducting the review?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

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5 CONSIDERAÇÕES FINAIS

Os nossos achados revelaram que as concentrações das NETs na saliva, no plasma e na cultura de neutrófilos estão diretamente associadas a parâmetros clínicos periodontais e reumatológicos e participam na progressão de ambas as doenças.

Polimorfismos associados a enzima PAD4 têm impacto na produção de NETs por neutrófilos do sangue periférico e na progressão da inflamação periodontal. Por fim, o tratamento periodontal não cirúrgico além de se mostrar eficiente na redução das concentrações de NETs, contribui também para melhorar a atividade da doença AR e pode representar uma terapia adjuvante eficiente ao tratamento antirreumático. A avaliação dos níveis circulantes de NETs evidencia o diálogo entre periodontite e AR por esta via, um resultado que abre perspectivas para a utilização de terapias complementares benéficas para ambas as doenças.

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