Received: 10 July 2023 | Revised: 7 February 2024 | Accepted: 18 March 2024

DOI: 10.1111/cid.13325

ORIGINAL ARTICLE

WILEY

Detection and comparison of neutrophil extracellular traps in tissue samples of peri-implantitis, periodontitis, and healthy patients: A pilot study

Sarmad Muayad Rasheed Al-Bakri[1,2] | Antonio Magan-Fernandez[1] | Pablo Galindo-Moreno[3,4] | Francisco O'Valle[4,5] | Natividad Martin-Morales[3,4,5,6] | Miguel Padial-Molina[3,4] | Francisco Mesa[1]

[1]Department of Periodontics, School of Dentistry, University of Granada, Granada, Spain

[2]PhD Program in Clinical Medicine and Public Health, University of Granada, Granada, Spain

[3]Department of Oral Surgery and Implant Dentistry, School of Dentistry, University of Granada, Granada, Spain

[4]ibs.GRANADA - Instituto de Investigación Biosanitaria, Granada, Spain

[5]Department of Pathology, School of Medicine and IBIMER, University of Granada, Granada, Spain

[6]PhD Program in Biomedicine, University of Granada, Granada, Spain

Correspondence
Antonio Magan-Fernandez, Facultad de Odontología, Campus de Cartuja, s/n, Universidad de Granada, 18071 Granada, Spain.
Email: amaganf@ugr.es

Funding information
Junta de Andalucía

Abstract

Objective: The aim of this study was to detect and compare the tissular expression of neutrophil extracellular traps (NETs) in peri-implant and periodontal samples of patients with peri-implantitis, periodontitis, and controls.

Materials and Methods: An observational study was performed on patients with peri-implantitis, periodontitis, and controls. Peri-implant and/or periodontal clinical examinations were performed on each participant. Tissue samples were collected during tooth/implant extraction for clinical reasons. Electron microscopy analysis, Picro-Sirius red staining, immunohistochemical (CD15), and immunofluorescence (citrullinated H3 and myeloperoxidase) techniques were performed to detect NET-related structures and the degree of connective tissue destruction, between the study groups.

Results: Sixty-four patients were included in the study: 28 peri-implantitis, 26 periodontitis, and 10 controls, with a total of 51 implants, 26 periodontal teeth, and 10 control teeth. Neutrophil release of nuclear content was observed in transmission electron microscopy. Immunohistochemical analysis showed a greater CD15 expression in both peri-implantitis and periodontitis compared to controls ($p < 0.001$), and peri-implantitis presented lower levels of connective tissue and collagen compared to both periodontitis ($p = 0.044$; $p < 0.001$) and controls ($p < 0.001$). Immunofluorescence showed greater citH3 expression in peri-implantitis than the one found in both periodontitis ($p = 0.003$) and controls ($p = 0.048$).

Conclusions: A greater presence and involvement of neutrophils, as well as a greater connective tissue destruction were observed in cases of peri-implantitis. A higher expression of NET-related markers was found in mucosal samples of peri-implantitis compared to periodontitis and controls.

KEYWORDS
extracellular traps, fluorescent antibody technique, immunohistochemistry, microscopy, neutrophils, peri-implantitis, periodontitis

Summary Box

What is known

- The release of neutrophil extracellular traps (NETs) has been reported as an antibacterial mechanism by neutrophils and has also been related to autoimmune damage.
- The association of NETs with periodontitis and gingivitis has been reported, but mostly on peripheral blood neutrophils and not tissue samples.
- The role of NETs in the pathogenesis of peri-implantitis has not been studied to date.

What this study adds

- This is the first study that assesses the role of NETs in peri-implantitis.
- Our results showed that a higher expression of neutrophils and NET formation biomarkers was found in samples of peri-implantitis compared to periodontitis and controls.
- These findings suggest a greater involvement of neutrophils in the inflammatory pathogenesis of peri-implantitis.

1 | INTRODUCTION

With the significant expansion of implant dentistry in recent years and an increasing number of implants placed annually, the prevalence of peri-implant diseases has also risen.[1] The prevalence of peri-implantitis exhibits considerable variability, depending on the definition used, ranging from 6.9% to 29.6% after a mean follow-up of 18.9 years, as reported in recent studies.[2] More recent investigations indicate an incidence of 10.4% after 5 years and 19.5% after 10 years, suggesting that one in five patients with implant-supported overdentures will develop peri-implantitis within a decade.[3]

Peri-implantitis represents the most severe form of these diseases and is clinically defined by signs of inflammation such as bleeding on probing, erythema, swelling, and loss of supporting bone tissues around the implant.[4] While peri-implantitis shares several clinical and radiologic features with periodontitis, significant differences in histopathological characteristics have been reported, showing clinical distinctions in disease onset and progression. Among these differences, the absence of a periodontal ligament in implants, variations in surrounding tissues compared to natural teeth, lower vascularization, and collagen fibers arranged parallel to the implant axis, are included.[5] Additionally, distinct bacterial species play a role in peri-implantitis compared to those typically associated with periodontitis.[6]

Previous studies on periodontitis and peri-implantitis lesions reported that peri-implant tissues are more susceptible to inflammation,[7] although other studies have reported histopathologically low differences.[8] Despite peri-implantitis being considered an inflammatory plaque-associated disease,[4] some authors have proposed alternative etiological factors in the onset and progression of peri-implant diseases.[9] Besides surgical-related factors, such as vestibular implant placement, other factors like the presence of excess cement in the peri-implant soft tissue can induce inflammation triggering biological responses associated with peri-implant diseases. The release of ions and metal particles from implants and metallic abutments has also been linked with inflammatory processes in oral soft tissues, as observed in the case of inflammasome-mediated inflammation.[10]

Neutrophils have been traditionally considered one of the main defense mechanisms against microbes, and a key cell type in initiating the inflammatory response.[11] In addition to their antimicrobial mechanisms through phagocytosis, activated neutrophils generate a response in which neutrophil DNA forms web-like structures that are expelled in the extracellular medium, known as neutrophil extracellular traps (NETs).[12] NETs are mainly composed of DNA and DNA-related proteins, including histones, and also contain granules with neutrophil antimicrobial peptides such as elastase, myeloperoxidase (MPO), and cathepsin G.[13] NET release can be triggered by pathogens or their products, as well as a range of agents including reactive oxygen species and certain periodontal bacterial species.[14–16]

The role of NETs in several autoimmune diseases has been proposed, as tissue accumulation of NETs and NETs-related components,

along with impaired clearance of them, have been associated with tissue damage.[17,18] Previous studies have reported a higher expression of NETs in gingival tissue of periodontitis and gingivitis patients compared to healthy samples.[19] Elevated NET formation in peripheral blood neutrophils has also been observed in patients with experimental gingivitis.[20] However, the potential role of NETs in the pathogenesis peri-implantitis and the comparison of NET expression between peri-implantitis and periodontitis have not been reported yet.

The hypothesis of our study is that polymorphonuclear neutrophils (PMN) and NETs play a more significant role in the pathogenesis of peri-implantitis, leading to higher NET expression in this inflammatory process. Therefore, the objective of our study was to detect and compare NET expression in gingival and mucosal tissue biopsies from patients with peri-implantitis, periodontitis, and controls.

2 | MATERIALS AND METHODS

2.1 | Subjects

An observational cross-sectional study was designed involving patients diagnosed with peri-implantitis, periodontitis, and healthy controls. Participants were consecutively recruited from a dental surgery clinic in Granada, Spain, between January 2019 and July 2020. All individuals accepted to participate and provided written informed consent before any procedure was conducted, and the study was conducted in agreement with the Declaration of Helsinki, according to its seventh revision in 2013. Approval was obtained from both the Human Research Ethics Committee of the University of Granada (ref. 639/CEIH/2018) and the Granada Research Ethics Committee (CEI Granada. ref. 0337-N-20). "Strengthening the Reporting of Observational Studies in Epidemiology" (STROBE) guidelines were followed in the preparation of this manuscript.[21]

Peri-implantitis was defined based on the 2018 World Workshop case definition, as bleeding and/or suppuration upon gentle probing, probing depths ≥6 mm, and bone levels ≥3 mm apical to the most coronal portion of the intra-osseous part of the implant.[4] Cases with more than 50% bone loss in short implants (<9 mm in length) were also considered peri-implantitis. Peri-implantitis patients were also required to be periodontally healthy or in stable supportive periodontal therapy. Periodontitis was defined as the presence of detectable interdental clinical attachment loss (CAL) at ≥2 non-adjacent teeth, or buccal or oral CAL ≥3 mm with pocket probing depth (PPD) >3 mm.[22] Controls were subjects with no history or clinical signs of periodontitis or peri-implantitis (probing depth ≤3 mm and no radiographic signs of bone loss), requiring extractions for other clinical reasons, such as orthodontic treatment. Exclusion criteria included age under 18 years old, implant or tooth loss unrelated to peri-implantitis or periodontitis (such as trauma or prosthetic reasons), pregnancy, breastfeeding, neoplastic diseases, or severe infections, use of antibiotics or anti-inflammatory therapy in the last 3 months, history of bisphosphonate or high-dosage corticoid therapy, or radiotherapy.

2.2 | Clinical examination and biopsy collection

Clinical examinations were performed in each implant/tooth before extraction using a specific periodontal probe (PCPUNC15, Hu-Friedy, Chicago, IL, USA) and an exploration dental mirror (SE plus® mouth mirror, Hahnenkratt E. GmbH, Königsbach-Stein, Germany). Probing pocket depth was measured in millimeters in four sites per implant/tooth (vestibular, medial, lingual, and distal). Bleeding on probing and visible plaque were recorded for each implant/tooth. Peri-implant or periodontal soft-tissue samples were collected during the extraction of failed implants due to peri-implantitis following the criteria from the International Congress of Oral Implantologists (ICOI) consensus (any of the following: pain on function, mobility, >50% bone loss or uncontrollable exudate),[23] or extraction of hopeless teeth due to periodontitis (any of the following: probing depths >8 mm, >75% bone loss, class III mobility or furcation defects).[24] In the control group, soft-tissue samples were gathered after extraction of the teeth for other reasons, as previously stated. A band of soft tissue was obtained from the vestibule/lingual aspect of the lesion through an internal incision that extended apically beyond the bottom of the peri-implant sulcus. The samples were collected from each implant/tooth, and then divided in two fragments, one for histological and immunohistochemical (IHC) analyses and the other for immunofluorescence (IF) analysis.

2.3 | Transmission electron microscopy study

Several 1 mm^2 sections of gingival papilla were fixed in 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded following a conventional protocol. Ultrathin (~70 nm-thick) sections were obtained in a Reichert Jung ULTRACUT ultramicrotome (Leica, Wetzlar, Germany), and staining with lead citrate and uranyl acetate were performed. Sections were then examined in a Libra 120 Plus TEM (Zeiss, Oberkochen, Germany).

2.4 | Histopathological analysis

For histological analyses, gingival papilla samples, including sulcular and oral epithelium in each one, were obtained from all participants in the study and were immediately fixed in 10% buffered formalin at room temperature for 48 h. Paraffin-embedded samples were deparaffinized in xylol (3 passes of 5 min) and then re-hydrated in ethanol following a decreasing gradation procedure (absolute, 96%, and 70%, 2 passes of 3 min, respectively). Tissue sections were stained with hematoxylin–eosin (H–E), and Picro-Sirius red. The morphological study was then performed by a blinded researcher on 4-μm sections with a BX42 light microscope (Olympus Optical Company, Ltd., Tokyo, Japan). Ten random images stained with Picro-Sirius red and normal light microscope or polarized light were captured from each sample with a 20× objective in a microscope with a digital camera attached

(DP70, Olympus Optical Company). Images were then analyzed with the software ImageJ (NIH, http://imagej.nih.gov/ij/) to quantify the percentage of area occupied by connective tissue (CT) and collagen in lamina propria, respectively.

2.5 | Immunohistochemical analysis

Paraffin-embedded sections were deparaffinized, hydrated, and heat-treated in 1 mM EDTA (pH 8) for antigenic unmasking in an antigen retrieval PT module (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 95°C for 20 min. Sections were incubated for 1 h at room temperature with prediluted monoclonal Anti-CD15, a surface marker expressed by neutrophils, determined to identify and observe neutrophil distribution in the gingival tissue samples[25] (Vitro-Master Diagnóstica, Granada, Spain). The immunocytochemical study was conducted using the micropolymer-peroxidase-based method (Master Polymer, Vitro-Master Diagnóstica) with an automatic immunostainer (Autostainer 480S, Thermo Fisher Scientific Inc., Waltham, MA) followed by development with diaminobenzidine. Appropriate positive (tonsil) and negative (non-immune serum) controls were performed concurrently. Hematoxylin was used for nuclear counterstaining. The IHC study was done in a blinded fashion on 4 µm sections with BX42 light microscopy (Olympus Optical Company, Ltd., Tokyo, Japan), by using the 40× objective in a microscope with an attached scale (BH2, Olympus Optical Company), the number of positive cells was quantified per mm^2. Results were also expressed as percentages of positive cells for each antibody, counting 100 cells per high-magnification field (40× objective) in three independent experiments. Histomorphometrical quantification was performed semiautomatically using each immunostain. Ten random images were captured from each sample with a 40× objective in a microscope with a digital camera attached (DP70, Olympus Optical Company). Images were then analyzed with the software ImageJ (NIH, http://imagej.nih.gov/ij/) to quantify the percentage of area occupied by both immunostains.

2.6 | Immunofluorescence analysis

For IF staining, frozen sections were defrosted at room temperature for 30 min and putting in acetone for 5 min. Then sections were blocked with 5% Bovine Serum Albumin (BSA) and permeabilized with 0.5% Triton in Phosphate Buffered Saline (PBS) for 5 min. The sections were then incubated overnight at 4°C with anti-acetyl-histone H3 antibody, Alexa Fluor® 647 Conjugate (1:100, 06-599-AF647, Invitrogen, Waltham, MA, USA). The sections were then incubated for 1 h at 4°C with goat anti-human MPO polyclonal antibody (1:100, AF 3667, R&D Systems, Minneapolis, MN, USA). The slides were then rinsed three times for 5 min with PBS and incubated with a secondary antibody (Alexa Fluor 488 donkey anti-goat Immunoglobulin G (IgG) (H + L) (A11055), Invitrogen, Waltham, MA, USA) for 1 h at room temperature. Slices were counterstained with100 µL of aqueous Hoechst 33342 (1:1000 dilution) for 15 min in the dark at room temperature. Imaging was performed with a Leica TCS SP5 and SP8 confocal microscope (Leica, Wetzlar, Germany).

2.7 | Statistical analysis

Kruskal–Wallis and Fisher's exact tests were used to compare the overall differences among all groups. Dunn's test for non-parametric pairwise comparisons was employed to independently assess all possible pairs and identify the specific groups where statistical differences were observed. All procedures of the statistical analysis were performed with Stata 14 (StataCorp LLC, College Station, TX, USA). A value of $p \leq 0$.was considered statistically significant in all tests. Statistical tests used are described in each table's footnotes.

3 | RESULTS

Our sample comprised 64 individuals, including 23 men (36%) and 41 women (64%), with a mean age of 57.8 ± 13.3 years. The sample was divided into three groups: 28 subjects (45%) with peri-implant disease, 26 individuals (40%) with periodontitis, and 10 healthy subjects (15%). Sociodemographic data for the three groups are presented in Table 1.

A total of 51 implants affected with peri-implantitis were diagnosed in the peri-implantitis group. Table 2 provides data of these implants and other implant-related variables. Clinical examination results performed in all implants and teeth included in the study are presented in Table 3. As expected, controls exhibited statistically significant lower probing pocket depths compared to implants with peri-implantitis and teeth with periodontitis. Additionally, controls showed a higher presence of plaque and bleeding on probing (Table 3).

Transmission electron microscopy analysis of periodontitis (Figure 1A) and peri-implantitis (Figure 1B) samples revealed widespread damage in CT, including substantial presence of dead and inflammatory cells. Neutrophils were abundant, with some of them displaying NET-related phenomena, such as empty nuclei and broken nuclear membranes, associated with chromatin expulsion to the extracellular medium.

Results of IHC and IF analyses are presented in Table 4. CD15 expression was significantly lower in controls compared to both peri-implantitis ($p = 0.008$) and periodontitis ($p < 0.001$) (Figure 2A–C). Picro-Sirius red staining for both CT and collagen, revealed statistically significant differences between all groups. Peri-implantitis group exhibited lower expression of CT and collagen compared to periodontitis ($p = 0.044$ for CT and $p < 0.001$ for collagen) and to controls ($p < 0.001$ for CT and $p < 0.001$ for collagen). Periodontitis had greater CT and collagen expression that peri-implantitis, but lower CT ($p < 0.001$) and collagen ($p = 0.002$) compared to controls (Figure 3). Confocal microscopy analysis of IF images showed that peri-implantitis exhibited greater expression of citH3 compared to both periodontitis ($p = 0.003$) and controls ($p = 0.048$). However, there were no differences in citH3 expression between periodontitis and

TABLE 1 Sociodemographic variables of the patients included in the study ($n = 64$).

Variable	Peri-implantitis ($n = 28$)	Periodontitis ($n = 26$)	Controls ($n = 10$)	p-value
Total samples	51 implants	26 teeth	10 teeth	
Age, mean ± SD	59.0 ± 7.6	59.6 ± 13.3	51.1 ± 22.4	0.475[a]
Gender, *n* (%)				
Male	8 (28.6%)	10 (38.5%)	5 (50%)	0.416[b]
Female	20 (71.4%)	16 (61.5%)	5 (50%)	
Smoking, *n* (%)				
Yes	12 (42.9%)	8 (30.8%)	3 (30%)	0.685[b]
No	16 (57.1%)	18 (69.2%)	7 (70%)	
Alcohol, *n* (%)				
Yes	5 (17.9%)	3 (11.5%)	1 (10.0%)	0.889[b]
No	23 (82.1%)	23 (88.5%)	9 (90.0%)	
Diabetes, *n* (%)				
Yes	3 (10.7%)	7 (26.9%)	3 (30.0%)	0.221[b]
No	25 (89.3%)	19 (73.1%)	7 (70.0%)	
Hypertension, *n* (%)				
Yes	4 (14.3%)	4 (15.4%)	0 (0%)	0.610[b]
No	24 (85.7%)	22 (84.6%)	10 (100%)	
Other systemic diseases, *n* (%)				
Yes	0 (0%)	2 (7.7%)	2 (20.0%)	0.075[b]
No	28 (100%)	24 (92.3%)	8 (80.0%)	

[a]Kruskal–Wallis test.
[b]Fisher's exact test.

controls ($p = 0.138$). MPO expression showed no differences between peri-implantitis and periodontitis groups, and MPO expression in the control group was negative (Figure 4).

4 | DISCUSSION

There are no precedents in the literature for studies addressing NETs in patients with peri-implantitis. Most of the studies published to date have been performed in periodontitis or healthy controls, mostly in blood neutrophils of these patients. The results of the present study reveal distinct expressions of neutrophil and NET-related markers in mucosal samples of peri-implantitis, periodontitis and controls. Peri-implantitis showed greater CD15 expression than periodontitis and controls. Additionally, peri-implantitis samples showed decreased CT and collagen. IF analysis of NET-related markers showed higher citH3 expression in peri-implantitis compared to the other groups.

Peri-implantitis is characterized by inflammation in the peri-implant mucosa, leading to progressive bone loss around dental implants.[4] Microbial dysbiosis and an exacerbated immune response are among the main agents in its pathogenesis.[26] Notably, common periodontal pathogens have been identified in healthy, peri-implant mucositis, and peri-implantitis sites, raising doubts about their sole association with peri-implantitis. Other bacterial species not traditionally associated with periodontal disease, have been associated with peri-implantitis.[27] Clinically induced peri-implantitis should also be considered, as local factors such as incorrect implant placement, excess cement, or metal ions and particle release may contribute to peri-implantitis without microbial involvement.[28] The role of inflammasomes should also be taken into consideration. Although AIM2 inflammasome is activated by double-DNA factors, NLRP3 inflammasome may not only be activated by microbial species, but also by metal particles.[29] This mechanism allows to explore different novel pathogenic mechanisms and biological pathways in the progression of peri-implantitis, as an inflammatory process related to different cell death mechanisms, as pyroptosis (also promoted by inflammasomes) or NET formation.

Implants with peri-implantitis and teeth with periodontitis showed a greater number of neutrophils compared to controls. Neutrophil presence in tissue was determined by the number of CD15+ cells, a surface marker use that has been extensively used for identifying and observing the tissue distribution of neutrophils, as previously reported in several studies,[25] and there were no differences in the expression of this marker between both diseases in our series. Surface expression of CD15 has been used as a canonical neutrophil marker, and has been highly correlated with immune activity of these cells in cases of active and treated diseases, since its expression is higher in mature neutrophils,[30,31] and has been used together with MPO as a positive NETs marker.[32] CD15+ cell count was higher in peri-implantitis compared to periodontitis, but these differences did not

TABLE 2 Implant-related variables of the implants ($n = 51$) in patients with peri-implantitis included in the study ($n = 28$).

Variable	*n* (%)
Implant type, *n* (%)	
Branemark	2 (3.9%)
Astra Tech	29 (56.9%)
Microdent	20 (39.2%)
Location ant/post, *n* (%)	
Anterior	18 (35.3%)
Posterior	33 (64.7%)
Location upper/lower, *n* (%)	
Maxillary	8 (15.7%)
Mandibular	43 (84.3%)
Function time[a], *n* (%)	
0–5 years	7 (24.1%)
5–10 years	6 (20.7%)
>10 years	16 (55.2%)
Prosthesis, *n* (%)	
Single crown	3 (5.9%)
Fixed partial denture	39 (76.5%)
Overdenture	5 (9.8%)
Hybrid prosthesis	4 (7.8%)
Mobility, *n* (%)	
Yes	2 (3.9%)
No	49 (96.1%)
Pain, *n* (%)	
Yes	6 (11.8%)
No	45 (88.2%)
Peri-implantitis severity	
Early	25
Moderate	12 (23.5%)
Advanced	14 (27.5%)

[a]Function time data only available for 29 implants.

achieve statistical significance. CD15 expression at the sulcular zone of the gingival biopsies showed similar results to previous studies assessing this biomarker.[8] Neutrophil deficiency in their number and/or function has been associated with the development of disease. Neutrophil hyperactivity, excessive release of NETs, or inefficient removal of NETs from periodontal or peri-implant tissue can cause CT damage, which is the main consequence of periodontal disease.[33] Several studies have suggested that NETs may have a key role in the pathogenesis of periodontitis (including its severity), and a relationship between NET formation, periodontal disease, and other systemic diseases, such as atherosclerosis.[34]

Decreased CT and collagen levels in the tissue samples with peri-implantitis were found compared to periodontitis and controls. This difference was also found in collagen between periodontitis and control samples. This may be explained by the subjacent inflammatory process also present in periodontitis, but with a lower tissue breakdown compared to the one in peri-implantitis. An imbalance between matrix metalloproteinases and tissue inhibitors of metalloproteinases has shown to be the main cause of collagen breakdown in the extracellular matrix during periodontitis,[35] and these molecules are the main collagenases in peri-implantitis,[36] explaining the greater CT destruction observed in our results.

CitH3 is a form of histone H3 modified through citrullination, a post-translational modification that involves the conversion of arginine amino acids to citrulline. This modification alters the structure and function of H3. This marker has been mainly associated with inflammation and autoimmunity, and it is one of the main structural components of NETs. Our results showed a higher expression of citH3 in peri-implantitis compared to both periodontitis and control samples. A previous pilot study from our group determined the expression in biopsies of MPO and citH3 in gingivitis, periodontitis, and controls. NET expression was found to be higher in gingivitis than periodontitis, concluding that NETs could be associated with acute phases of the inflammatory process.[19] This finding supports the results of the present study, and we hypothesize that peri-implantitis lesions present a different inflammatory profile, with a higher acute inflammatory component, than periodontitis lesions.[8] In this type of inflammatory response, NETs would play a major role and therefore its expression is higher in peri-implantitis lesions. To the best of our knowledge, no other in vivo assessment of citH3 in mucosal tissue of patients with peri-implantitis has been published so far. Histones associate with DNA in the nucleus of neutrophils and help to condense it into chromatin.[37] Histones can also act as antimicrobial peptides through several mechanisms such as permeabilization of the bacterial membrane, binding to bacterial DNA, RNA or lipopolysaccharide, and pathogen entrapment as a component of NETs. All these characteristics make NETs bactericidal by themselves, and do not only rely on the antimicrobial peptides embedded in them.[38] According to White et al., several stimuli may induce NET formation such as Gram-positive and Gram-negative bacteria and their components.[15] NETs can also be harmful if they are produced in excess or if their clearance from tissue is impaired. Citrullinated and non-citrullinated components of NETs have an autoimmune potential, which have been associated with the development of periodontitis. Periodontitis may initiate the deamination of relevant histones such as H2A, H4, or H3. H3 epitope may become undetectable due to structural modifications, and therefore H3 activity may be affected.[38] It has also been reported that H3 may not be mandatory for NET formation since it is a product of PAD4 activation.[39]

MPO is one of the main antimicrobial enzymes produced by neutrophils during the inflammatory response. MPO has been shown to be a relevant mediator of tissue damage and the subsequent inflammation in a wide range of inflammatory processes and diseases. MPO binds to extracellular matrix proteins, plasma proteins, and glycosaminoglycans, and causes localized changes and site-specific in tissue through ionic interactions and oxidant production.[40] Recent studies have shown that MPO expression showed no significant differences between peri-implantitis and periodontitis. MPO has been associated

TABLE 3 Peri-implant and periodontal clinical variables of implants and teeth in patients included in the study.

Variable	Peri-implantitis ($n = 51$)	Periodontitis ($n = 26$)	Controls ($n = 10$)	p-value
Plaque, *n* (%)				
Yes	44 (86.3%)	21 (80.8%)	2 (20%)	<0.001[a]
No	7 (13.7%)	5 (19.2%)	8 (80%)	
Bleeding on probing, *n* (%)				
Yes	42 (82.4%)	23 (88.5%)	1 (10%)	0.005[a]
No	9 (17.6%)	3 (11.5%)	9 (90%)	
Mesial PPD (mm), mean ± SD	6.4 ± 2.5	4.4 ± 2.0	1.5 ± 0.7	<0.001[b]
Distal PPD (mm), mean ± SD	6.8 ± 2.8	4.2 ± 2.0	1.3 ± 0.7	<0.001[b]
Vestibular PPD (mm), mean ± SD	6.4 ± 2.8	3.8 ± 2.2	1.1 ± 0.3	<0.001[b]
Lingual PPD (mm), mean ± SD	6.2 ± 2.8	4.2 ± 1.9	1.1 ± 0.3	<0.001[b]
Average PPD (mm), mean ± SD	6.4 ± 2.5	4.1 ± 1.5	1.3 ± 0.4	<0.001[b]
Periodontitis stage				
I		4 (15.4%)		
II		13 (50.0%)		
III		4 (15.4%)		
IV		5 (19.2%)		
Periodontitis grade				
A		0 (0%)		
B		21 (80.8%)		
C		5 (19.2%)		

Note: Averages and standard deviations are reported for continuous variables. All regression analyses adjusted for several implants within patients.
Abbreviation: PPD, pocket probing depth.
[a]Multilevel logistic regression.
[b]Multilevel linear regression.

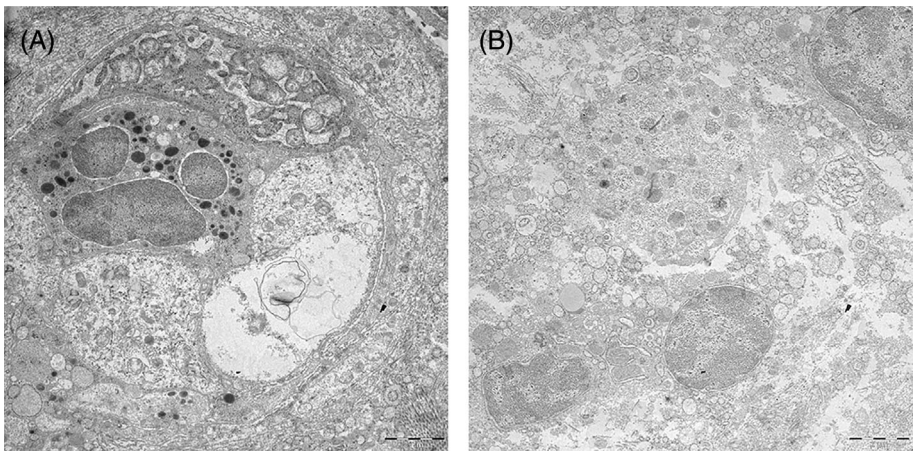

FIGURE 1 Transmission electron microscopy images. (A) Micrograph from a periodontitis sample showing degraded plasmatic cells with remains of the endoplasmic reticulum. Visible remains of a neutrophil showing irregularities and partial membrane degradation. (B) Micrograph from a peri-implantitis sample showing conserved neutrophils and remains of organelles with ultrastructural morphological alterations and lysosomal granules. Their degradative phenomena are compatible with neutrophil extracellular trap (NET) formation. Bar scale: 2 µm.

with periodontal/peri-implant tissue destruction, and its activity was significantly increased in the saliva of patients with periodontal disease compared to healthy individuals. These levels were reduced after non-surgical periodontal therapy.[41] According to Liskmann et al., comparing clinically healthy implants and with peri-implantitis, MPO levels were significantly higher in peri-implantitis lesions, and correlated with clinical variables. A similar inflammatory response in tissues around implants and natural teeth was expressed, and MPO was described as a potential surrogate inflammatory marker in implants.[42]

MPO has been associated with the destruction of peri-implant tissue. The study by Carcuac et al. conducted histopathological analyses in biopsies gathered from patients with severe generalized periodontitis and with severe peri-implantitis. They reported a greater number of MPO-positive cells in peri-implantitis compared to periodontitis, and peri-implantitis had higher lesion size than periodontitis.[43] Our results showed no differences in MPO expression between groups, and the reasons may be the different series of patients used and the variability that this marker may show, especially considering differences in terms

TABLE 4 Immunohistochemical and Immunofluorescence markers expressions of peri-implant and periodontal gingival biopsies.

Variable	Peri-implantitis [A] ($n = 38$)	Periodontitis [B] ($n = 22$)	Controls [C] ($n = 10$)	*p*-value			
				Global[a]	A vs. B[b]	A vs. C[b]	B vs. C[b]
CD15 total (cells/mm^2)	446.7 ± 580.8	495.4 ± 555.9	89.6 ± 81.3	0.044	0.009	0.008	<0.001
CD15 vestibular (cells/mm^2)	254.5 ± 219.8	192.7 ± 289.5	40.3 ± 30.6	0.015	0.066	<0.001	0.010
CD15 sulcular (cells/mm^2)	966.3 ± 561.6	649.0 ± 800.1	102.4 ± 130.0	<0.001	0.019	<0.001	0.006
Picro-Sirius red—CT (%)	18.3 ± 10.0	25.7 ± 18.5	50.4 ± 13.9	<0.001	0.044	<0.001	0.003
Picro-Sirius red—collagen/(%)[c]	7.3 ± 5.5	13.4 ± 7.6	15.3 ± 3.3	<0.001	<0.001	<0.001	0.002
IF—citH3 (%)	17.3 ± 5.7	1.7 ± 1.8	6.5 ± 5.6	0.007	0.003	0.048	0.138
IF—MPO (%)	12.3 ± 11.7	12.2 ± 16.6	0	0.944	0.403	-	-

Note: Averages and standard deviations are reported for continuous variables.
Abbreviations: citH3, citrullinated H3; CT, connective tissue; IF, immunofluorescence; MPO, myeloperoxidase.
[a]Multilevel linear regression analysis adjusted for several implants within patients.
[b]Dunn's test for pairwise comparisons.
[c]Picro-Sirius red with polarized light.

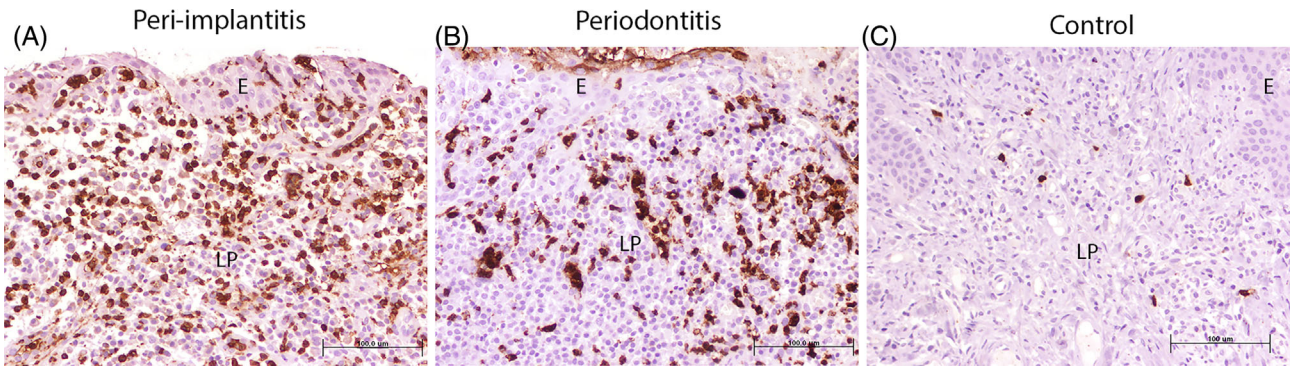

FIGURE 2 Immunocytochemical study in sulcular area of gingival tissue. Numerous neutrophil CD15 positives in peri-implantitis (A), periodontitis (B), and lower number in control (C) samples (arrows). (peroxidase-conjugated micropolymer detection. Original magnification: 20×). Bar scale: 100 μm. E, epithelium; LP, lamina propria.

of disease severity, making it a sample-dependent analysis. MPO deficiency results in an exacerbated inflammatory response, affecting neutrophil function.[44]

This study also presents some limitations. The sample size, albeit larger than previous NETs and periodontitis studies, remains relatively small. Though sufficient for detecting significant correlations among PMN count, CD15 expression level, and NETs in all three study groups, larger sample sizes could enhance the robustness of findings. The heterogeneity of implant characteristics among patients with peri-implantitis is also a potential limitation of the present study, specifically in terms of type of implant placed, type of prosthesis, and other variables, as seen in Table 2. Future studies with greater samples could apply more strict inclusion criteria, study specific type of prosthesis/implants, or even adjust for these variables in a multivariate analysis with a larger sample size. The cross-sectional design is also a limitation, and longitudinal designs should be performed in the future to confirm the results of this manuscript. Also, the reliance on tissue analyses makes results inherently sample-dependent, prompting the need for replication in diverse patient cohorts and settings.

To our knowledge, this is the first study evaluating in vivo the role of NETs in peri-implantitis. The colocalization of the components performed in the present study is the current most recommended for NET identification in tissue samples.[45,46] The results from this study provide new insights into the underlying mechanisms of peri-implantitis, giving relevance to the role of neutrophils and NET formation in its pathogenesis. These results also provide potential therapeutic targets for preventing and treating peri-implant diseases in the future.

In conclusion, a higher expression of NET-related markers was found in mucosal tissue of peri-implantitis compared to periodontitis and healthy controls, suggesting a more relevant role of these immune cells and their associated mechanisms in the pathogenesis of peri-implantitis. Further studies are needed to completely understand the role of NETs in this disease and its pathogenesis.

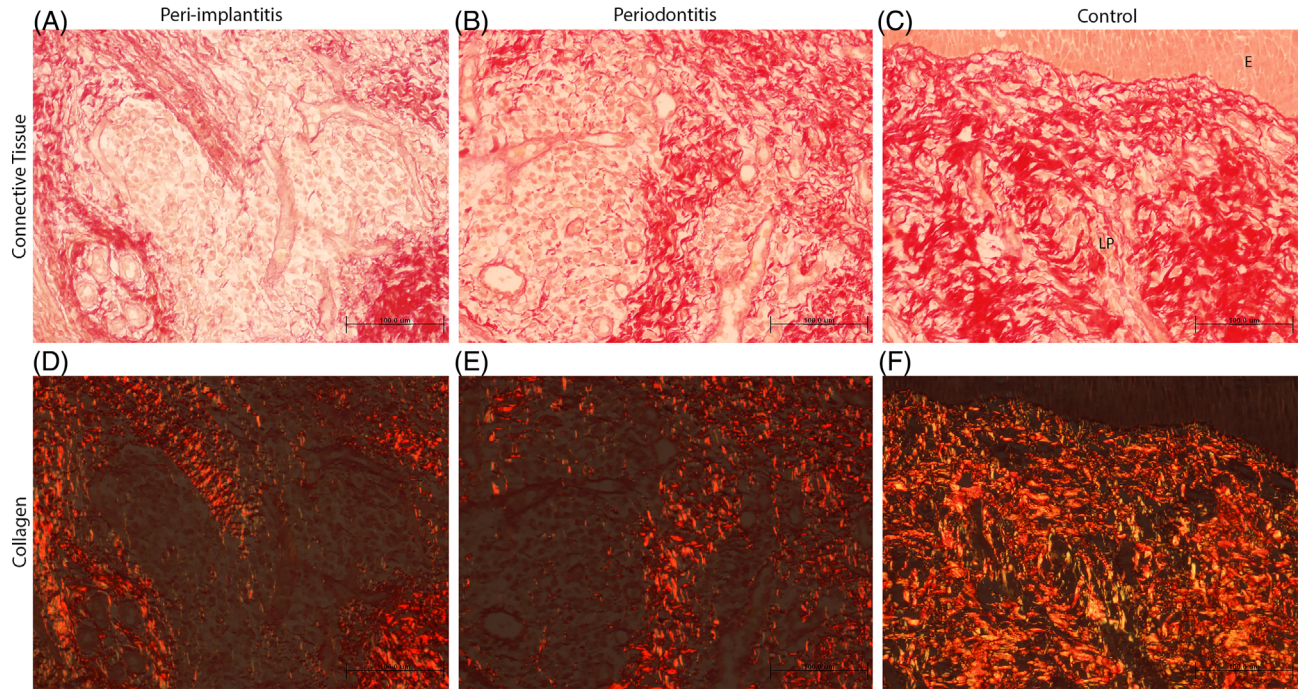

FIGURE 3 Picro-Sirius red staining images obtained with transmitted light microscope in peri-implantitis (A), periodontitis (B), and control (C), and polarized light (peri-implantitis D, periodontitis E, and control F). Lower connective tissue (deep red color) in peri-implantitis, and periodontitis compared with control. Morphological detection of collagen fiber (birefringent image area) using Picro-Sirius red with polarized light (original magnification 20×). Bar scale: 100 μm.

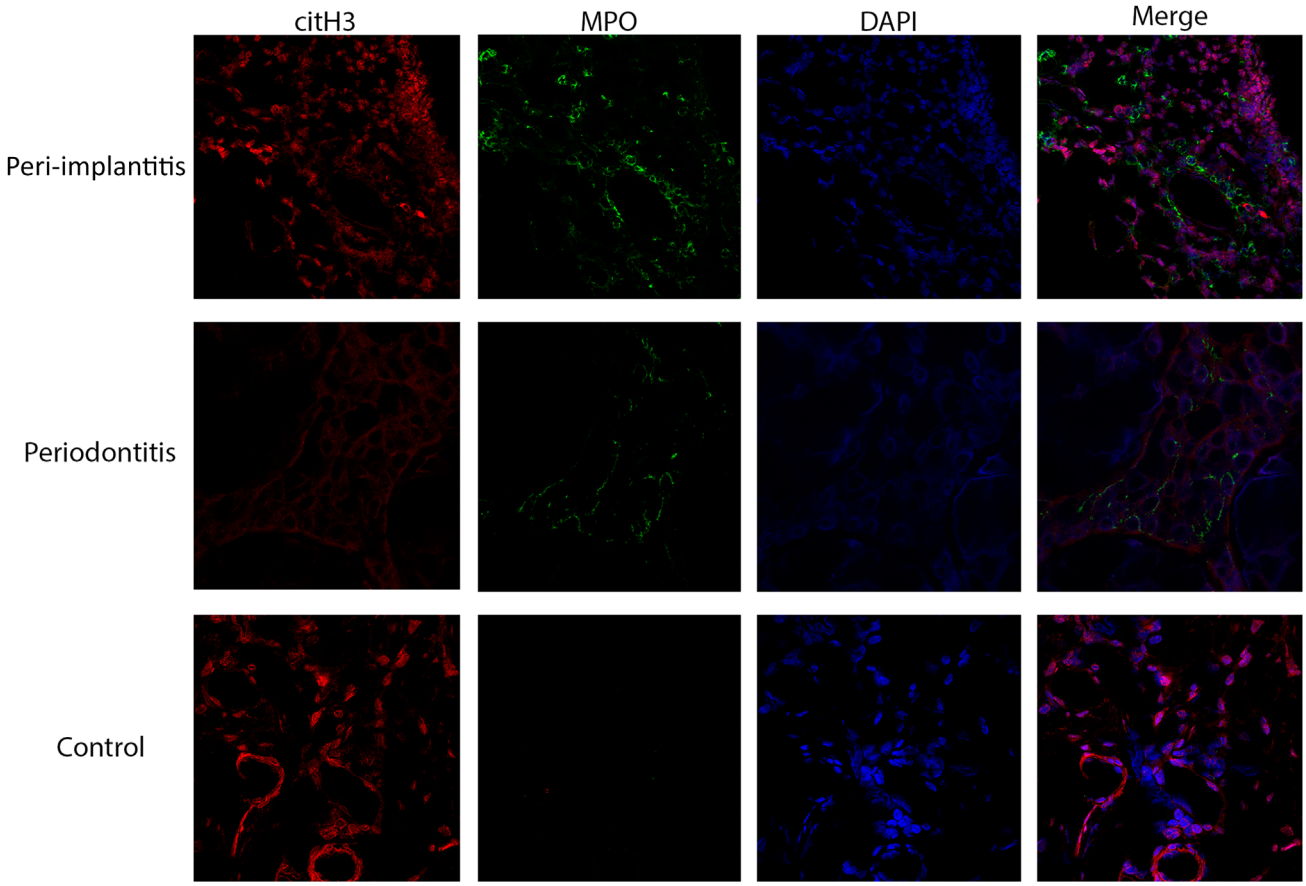

FIGURE 4 Confocal microscopy immunofluorescence images. Expression of H3 (shown in red), myeloperoxidase (MPO) (shown in green), and DAPI DNA staining in the three study groups. Structures compatible with neutrophil extracellular trap (NET) formation are found in cases of peri-implantitis and periodontitis. (Immunofluorescence, original magnification 40×).

AUTHOR CONTRIBUTIONS

Conceptualization: AMF, FO, and FM. *Data curation*: SMRA and MPM. *Funding acquisition*: FM. *Investigation*: SMRA and PGM. *Experiments*: SMRA, FO, and NMM. *Statistical analysis*: AMF. *Supervision*: FO and FM. *Validation*: FO and MPM. *Writing—original draft*: SMRA and AMF. *Writing—review and editing*: PGM, FO, MPM, and FM. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This study was supported by Research Groups #CTS 583, #CTS-1028, and #CTS-138 (Junta de Andalucía, Spain). The authors thank the staff of the laboratory of the Pathology Department of the University of Granada School of Medicine, for their technical assistance in this study. Funding for open access charge: Universidad de Granada / CBUA.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ORCID

Antonio Magan-Fernandez https://orcid.org/0000-0001-6430-2276
Miguel Padial-Molina https://orcid.org/0000-0001-6222-1341
Francisco Mesa https://orcid.org/0000-0002-8293-2527

REFERENCES

1. Mombelli A, Muller N, Cionca N. The epidemiology of peri-implantitis. *Clin Oral Implants Res*. 2012;23(Suppl 6):67-76.
2. Krebs M, Kesar N, Begic A, von Krockow N, Nentwig GH, Weigl P. Incidence and prevalence of peri-implantitis and peri-implant mucositis 17 to 23 (18.9) years postimplant placement. *Clin Implant Dent Relat Res*. 2019;21(6):1116-1123.
3. Onclin P, Slot W, Vissink A, Raghoebar GM, Meijer HJA. Incidence of peri-implant mucositis and peri-implantitis in patients with a maxillary overdenture: a sub-analysis of two prospective studies with a 10-year follow-up period. *Clin Implant Dent Relat Res*. 2022;24(2):188-195.
4. Berglundh T, Armitage G, Araujo MG, et al. Peri-implant diseases and conditions: consensus report of workgroup 4 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. *J Clin Periodontol*. 2018;45(suppl 20):S286-S291.
5. Berglundh T, Zitzmann NU, Donati M. Are peri-implantitis lesions different from periodontitis lesions? *J Clin Periodontol*. 2011;38(suppl 11):188-202.
6. Hashimoto Y, Okada S, Yasuda K, Kawagoe M, Kajiya M, Tsuga K. Microbial differences between active and remission peri-implantitis. *Sci Rep*. 2022;12(1):5284.
7. Degidi M, Artese L, Piattelli A, et al. Histological and immunohistochemical evaluation of the peri-implant soft tissues around machined and acid-etched titanium healing abutments: a prospective randomised study. *Clin Oral Investig*. 2012;16(3):857-866.
8. Galindo-Moreno P, Lopez-Martinez J, Caba-Molina M, et al. Morphological and immunophenotypical differences between chronic periodontitis and peri-implantitis – a cross-sectional study. *Eur J Oral Implantol*. 2017;10(4):453-463.
9. Albrektsson T, Tengvall P, Amengual-Penafiel L, Coli P, Kotsakis G, Cochran DL. Implications of considering peri-implant bone loss a disease, a narrative review. *Clin Implant Dent Relat Res*. 2022;24(4):532-543.
10. Canullo L, Tallarico M, Radovanovic S, Delibasic B, Covani U, Rakic M. Distinguishing predictive profiles for patient-based risk assessment and diagnostics of plaque induced, surgically and prosthetically triggered peri-implantitis. *Clin Oral Implants Res*. 2016;27(10):1243-1250.
11. Chapple ILC, Hirschfeld J, Kantarci A, Wilensky A, Shapira L. The role of the host-neutrophil biology. *Periodontol 2000*. 2023.
12. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004;303(5663):1532-1535.
13. Fousert E, Toes R, Desai J. Neutrophil extracellular traps (NETs) take the central stage in driving autoimmune responses. *Cells*. 2020;9(4):915.
14. Vitkov L, Hartl D, Minnich B, Hannig M. Janus-faced neutrophil extracellular traps in periodontitis. *Front Immunol*. 2017;8:1404.
15. White PC, Chicca IJ, Cooper PR, Milward MR, Chapple IL. Neutrophil extracellular traps in periodontitis: a web of intrigue. *J Dent Res*. 2016;95(1):26-34.
16. Hirschfeld J, White PC, Milward MR, Cooper PR, Chapple ILC. Modulation of neutrophil extracellular trap and reactive oxygen species release by periodontal bacteria. *Infect Immun*. 2017;85(12):e00297-17.
17. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med*. 2017;23(3):279-287.
18. Mutua V, Gershwin LJ. A review of neutrophil extracellular traps (NETs) in disease: potential anti-NETs therapeutics. *Clin Rev Allergy Immunol*. 2021;61(2):194-211.
19. Magan-Fernandez A, O'Valle F, Abadia-Molina F, Munoz R, Puga-Guil P, Mesa F. Characterization and comparison of neutrophil extracellular traps in gingival samples of periodontitis and gingivitis: a pilot study. *J Periodontal Res*. 2019;54(3):218-224.
20. Roberts HM, Yonel Z, Kantarci A, Grant MM, Chapple ILC. Impact of gingivitis on circulating neutrophil reactivity and gingival crevicular fluid inflammatory proteins. *Int J Environ Res Public Health*. 2022;19(10):6339.
21. von Elm E, Altman DG, Egger M, et al. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *BMJ*. 2007;335(7624):806-808.
22. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: framework and proposal of a new classification and case definition. *J Clin Periodontol*. 2018;45(Suppl 20):S149-S161.
23. Misch CE, Perel ML, Wang HL, et al. Implant success, survival, and failure: the International Congress of Oral Implantologists (ICOI) Pisa Consensus conference. *Implant Dent*. 2008;17(1):5-15.
24. Kwok V, Caton JG. Commentary: prognosis revisited: a system for assigning periodontal prognosis. *J Periodontol*. 2007;78(11):2063-2071.
25. Asman B, Gustafsson A, Bergstrom K. Gingival crevicular neutrophils: membrane molecules do not distinguish between periodontitis and gingivitis. *J Clin Periodontol*. 1997;24(12):927-931.
26. Zitzmann NU, Berglundh T. Definition and prevalence of peri-implant diseases. *J Clin Periodontol*. 2008;35(8 Suppl):286-291.
27. Padial-Molina M, Lopez-Martinez J, O'Valle F, Galindo-Moreno P. Microbial profiles and detection techniques in peri-implant diseases: a systematic review. *J Oral Maxillofac Res*. 2016;7(3):e10.
28. Monje A, Insua A, Wang HL. Understanding peri-implantitis as a plaque-associated and site-specific entity: on the local predisposing factors. *J Clin Med*. 2019;8(2):279.

29. Galindo-Moreno P, Montalvo-Acosta S, Martin-Morales N, et al. Inflammasomes NLRP3 and AIM2 in peri-implantitis: a cross-sectional study. *Clin Oral Implants Res*. 2023;34(12):1342-1353.
30. Ndlovu LN, Peetluk L, Moodley S, et al. Increased neutrophil count and decreased neutrophil CD15 expression correlate with TB disease severity and treatment response irrespective of HIV Co-infection. *Front Immunol*. 2020;11:1872.
31. Blanco-Camarillo C, Aleman OR, Rosales C. Low-density neutrophils in healthy individuals display a mature primed phenotype. *Front Immunol*. 2021;12:672520.
32. de Andrea CE, Ochoa MC, Villalba-Esparza M, et al. Heterogenous presence of neutrophil extracellular traps in human solid tumours is partially dependent on IL-8. *J Pathol*. 2021;255(2):190-201.
33. Magan-Fernandez A, Rasheed Al-Bakri SM, O'Valle F, Benavides-Reyes C, Abadia-Molina F, Mesa F. Neutrophil extracellular traps in periodontitis. *Cells*. 2020;9:6.
34. Irwandi RA, Chiesa ST, Hajishengallis G, Papayannopoulos V, Deanfield JE, D'Aiuto F. The roles of neutrophils linking periodontitis and atherosclerotic cardiovascular diseases. *Front Immunol*. 2022;13:915081.
35. Seguier S, Gogly B, Bodineau A, Godeau G, Brousse N. Is collagen breakdown during periodontitis linked to inflammatory cells and expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human gingival tissue? *J Periodontol*. 2001;72(10):1398-1406.
36. Arakawa H, Uehara J, Hara ES, et al. Matrix metalloproteinase-8 is the major potential collagenase in active peri-implantitis. *J Prosthodont Res*. 2012;56(4):249-255.
37. Thalin C, Daleskog M, Goransson SP, et al. Validation of an enzyme-linked immunosorbent assay for the quantification of citrullinated histone H3 as a marker for neutrophil extracellular traps in human plasma. *Immunol Res*. 2017;65(3):706-712.
38. Neeli I, Dwivedi N, Khan S, Radic M. Regulation of extracellular chromatin release from neutrophils. *J Innate Immun*. 2009;1(3):194-201.
39. Kenny EF, Herzig A, Kruger R, et al. Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife*. 2017;6:e24437.
40. Hawkins CL, Davies MJ. Role of myeloperoxidase and oxidant formation in the extracellular environment in inflammation-induced tissue damage. *Free Radic Biol Med*. 2021;172:633-651.
41. Dagar M, Deepa DK, Molly M, Sharma A, Khattak BP. Effect of non-surgical periodontal therapy on salivary myeloperoxidase levels: a biochemical study. *J Indian Soc Periodontol*. 2015;19(5):531-536.
42. Liskmann S, Zilmer M, Vihalemm T, Salum O, Fischer K. Correlation of peri-implant health and myeloperoxidase levels: a cross-sectional clinical study. *Clin Oral Implants Res*. 2004;15(5):546-552.
43. Carcuac O, Berglundh T. Composition of human peri-implantitis and periodontitis lesions. *J Dent Res*. 2014;93(11):1083-1088.
44. Aratani Y. Myeloperoxidase: its role for host defense, inflammation, and neutrophil function. *Arch Biochem Biophys*. 2018;640:47-52.
45. Nakazawa D, Kumar S, Desai J, Anders HJ. Neutrophil extracellular traps in tissue pathology. *Histol Histopathol*. 2017;32(3):203-213.
46. Abu Abed U, Brinkmann V. Immunofluorescence labelling of human and murine neutrophil extracellular traps in paraffin-embedded tissue. *J Vis Exp*. 2019;151:e60115.

How to cite this article: Al-Bakri SMR, Magan-Fernandez A, Galindo-Moreno P, et al. Detection and comparison of neutrophil extracellular traps in tissue samples of peri-implantitis, periodontitis, and healthy patients: A pilot study. *Clin Implant Dent Relat Res*. 2024;1-11. doi:10.1111/cid.13325