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Complete List of Authors:	Magán-Fernández, Antonio; University of Granada, Periodontology Department, School of Dentistry O'Valle, F.; University of Granada, Pathology Abadía-Molina, Francisco; Universidad de Granada Facultad de Ciencias, Department of Cell Biology Muñoz, Ricardo; Junta de Andalucia Servicio Andaluz de Salud, UGC Bucodental Metropolinato-Granada Puga, Patricia; Junta de Andalucia Servicio Andaluz de Salud, Unidad Intercentros Salud Bucodental Mesa, Francisco
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CHARACTERIZATION AND COMPARISON OF NEUTROPHIL EXTRACELLULAR TRAPS IN GINGIVAL SAMPLES OF PERIODONTITIS AND GINGIVITIS. A PILOT STUDY

Antonio Magán-Fernández[1], Francisco O'Valle[2,3], Francisco Abadía-Molina[4,5], Ricardo Muñoz[6], Patricia Puga-Guil[7], Francisco Mesa[1].

[1] Periodontology Department, School of Dentistry, University of Granada, Granada, Spain.

[2] Pathology Department, School of Medicine, (IBIMER, CIBM), University of Granada, Spain.

[3]Biosanitary Research Institute (ibs.GRANADA). University of Granada, Granada, Spain.

[4]Department of Cell Biology, University of Granada, Granada, Spain.

[5]INYTA, Institute of Nutrition and Food Technology "José Mataix", University of Granada, Granada, Spain.

[6]UGC Bucodental Metropolinato-Granada, Andalusian Public Health System, Granada, Spain.

[7]Unidad Intercentros Salud Bucodental, Andalusian Public Health System, Jaen, Spain.

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Corresponding author:
Antonio Magán-Fernández
Facultad de Odontología, Campus de Cartuja s/n,
Universidad de Granada, 18071, Granada, Spain.
Tel +34-958243804 Fax +34-958240908
E-mail: amaganf@ugr.es

Abstract

Background and objective: Neutrophil extracellular traps are a recently discovered antimicrobial mechanism used by neutrophils that have been proposed as an intervention in the pathogenesis of periodontitis. The objective of our study was to characterize the expression of neutrophil extracellular traps in gingival tissues with periodontitis and controls, and to compare the expression of these traps in gingival tissue samples of patients with gingivitis and periodontitis.

Material & methods: An observational cross-sectional study was conducted on patients with periodontitis, gingivitis and controls that needed tooth extraction. Gingival tissue biopsies were gathered after clinical examination and tooth extraction. Electron microscopy and immunofluorescence were performed to characterize neutrophil extracellular traps, comparing periodontitis and control patients. Immunohistochemical analysis was performed to quantify neutrophil extracellular trap expression through extracellular citrullinated histone H3 and myeloperoxidase in biopsies from patients with gingivitis and periodontitis.

Results: 13 biopsies were gathered from 13 patients: 5 gingivitis, 6 periodontitis and 2 controls. Electron microscopy and immunofluorescence imaging showed greater expression of neutrophils present in periodontal inflamed tissue compared to controls. Release of nuclear content to the extracellular space was observed, compatible with the formation of neutrophil extracellular traps. The expression of citrullinated histone H3 was higher in gingivitis samples than periodontitis samples (p=0.0106). Myeloperoxidase expression was higher in periodontitis than gingivitis, but without achieving statistical significance.

Conclusion: Neutrophil extracellular traps were found in tissue samples of periodontitis as extracellular components of chromatin, along with neutrophil enzymes, that were not present in healthy controls. The comparison of neutrophil extracellular traps expression in periodontitis and gingivitis showed higher expression in gingivitis, associating them to acute phases of the periodontal inflammatory process.

Keywords: Neutrophils; Extracellular traps; Gingivitis; Chronic Periodontitis; Immunohistochemistry.

Introduction

Periodontitis, an infectious disease affecting the tooth-supporting tissues with a wide range of clinical, microbiological and immunological manifestations. It is associated with and probably caused by dynamic interactions among infectious agents, host immune responses, hazardous environmental exposure and genetic propensity (1). Neutrophils are key inflammatory cells that play a role in periodontal lesions. Neutrophils are the most abundant innate immune effector cells of the human immune system. They are armed with broadly effective antimicrobials that are stored predominately in specialized granules (2). Peripheral blood neutrophils are recruited from the bloodstream into the site of the infection. They reach the endothelial cells to interact with selectin and integrin receptors through extravasation (3, 4). Subsequently, they transit through the tissue to the infected site by chemotaxis (5). Neutrophil action is regulated through three major mechanisms: phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs) (6).

NETs are large, extracellular, web-like structures composed of cytosolic and granule proteins that are assembled on a scaffold of decondensed chromatin, described for the first time by Brinkmann et al. (7). NETs are composed of nuclear constitutes such as histones, and neutrophil antimicrobial peptides as neutrophil elastase (NE) or myeloperoxidase (MPO). Their main role is to contain pathogenic organisms by a non-phagocytic mechanism, but their ability to kill bacteria remains to be proven (8). The process in which NETs are released, known as NETosis, can be mediated by several stimuli, including various types of microbes, microbial products like lipopolysaccharide, host-derived inflammatory factors and external substances (2, 8). This process is regulated by a complex signaling pathway, which includes multiple receptors for signal transduction (Toll-like receptors, receptors for advanced glycation end products and cytokines) that mediate the production of reactive oxygen species (ROS) and the activation of protein-arginine deiminase type 4 (PAD4). Hypochlorous acid has also been reported to be an important agent in this process, as a downstream product derived from the action of MPO on hydrogen peroxide (9). These events cause chromatin citrullination, nuclear de-condensation,

nuclear membrane disintegration, mixing of chromatin with antimicrobial peptides, cytoskeletal re-organisation, and plasmatic membrane breakdown (2, 10, 11). The de-condensed chromatin formed of histones and DNA exits from the nucleus and is mixed with the cytoplasm, and eventually extrudes their content outside the cell (12). NETs main antimicrobial actions consist on trapping and immobilizing pathogens, while the antimicrobial peptides embedded in the structure, such as NE or MPO kill the microorganisms (10).

However, NETosis might have a potential adverse side-effect and can cause collateral tissue damage due to an autoimmune mechanism. This has been shown in lupus patients, where an autoimmune reaction was caused by high concentration of NETs and NETs-associated proteins due to an impaired clearance (13). NETs have also been associated with periodontitis in several circumstances. The expression of NETs was found to be higher in gingival purulent exudate samples (14). Key periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia* have been associated with the induction of NETs. They have also been demonstrated to be mechanisms to avoid NETs through the expression of bacterial nucleases (15, 16). Patients with untreated chronic periodontitis showed impaired NET degradation due to decreased plasma levels of DNAse. This impaired NET degradation improved and became similar to that of controls after non-surgical periodontal therapy (17). Such high and concentrated levels of neutrophil extracellular trap-associated molecules in gingival tissue could potentially lead to an autoimmune reaction, mediated by citrullinated histones or extracellular DNA. This leads to significant damage, characteristic of periodontal pathogenesis (10).

To date, the role of NETs in gingival tissue biopsies have only been reported by the article of Cooper et al., which showed the first evidence of this fact (10). Studies have been mainly conducted on peripheral blood leucocytes, samples of purulent gingival exudate or supragingival plaque (16). However, a comparison between two different inflammatory entities, such as gingivitis and periodontitis, has not been performed to date.

The objective of our study was to characterize the expression of NETs in gingival tissues with periodontitis and controls, and to compare the expression of NETs in gingival tissue samples of patients with gingivitis and periodontitis.

Material and methods

An observational case-control study was designed on patients that attended public healthcare centers in the metropolitan area of Granada (Spain) during the year 2016. Gingival biopsies involving the interproximal papilla and the periodontal sulcus were obtained from patients receiving tooth extractions due to periodontal reasons. All periodontal teeth showed high mobility, bleeding on probing and at least one site with probing pocket depth (PPD) ≥ 5mm. Biopsies of gingivitis were selected from cases of tooth extraction due to caries, and presented bleeding on probing (BoP) with PPD < 3 mm. Two healthy gingival biopsies were taken from teeth, extracted for orthodontic reasons, for NETs characterization. All patients included in the study were above 18 years of age. Patients were excluded if they had received previous periodontal treatment, if they had received antibiotic or anti-inflammatory therapy within two months prior to the examination, or if they had neoplastic diseases, HIV or other severe systemic infection. Sociodemographic data were gathered from each patient. Periodontal examination was performed on each tooth prior to extraction using SE plus® mouth mirror (Hahnenkratt E. GmbH, Königsbach-Stein, Germany) and PCPUNC15 periodontal probe (Hu-Friedy, Chicago, IL, USA). PPD was assessed in millimeters on 6 sites per tooth (mesiovestibular, vestibular, distovestibular, mesiolingual, lingual and distolingual) to properly assess the periodontal status. BoP was also registered individually for each tooth to determine the presence of gingival inflammation. All procedures in this study were performed in accordance with the 1964 Helsinki declaration and its latest amendment in 2013. The study was approved by the Human Research Ethics Committee of the University of Granada (639/CEIH/2018). All participants signed written informed consent when recruited.

Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines were followed for the preparation of this manuscript (18).

Transmission Electron Microscopy (TEM) study

Several 1-mm^2 fragments of gingival papilla were fixed in 2.5% glutaraldehyde solution and then postfixed in 1% OsO4 at 4°C for 2 h, washed in distilled water, dehydrated in increasing concentrations of acetone, and embedded in Epon following the conventional protocol. Semithin sections were stained with toluidine blue solution. Ultrathin (~70 nm-thick) sections were obtained in a Reichert Jung ULTRACUT ultramicrotome (Leica, Wetzlar, Germany) and stained with lead citrate and uranyl acetate. In order to identify NETs, ultrathin sections were examined under a Libra 120 Plus TEM (Zeiss, Oberkochen, Germany).

Histopathological analysis

Gingival papilla samples including sulcular and oral epithelium were obtained from all participants and immediately fixed in 10% buffered formalin for 48 h at room temperature. Paraffin-embedded samples were deparaffinized in xylol (3 passes of 5 minutes) and re-hydrated in ethanol of decreasing gradation (absolute, 96%, and 70%, 2 passes of 3 min, respectively). Tissue sections were stained with hematoxylin-eosin (H-E) and the inflammatory infiltrate was morphologically assessed. The morphological study was done in blinded fashion on 4-micrometer sections with light microscopy.

Immunohistochemical analysis

Paraffin-embedded sections were dewaxed, 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) at 95 °C for 20 min. Sections were incubated for 1 hour at room temperature with prediluted polyclonal antibody (rabbit anti-human) against myeloperoxidase (Master Diagnóstica, Granada, Spain) and polyclonal (rabbit anti-human) anti-citH3 (Abcam ab5103, Abcam plc, Cambridge, UK) at 1:100 dilution. The immunohistochemical study was conducted

using the micropolymer-peroxidase-based method (Master Polymer, Master Diagnóstica) with automatic immunostainer (Autostainer 480S, Thermo Fisher Scientific Inc., Waltham, MA) followed by development with diaminobenzidine. Appropriate positive (tonsil) and IgG isotype (non-immune serum) as negative controls were run concurrently. Hematoxylin was used for nuclear counterstaining.

The immunohistochemical study was done in blinded fashion on 4-micrometer sections with BX42 light microscopy (Olympus Optical Company, Ltd., Tokyo, Japan), using 40x objective in a microscope with an attached scale (BH2, Olympus Optical Company, Ltd., Tokyo, Japan). The number of positive cells were quantified per mm^2. Results were also expressed as the percentages of positive cells for each antibody, counting 200 cells per high-magnification field (40x objective) in three independent experiments. Histomorphometrical quantification was performed semiautomatically using each immunostain. 10 random images were captured from each sample with 40x objective in a microscope with an attached digital camera (DP70, Olympus Optical Company, Ltd., Tokyo, Japan). Images were then analyzed with the software ImageJ (NIH, http://imagej.nih.gov/ij/) to quantify the percentage of area occupied by both immunostains.

Immunofluorescence confocal microscopy analysis

Paraffin-embedded sections were dewaxed, hydrated, and heat-treated as specified in the previous section. Tissue sections were blocked with 10% normal goat serum for 30 min and permeabilized with 0.1% Triton X-100 and 0.3% Tween-20 for 30 min. The sections were then incubated overnight at 4 ºC with a combination of the following primary antibodies, both at a dilution of 1/100: citH3 (polyclonal rabbit anti-human) (abcam ab5103) and CD177 (IgG1 mouse anti-human) (abcam ab8092) (both from Abcam plc, Cambridge, UK). The slides were then rinsed 3 times for 5 min with PBS and incubated for 1h at room temperature with the secondary antibodies; Alexa Fluor 488 goat anti-rabbit 1:500 for citH3 detection and Alexa Fluor 568 goat anti-mouse 1:500 for CD177 detection (A-11008 and A-11004 respectively,

Invitrogen, San Diego, CA, USA). The slides were counterstained with Hoechst 33342 (Invitrogen, San Diego, CA, USA), diluted at 10µg/ml in PBS and mounted with Dako (#S3023, Dako Denmark A/S, Glostrup, Denmark) fluorescence mounting medium. Unlabeled mouse IgG1 isotype (non-immune serum) was used as negative control. Imaging was performed with a Leica TCS SP5 confocal microscope (Leica, Wetzlar, Germany).

Statistical analysis

Stata 14 (StataCorp LLC, College Station, TX, USA) was used for the statistical analysis. A non-parametric test (Mann-Whitney U-test) was applied to compare positive cells between periodontitis and gingivitis samples. A $p \leq 0.05$ significance level was considered in all tests. Statistical tests used are described in table footnotes.

Results

A total of 13 biopsies were gathered from 13 patients: 5 gingivitis, 6 periodontitis and 2 controls. Controls were only used for the characterization of NETs.

Confocal microscopy analysis of immunofluorescence images of the healthy control biopsies showed small citH3 staining and no CD177 positive staining (Figure 1a). The periodontitis biopsies showed positive staining of citH3 together with CD177 neutrophil marker in the extracellular medium, characteristic of NETosis as shown by previous studies in other tissues (Figure 1b) (19).

TEM study of healthy controls showed a clear and organized tissue structure without the presence of inflammatory infiltrate (Figure 2a). In contrast, TEM images of gingival periodontitis biopsies revealed damaged tissue with large amounts of dead and inflammatory cells in the gingival disrupted tissue. Neutrophils were found showing typical NETosis

phenomena with empty nuclei and broken nuclear membranes associated with expulsion of the chromatin to the extracellular medium (Figure 2b and 2c).

Table 1 describes the results from the comparison of immunohistochemical markers between gingivitis and periodontitis. CitH3 and MPO expression are presented as total and separated measurements of sulcular and oral epithelium. Both markers showed greater expression in the sulcular epithelium. There was a generalized nuclear staining of citH3 in both epitheliums of all samples, and no statistically significant differences (p=0.3043). Nuclear staining was excluded from the assessment since the extracellular staining is the one of interest for NETosis. Extracellular citH3 expression was found to be higher in gingivitis samples (p=0.0106) in both vestibular and sulcular epitheliums. MPO expression was higher in periodontitis samples but did not achieve statistical significance (Figure 3). Comparison of mean grey values expressed by both markers showed no statistically significant differences.

Discussion

To our knowledge, our findings report for the first time a different *in vivo* expression of NETs in human samples of gingivitis and periodontitis.

There is great diversity in markers and methodologies among the studies that have investigated the role of NETs in tissue pathology (19). Neutrophils are converted from a non-active cell to a cell primed by bacterial components (endotoxins) and host mediators like cytokines. To eliminate the invading periodontal pathogens, neutrophil granulocytes have an immune armamentarium at their disposal: phagocytosis, ROS, intracellular and extracellular degranulation. However, the neutrophil-bacteria reaction during periodontal inflammation occurs mainly outside the gingival tissue, and significantly within the gingival crevice (14). Supporting this fact, gingival exudates harvested from periodontal pockets showed lack of phagocytized bacteria, but an abundance of NETosis trapping the bacteria in the gingival

crevice (20). The gingival pocket provides an oxygen-rich environment and a natural pH that favors the action of ROS. Chronic periodontitis causes a hyper-responsivity that also involves ROS, which are inducers of NETosis, especially hydrogen peroxide (9). In addition, IFNα is one of the mediators that stimulate NET release and was found in significant amounts in the sera of patients with periodontitis (21). Thus, it is biologically plausible that NETosis could also be upregulated and play a role in the pathogenesis of periodontitis.

Immunofluorescence imaging of periodontitis samples compared to healthy control biopsies showed greater citH3 staining and CD177 positive staining. CD177 is a glycosylphosphatidylinositol-anchored protein expressed by a variable proportion of human neutrophils that mediates surface expression of the ant neutrophil cytoplasmic antibody antigen proteinase 3. CD177 is expressed in neutrophils specifically and is upregulated during inflammation. CD177+ neutrophils represent functionally activated population (22) and CD177 signals in a β2 integrin-dependent manner to orchestrate a set of activation-mediated mechanisms that impair human neutrophil migration (23). Previous studies have used CD177 as a specific neutrophil marker for the characterization of neutrophil extracellular traps, including studies on supragingival biofilms (24).

Our results showed a higher citH3 expression in gingivitis samples in comparison with periodontitis. The chronic nature of periodontal infection might initiate deamination of other core histones like H2A or H4, concealing the activity of H3, or the histone itself might modify its structure, making the antibody assay unable to detect the epitope of H3 (25). In addition to these findings, some authors have also stated that H3 is not necessary for NETosis, but it is merely a product of the process after the activation of PAD4 (25, 26). Histones are highly rich proteins that represent one the main constituents of casted neutrophil contents (7, 12, 27). It has also been shown that the increased rate of citH3 coincides with an increase in NET activity when preliminarily validating an enzyme-linked immunosorbent assay (27). Histones have also shown possible antibacterial activity, since the association of citrullinated histones and NETs may have the role of killing trapped microbes, affecting the interactions with the bacterial cell

wall. This leads to the idea that NETs may also be bactericidal, independently of the neutrophil antimicrobial peptides (28). Higher citH3 levels in gingivitis biopsies indicate that NETosis reached the maximum activity during this phase, since histones are degenerated 2 to 4 hours before cell death and citrullinated levels reach their peak during NETosis production (29). This is in accordance with our results. NETs may show more bactericidal activity during gingivitis progression. Neutrophil elastase (NE) is the main protease causing nuclear de-condensation and releasing of NETs *in vivo* (28). Conversely, an auto-inhibition can be caused by local high concentrations of NE, which may reduce nuclear de-condensation, subsequently inhibiting histone degradation with the progression of the inflammatory process (29). This could explain the reduction of citH3 expression in the periodontitis samples.

Not all histones that are present during the pathogenesis of the disease are antimicrobial. Some stimuli produce the release of histones but they are not necessarily caused by NETosis. Histones are also related to a different neutrophil death modality called leukocyte hypercitrullination (LTH) (25). Other microbial stimuli like DNA from *P. aeruginosa* can be detected as neutrophil DNA through a mimicking mechanism (30). DNAse produced by periodontal pathogens inhibit the formation of NETs and might augment the probability of bacterial colonization in the periodontal tissue. Destruction of NETs may result in the release of their antimicrobial peptides to the medium, these can potentially have a harmful widespread effect on the tissue.

MPO is another enzyme that forms a part of a NET and is commonly used to assess the NETs activity. In contrast to citH3, a higher expression of MPO was found in periodontitis samples although it did not reach statistical significance. The absence of statistical significance may be explained by the NETosis independent MPO release through regular neutrophil degranulation. Future studies should address only MPO attached to DNA, in order to ensure that the measured MPO comes from a NETosis process. These results could also be explained by the fact that MPO, along with other NETs-related enzymes, cause rapid degradation of free histones in plasma blood. Hence with the increase of MPO, citH3 levels decrease as gingivitis progress to periodontitis. CitH3 bound to DNA could resist this degradation and subsequently resist binding

to the assay (27, 30). Taken together, our data supports the hypothesis that MPO levels are not related to NETosis. This assumption coincides with recent experiments done with certain chemical inducers (calcium ionophore A23187, bacterial toxin nigericin) which did stimulate NETosis in the presence of an MPO inhibitor. They also showed that MPO was not essential for NETosis since different stimuli, such as bacterial stimuli, produce NETosis independently of the MPO expression or inhibition (26, 30). NETs were produced when partial or complete MPO deficient neutrophils were stimulated with bacteria (26, 30-32). In agreement with our results, reports identify that the presence of MPO is not always necessary during chromatin de-condensation. This discrepancy might account for the fact that MPO is stimuli-dependent and the proteins involved in NETosis yield different results between studies due to the high diversity of stimuli used (33).

The principal limitation of this study would be its limited sample size, although significant results were found. These results could serve as a proof-of-concept for further studies conducted in the future. A deeper knowledge on the regulatory mechanisms and the *in vivo* role of NETs in tissue samples is still needed, especially regarding the pathogenesis of periodontal disease.

Conclusions

NETs are characterized in tissue samples of periodontitis and gingivitis as extracellular components of DNA and histones along with neutrophil enzymes, showing images compatible with NETosis. The comparison of periodontitis and gingivitis showed that NETs composition changed, and the expression was higher in gingivitis. This was associated with acute phases of the inflammatory process.

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Conflict of interest

The authors report no conflicts of interest related to this study.

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Table 1. Immunohistochemical marker expressions

	Gingivitis (n=5)	Periodontitis (n=6)	*p*-value*
Total citH3 (cells/mm^2)	287.10 ± 93.19	89.25 ± 66.79	0.0106
Vestibular citH3 (cells/mm^2)	52.90 ± 33.41	16.13 ± 13.53	0.0441
Sulcus citH3 (cells/mm^2)	234.19 ± 85.43	73.12 ± 68.72	0.0104
Total MPO (cells/mm^2)	361.94 ± 319.05	607.53 ± 394.45	0.3142
Vestibular MPO (cells/mm^2)	30.97 ± 40.97	90.86 ± 85.09	0.1971
Sulcus MPO (cells/mm^2)	330.97 ± 286.87	516.67 ± 328.31	0.4102
citH3 Intensity (mean grey value)	37.69 ± 5.00	41.54 ± 8.39	0.5839
MPO Intensity (mean grey value)	10.73 ± 5.91	15.93 ± 5.29	0.1441

Values expressed as mean ± standard deviation; *Mann-Whitney U-test

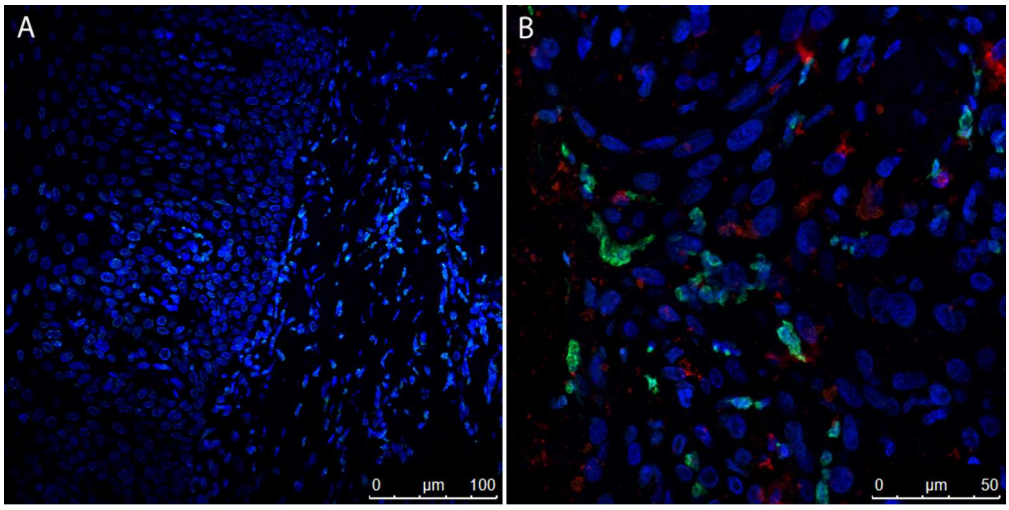

Figure 1. Confocal microscopy. H3 (Alexa Fluor-488 shown in green), CD177 (Alexa Fluor-568 shown in red) and Hoechst nuclear DNA staining merged channels. (a) Representative immunofluorescence from a control sample showing little citrullinated histone H3 staining and none neutrophil CD177 staining. (b) Representative immunofluorescence from a periodontitis sample showing H3 extracellular staining in the gingival corium and numerous CD177 positive neutrophils.

135x67mm (192 x 192 DPI)

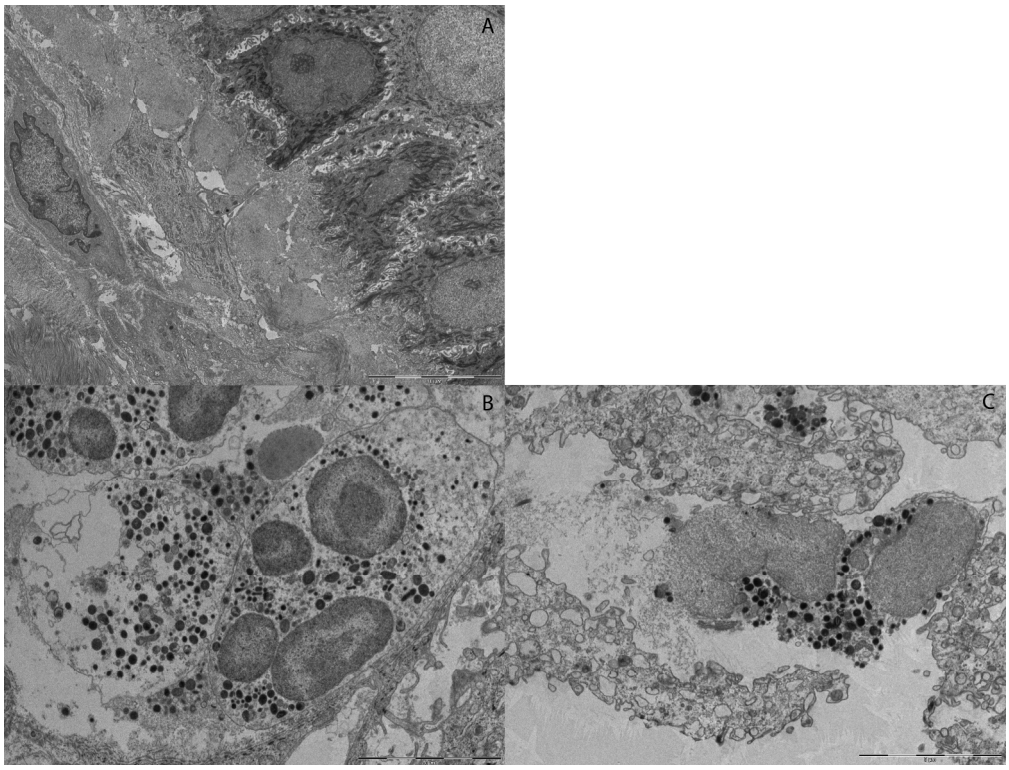

Figure 2. Transmission electron microscopy. (a) Micrograph from a control sample showing intact gingival epithelium and corium. (b, c) Micrographs from a periodontitis sample showing typical NETosis broken neutrophils in the gingival corium and disrupted connective tissue.

478x362mm (144 x 144 DPI)

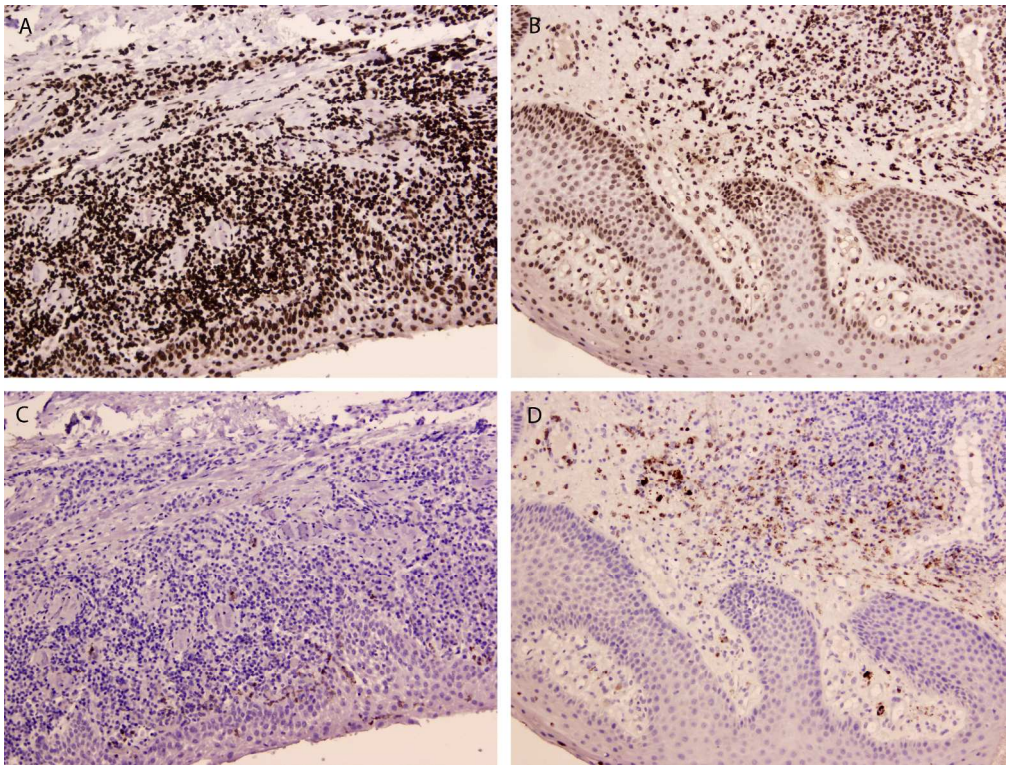

Figure 3. Morphological and immunocytochemical study. Higher extracellular expression of citH3 in gingivitis samples (A) compared to periodontitis (B). Lower MPO expression in gingivitis samples (C) compared to periodontitis (D) (original magnification x40).

460x348mm (152 x 152 DPI)

Figure Legends

Figure 1. Confocal microscopy. H3 (Alexa Fluor-488 shown in green), CD177 (Alexa Fluor-568 shown in red) and Hoechst nuclear DNA staining merged channels. (a) Representative immunofluorescence from a control sample showing little citrullinated histone H3 staining and none neutrophil CD177 staining. (b) Representative immunofluorescence from a periodontitis sample showing H3 extracellular staining in the gingival corium and numerous CD177 positive neutrophils.

Figure 2. Transmission electron microscopy. (a) Micrograph from a control sample showing intact gingival epithelium and corium. (b, c) Micrographs from a periodontitis sample showing typical NETosis broken neutrophils in the gingival corium and disrupted connective tissue.

Figure 3. Morphological and immunocytochemical study. Higher extracellular expression of citH3 in gingivitis samples (A) compared to periodontitis (B). Lower MPO expression in gingivitis samples (C) compared to periodontitis (D) (original magnification x40).

RESPONSES TO REVIEWERS' COMMENTS

Manuscript Title: "Characterization and comparison of neutrophil extracellular traps in gingival samples of periodontitis and gingivitis. A pilot study" **(JRE-07-18-4616.R2)**.

We are grateful for all of the helpful comments and suggestions.

Referee(s)' Comments to Author:

Reviewer: 1

Query 1. This manuscript is much improved. However, the authors must add "pilot study" to their title due to the small numbers.

Response: We would like to thank the reviewer for their comments and suggestions. As requested, pilot study has been added to the article title accordingly.

Query 2. The paper needs very careful proof reading during type setting – the grammar is still poor.

Response: The manuscript has been now revised by a native English speaker to improve the grammar.

Query 3. The statement added p5 line 38 does not address my issue as "destroyed" implies NETs kill bacteria but this remains to be proven – please state this.

Response: The sentence has been modified following the request of the reviewer.

Query 4. The statement added on p4 line 44 does not acknowledge that Cooper et al demonstrated NETs for the 1st time in inflamed gingival tissues – this needs stating correctly.

Response: The sentence has been rewritten to state more clearly the fact requested by the reviewer.