ORIGINAL RESEARCH

Tissue-invasive Pathogens in Periodontitis Patients and Their Correlation with Pro-inflammatory Markers: An Analytical Case-control Study

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Abstract

Aim: Tissue-invasive bacteria have been proposed to be a crucial factor in the etiopathogenesis of periodontitis, with the probable interaction of tissue-invasive bacteria with the innate immune response through inflammasomes, perpetuating periodontal attachment loss. This study aims to reveal the correlation between such tissue-invasive bacteria in upregulating inflammasomes and pro-inflammatory cytokines.

Materials and methods: This study recruited a total of 10 patients with stage III/IV and grade C periodontitis based on the bone loss to age ratio. Patient sites were grouped into group I: healthy sites with no clinical attachment loss (CAL); group II (mild-to-moderate): 1–4 mm of CAL; group III: severe (≥ 5 mm of CAL). Tissue samples were collected in these sites during periodontal flap surgery and assessed for both the bacterial genomic DNA and assessed for the upregulation of pro-inflammatory markers NOD-like receptor – Pyrin domain containing protein 3 (NLRP3), Human AIM2, Human Pro-TNFα, Human Pro-IL-1β, Human Pro-IL 6 and Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes.

Results: The levels of NLRP3, AIM2, and pro-inflammatory cytokine levels were all higher in the severe sites when compared with the other two sites. The tissue invasive bacterial phylotypes in these sites were thereafter compared with the levels of the pro-inflammatory markers in the various groups. The fold changes in the pro-inflammatory markers evaluated in this study all hovered around 1, indicating not much difference in the upregulation of these markers of inflammation. Statistically, significant correlation between bacterial phenotypes in the healthy sites group and the pro-interleukin-6 (IL-6) cytokine expression was observed ($r = 0.68$; $p < 0.04$).

Conclusions: This study has highlighted the presence of tissue-invasive bacteria in sites with or without CAL. The fact that these healthy sites, after non-surgical therapy, have comparable levels of pro-inflammatory markers in the tissues may be explained by immune priming, by tissue-invasive periodontal pathogens.

Clinical significance: Tissue-invasive bacteria are present in periodontally healthy sites too, and non-surgical periodontal therapy is inadequate to eliminate them. Greater importance should be given to the soft tissue walls of the periodontal pocket in clinical management of periodontitis.

Keywords: Inflammasomes, Immune priming, Pro-inflammatory markers, Tissue-invasive bacteria.

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Introduction

Periodontitis is an inflammatory state of the periodontium that is initiated and perpetuated by microorganisms that make up the dental biofilm. The periodontist therefore seeks to treat this inflammatory state by a process of mechanical removal of the biofilm present on the tooth surface, along with factors such as calculi and correction of plaque retentive factors on teeth and dental restorations to prevent the formation and maturation of the dental biofilm. However, there is a further domain wherein microorganisms when present, may potentially have a larger role in modulating the inflammatory state of the periodontium.[1] This alternate domain pertains to the ability of the dental biofilm bacteria to pass through the tissue to reside and proliferate within the gingival epithelium and connective tissue, where they are seen to be present both intracellular, as well as paracellular, as they seek out niches within the tissues where they may be able to survive and attempt to evade the effectors of the innate immune response.[2]

Periodontitis is in essence caused by a dysregulated immune-inflammatory response of the host to microflora present at the tissue-tooth interface.[3] The possible ability of these bacteria to become tissue invasive, and thereby upregulates the activation of pattern recognition receptors and activate cell-mediated immune system against these tissue-invasive pathogens is a critical possibility that needs to be investigated.[4] While recent studies have quite firmly established the fact that tissue-invasive pathogens are present in periodontal disease, the true essence of their role while they are in the periodontium, as tissue-invasive bacteria has not been thoroughly elucidated. It has been hypothesized that tissue-invasive bacteria could create a greater level of bacterial interaction

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with the innate immune response, creating a hyperinflammatory state in the periodontium, thereby perpetuating periodontal attachment loss.[5]

Critical in the understanding of the innate immune system in its ability to act against tissue-invasive pathogens are inflammasomes, which are oligomeric molecules that are part of the innate immune system of the host, which are upregulated in the cytosol of cells in response to infection or cellular stressors.[6] These complex molecules serve as pathogen recognition receptors that are also capable of activating a caspase-1-driven inflammatory response, which in turn leads to the production of pro-inflammatory cytokines in the tissues. These inflammasomes function as sentinels that are either present on cell membranes or within the cellular cytoplasm, and activation of these inflammasomes results in the upregulation of downstream pro-inflammatory cytokines, which when unregulated leads to tissue destruction. Among the more frequently upregulated inflammasomes during periodontal disease are the NOD-Like Receptor – Pyrin domain containing protein 3 inflammasome (NLRP3) and the absent in melanoma-2 (AIM2) inflammasome, that have been shown to be upregulated in the periodontium during periodontal disease.[7] These inflammasomes that are frequently upregulated by intra-tissue bacteria are in turn mediators of pro-inflammatory cytokines such as Interleukin-1-beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and Interleukin-6 (IL-6), that are upregulated in sites with periodontal disease.

While earlier studies have highlighted the presence of intra-tissue bacteria, none of the earlier studies had correlated the copy numbers of tissue-invasive pathogens with the levels of pro-inflammatory markers. A further lacuna has been the evaluation and correlation of the levels of tissue-invasive pathogens and pro-inflammatory markers in sites with varied levels of periodontal destruction. The current study was designed as a preliminary trial to scope the possibility that tissue-invasive pathogens might be present in sites with varied levels of clinical attachment loss (CAL), even after the completion of root surface debridement, and that the copy numbers of these putative periodontal pathogens may correlate with markers of inflammation within the tissues. The study was envisaged as an analytical case-control study, with the goal of correlating the levels of tissue invasive bacteria and markers of inflammation, in periodontitis patient sites with varied levels of tissue destruction, who had initially been diagnosed with stage III and grade C periodontitis. The study hypothesized that the presence of tissue-invasive bacteria would correlate with markers of inflammation in the tissue samples evaluated after the completion of non-surgical periodontal therapy. The aim of this study therefore was to evaluate the presence of tissue-invasive bacterial phylotypes and correlate them with markers of inflammation – NLRP3 and AIM 2 inflammasomes and pro-inflammatory cytokines – IL-1β, TNF-α, and IL-6.

Materlals and Methods

The study protocol was evaluated and approved by the institutional clinical trials and ethics review board SRMDC/IRB/2017/PhD/No.140 and was enrolled into the clinical trials registry of India – CTRI/2021/01/030226. This analytical case-control study was carried out was from February of 2022 to February of 2024. The study subjects were recruited from the outpatient clinics of the Department of Periodontics, SRM Dental College, Ramapuram, India. Ten patients aged between 30and 60 years, with 20 remaining natural teeth and who were diagnosed with stage III/IV and grade C periodontitis (that was categorized based on the bone loss to age ratio) and who had varied levels of destruction in the different sites to allow the patient sites to be categorized into (a) severe – ≥5 mm of CAL (group III); (b) mild-to-moderate – 1–4 mm of CAL (group II); and (c) healthy sites with no CAL (group I) were included in the study. All patients were recruited from a teaching hospital. Patients excluded were those who had uncontrolled diabetes mellitus, those who were current smokers, and those who had received systemic antimicrobial therapy in the last 6 months. Further, those who were pregnant or lactating as well as those who were chewing tobacco, pan, or other oral non-tobacco chewing habits were excluded. All the patients received the preliminary phase management of scaling and root surface debridement, along with the management of factors that promote plaque accumulation, such as carious lesions, overhanging margins, over-contoured crowns, plunger cusp, splinting of mobile teeth etc. The patients were then reviewed, and the sites selected were those that were indicated for pocket reduction surgery in the posterior teeth and had been designated to be taken up for modified Widman flap surgery. The mild-to-moderate and healthy sites were selected from the interproximal gingiva of teeth adjacent to sites taken up for pocket reduction, which needed to be included for the sake of access to tissues. All the tissue samples were thus procured through internal bevel incisions and tissues so procured were rinsed in normal saline and placed into aliquots with "RNA Save" and refrigerated at –80°C, until they were to be taken up for analysis.

After all the samples were collected, the tissue samples were split into two halves, one for the assessment of tissue-invasive pathogens and the other for assessment of markers of inflammation. For the assessment of the tissue-invasive bacterial phylotypes, the first step was to eliminate the surface adherent tissue-invasive bacteria. This was performed in accordance with the technique validated by Baek et al.[9] These pretreated samples were then taken up for assessment of bacterial genomic DNA. Briefly the technique for elution of the bacterial DNA started with the homogenizing of the tissue sample by placing the pretreated sample in 200 µL of Proteinase K, in which it was vortexed and then incubated at 55°C for 10–15 minutes for the freeing of the genomic DNA. The samples were then placed in an elution buffer to collect the purified DNA. These samples were then analyzed using a 16S rRNA primer to identify bacterial amplicons user a SYBR Green Master Mix-based RT-PCR. The forward primer used was 5′-CGGTGAATACGTTCYCGG-3′ and the reverse primer used was 5′-GGWTACCTTGTTACGACTT-3′ (Barcode Biosciences, Bengaluru, India). The bacterial copy numbers for the three groups were then tabulated.

Alongside, the other half of the tissue procured were evaluated for the presence of pro-inflammatory markers that were upregulated in the tissues. The RNA was extracted using the Trizol method that included an initial process of vortexing and incubation of chopped tissue samples for complete disassociation of nucleic acid proteins. Further processing was carried out until RNA pellets were procured and they were then taken up for cDNA conversion(Lupex Biotechnologies Pvt Ltd., India). After the cDNA was done, the samples were evaluated for the following pro- inflammatory genes (a) Human NLRP3 inflammasome gene, (b) Human AIM2 inflammasome gene, (c) Human pro-TNF-α gene, (d) Human pro-IL-1β gene, (e) Human pro-IL 6 gene, (f) Human GAPDH gene (housekeeping). The primers for the pro-inflammatory markers are given in Table 1. The RT-qPCR was then carried out for assessment of the upregulation of these genes in the sample using

a SYBR Green Master Mix-based protocol (Lupex Biotechnologies Pvt Ltd, India). The RT-qPCR data were collected on Aria 2.0 software from Agilent Technologies.

The raw data were entered into excel sheet and the data were analyzed using SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY: IBM Corp. Released 2019). The Normality tests Kolmogorov–Smirnov and Shapiro–Wilks tests results reveal that the values followed normal distribution. Therefore, to analyze the data, parametric tests were applied. Descriptive statistics was expressed using mean and standard deviation. Inferential statistics was done using one-way analysis of variance (ANOVA) followed by *post hoc* test. Pearson correlation analysis was done to correlate study groups with biomarkers. Significance level was fixed as 5% ($\alpha = 0.05$) and p-value < 0.05 was considered to be statistically significant.

Results

The study samples were divided into three males and seven females. All of the tissue samples revealed tissue-invasive pathogens and the mean values of the evaluated bacterial genomic amplicon levels in the various patient groups were evaluated and their mean values was then correlated with the levels of pro- inflammatory markers.

The mean copy numbers of tissue-invasive bacterial phylotypes in the healthy sites was 157.20 ± 122.37 copies/µL. In the mild-moderate sites, the mean copy numbers were 232.50 ± 133.064 copies/µL and in the sites with severe destruction, it was 340.70 ± 177.18 copies/µL (Table 2).

The mean fold change of NLRP3 levels in the healthy sites was 0.98 ± 0.006, whereas in the sites with mild-to-moderate CAL the NLRP3 levels was 0.95 ± 0.064 while the mean NLRP3 levels with severe CAL was 0.86 ± 0.165. The results indicate lesser values of NLRP3 in the sites with severe destruction, when compared with the healthy and milder CAL sites with the other two groups. This inflammatory profile was similar when evaluating for the levels of AIM2 inflammasome as well as pro-TNF-α, IL-1β and IL-6, wherein all these markers were higher in the healthy groups, which was however not statistically significant. When the ANOVA test was applied to the expression of these five pro-inflammatory markers, it did not reveal a statistically significant difference in any of the three groups, with f values were not indicating a large variance and the p-values not being statistically significant (Table 3). This analysis of variance between the different groups was followed up by a pairwise *post hoc* comparison between each of the markers that were evaluated. This *post hoc* pairwise comparison also revealed no statistically significant difference, on comparison between the three groups (Table 4).

This study thereafter tried to correlate between tissue-invasive bacterial phylotypes in the various groups with the pro- inflammatory markers. The Pearsons correlation between the copy numbers in the healthy sites and the relative units of the pro-IL-6 cytokine expression showed statistical significance ($r = 0.68$; $p < 0.04$) (Table 5). The results therefore indicate that the levels of pro-inflammatory markers among the three different groups are comparable.

Table 1: Primers used for the pro-inflammatory markers

Pro-inflammatory markers	*Primers*
NLRP3	F: 5′-AGCCCCGTGAGTCCCATTA-3′ R: 5′-ACGCCCAGTCCAACATCATCT-3′
AIM2	F: 5′-ATCTCCTGCTTGCCTTCTTGG-3′ R: 5′-AAGTCTCTCCTCATGTTAAGCCTG-3′
IL-1β	F: 5′-AGATGATAAGCCCACTCTACAG-3′ R: 5′-ACATTCAGCACAGGACTCTC-3′
TNF-α	F: 5′-CCCGAGTGACAAGCCTGTAG-3′ R: 5′-GATGGCAGAGAGGAGGTTGAC-3′
IL-6	F: 5′-ACAGCCACTCACCTCTTCAG-3′ R: 5′ -CCATCTTTTTCAGCCATCTTT-3′
GAPDH	F: 5′-GTCTCCTCTGACTTCAACAGCG-3′ R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′

Discussion

The role of tissue-invasive pathogens in periodontitis has not been appropriately investigated, even though we understand through well-documented cell culture studies, the ability of bacteria considered to be periodontopathic to invade the cells that arise from the periodontium. However, the actual role of tissue-invasive pathogens in the initiation and progression of periodontitis has not been suitably investigated. Baek et al.[9,10] Rajakaruna et al.,[11] in a larger cohort of patients reported the presence of tissue invasive bacteria in the granulation tissue of periodontitis patients undergoing periodontal surgery, where they commented on the tissue localization of bacteria considered to be periodontopathic. Baek et al.,[9] assessed tissue samples arising from the treatment of

Table 2: Descriptive statistic of bacterial phylotypes in the varied patient sites

	Groups		
PCR variables	*Group I/healthy samples*	*Group II/mild–mod. sites*	*Group III/severe sites*
Copy number copies/µl	157.20 ± 122.37	232.50 ± 133.064	340.70 ± 177.18

Table 3: Descriptive statistics of the pro-inflammatory markers expression in the tissue sites

Groups	*NLRP3*	*AIM2*	*TNF-α*	*IL-1β*	*IL-6*
Fold change (Mean ± SD)					
Group I/Healthy samples	0.98 ± 0.006	0.97 ± 0.028	0.99 ± 0.006	0.98 ± 0.022	0.99 ± 0.001
Group II/Sites with mild to mod. CAL	0.95 ± 0.064	0.95 ± 0.061	0.99 ± 0.006	0.95 ± 0.060	0.99 ± 0.005
Group III/Sites with Severe CAL	0.86 ± 0.165	0.84 ± 0.198	0.97 ± 0.036	0.86 ± 0.181	0.99 ± 0.012
ANOVA test					
F value	$F = 2.83$	$F = 3.02$	$F = 3.29$	$F = 2.61$	$F = 1.70$
p-value	$p = 0.08$	$p = 0.07$	$p = 0.05$	$p = 0.09$	$p = 0.20$

ANOVA, analysis of variance

Table 4: Comparison of inflammatory marker levels between the different groups with *post hoc* analysis

Inflammatory markers	*Groups*	*Mean difference*	*p-value*
NLRP3	Group I vs II	0.03665	1.000
	Group I vs II	0.11929	0.091
	Group II vs III	–0.08264	0.367
AIM2	Group I vs II	0.01984	1.000
	Group I vs II	0.13772	0.100
	Group II vs III	–0.11787	0.195
TNF-α	Group I vs II	0.00157	1.000
	Group I vs II	0.02457	0.097
	Group II vs III	–0.02300	0.132
IL-1β	Group I vs II	0.02526	1.000
	Group I vs II	0.12062	0.125
	Group II vs III	–0.09536	0.304
IL-6	Group I vs II	0.00274	1.000
	Group I vs II	0.00731	0.246
	Group II vs III	–0.00456	0.800

Table 5: Correlation between bacterial copy numbers and pro-inflammatory markers

Groups	*Pearson correlation*	*NLRP3*	*AIM2*	*TNF-α*	*IL-1β*	*IL-6*
Group I/Healthy samples	*r* value	0.38	–0.02	–0.02	–0.02	0.68
	p-value	0.19	0.47	0.47	0.48	0.04*
Group II/Sites with mild-mod periodontitis	*r* value	–0.62	–0.44	–0.58	–0.45	–0.19
	p-value	0.06	0.15	0.08	0.15	0.33
Group III/Sites with severe periodontitis	*r* value	–0.06	–0.006	0.05	0.03	0.07
	p-value	0.44	0.49	0.45	0.46	0.44

*$p < 0.05$, statistically significant

pockets in sites with severe periodontitis, arising from seven patients, to evaluate the intra-tissue complexity of bacterial organisms as well as their numbers. However, they did not try to correlate these organisms with any pro-inflammatory markers. Rajakaruna et al.,[11] assessed the presence of Porphyromonas gingivalis and Tannerella forsythia in the gingiva and connective tissue of eleven patients with periodontitis with immunohistochemistry and qPCR, but once again did not attempt to correlate the levels of these bacteria with pro-inflammatory marker upregulation.

Bartold PM and Van Dyke TE [12] have highlighted the necessity to study the role of tissue-invasive pathogens in varied states of health and disease, to accurately understand the actual role of tissue-invasive pathogens in periodontal disease. The current study has been unique in that it studied the numbers of tissue-invasive bacterial phylotypes from sites that had varied levels of CAL—with sites being designated as healthy, mild-moderate and severe. All the patients selected for this preliminary study were patients who had been diagnosed with stage III/IV and grade C periodontitis with varying levels of CAL, who had received non-surgical periodontal therapy as part of the preliminary phase of periodontal therapy. As part of this preliminary study following the assessment of total bacterial phylotypes, we then assessed the levels of pro-inflammatory markers in the tissues, and thereafter correlated the tissue-invasive bacterial phylotypes with the mRNA levels of pro-inflammatory markers, in the tissues that were procured from these patient sites during periodontal surgery.

The results that arise from this study were quite remarkable, in two significant aspects. First and foremost, this study has highlighted that not only do sites with CAL harbor tissue-invasive bacteria, but also in sites which have not hitherto experienced CAL. We have understood from previous studies that the subgingival flora in the sulcus at sites with periodontal health, harbors bacteria that are known to be periodontopathic.[13] However, this study is the first to highlight the data that that tissue-invasive bacteria are also present within the tissue in periodontally healthy sites, and that non-surgical periodontal therapy is inadequate to eliminate these tissue-invasive bacteria. What is, however, more remarkable is the fact that these sites, following non-surgical therapy, have comparable levels of pro-inflammatory markers in the tissues.

This is probably a reflection of immune priming of the tissues in health, even as the tissues following non-surgical therapy in the sites with precedent CAL attempt to correct the dysregulation to the immune-inflammatory process, due to periodontal disease. Perhaps, significant to this immune priming that we see at the healthy sites, was the statistically significant correlation between IL-6 and the tissue-invasive phylotypes in the healthy sites.[13–16] This study has limitations as evidenced in the narrow pro-inflammatory marker profile that was evaluated in this study. The study could have also attempted to assess the intra-tissue bacterial and pro-inflammatory marker profile of patients who did not exhibit periodontal disease, so as to configure the role of chronic bacterial inflammation of the tissues in promoting the invasion of bacteria into the tissues, as well

in the upregulation of markers of inflammation. Further studies that attempt to throw greater light on these factors may be of great essence to our understanding of periodontitis, as well as the need to include techniques in the management of periodontal disease, that give importance to the management of the soft tissue walls that make up the walls of a periodontal pocket, so as to eliminate intra-tissue periodontal pathogens.

Conclusions

These results, while yet preliminary, need to be re-evaluated with a larger sample size, with attempts perhaps to identify specific periodontopathic bacterial species and to correlate their relationship to these pro-inflammatory markers. However, the intra-tissue course of the bacteria that make up the dental biofilm is at the least highly intriguing, as to their possible import in periodontal pathogenesis and periodontal therapy.

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