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Expression of NLRC4 Inflammasome and Its Correlation with *Treponema denticola* in Stage III/IV Periodontitis with Type II Diabetes Mellitus

Tip II Diabetes Mellituslu Evre III/IV Periodontitiste NLRC4 İnflamasom Ekspresyonu ve *Treponema denticola* ile Korelasyonu

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Abstract

Objective: To evaluate the expression of NLRC4 inflammasome and correlate with *Treponema denticola* (*T. denticola*) levels, so as to comprehend their role in mediating chronic inflammation in individuals with periodontitis (PD) and Type 2 Diabetes Mellitus (T2DM).

Materials and Methods: A total of fifty-one subjects were recruited and grouped as those systemically and periodontally healthy (PH, n=17), those systemically healthy with PD (PD, n=17), and those with T2DM with PD (PD+T2DM, n=17). Site specific probing pocket depth, clinical attachment level, plaque index, and gingival index were recorded. Thereafter, samples of subgingival plaque and gingival tissue taken with biopsy using an internal bevel incision were procured at sites evidencing the disease state. *T. denticola* was quantified using qPCR, and NLRC4 expression was evaluated with immunohistochemistry.

Results: Compared to the PH controls, significantly higher expression and intensity of NLRC4 inflammasome was observed in the PD and PD with T2DM groups, with a significantly greater expression in the PD+T2DM group ($p<0.05$). In all three groups, NLRC4 expression (mean percentage, intensity) and *T. denticola* levels showed a significant positive correlation ($p<0.05$).

Conclusion: The subgingival plaque, *T. denticola* levels in PD were significantly related to NLRC4 expression, both in the presence and absence of T2DM. NLRC4 activation possibly plays a role in establishing a hyperinflammatory state in diabetes mellitus modified PD.

Keywords: NLRC4 inflammasome, *Treponema denticola*, Type 2 diabetes mellitus, immunohistochemistry, qPCR, periplasmic flagella

Öz

Amaç: Bu çalışmada, periodontitis (PD) ve Tip 2 Diabetes Mellitus (T2DM) olan bireylerde, kronik enflamasyondaki rollerini anlamak amacıyla NLRC4 inflamasom ekspresyon ve *Treponema denticola (T. denticola)* seviyeleri ile korelasyonunun değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Toplam elli bir denek dahil edilen çalışma şu şekilde gruplandırılmıştır: Sistemik ve periodontal olarak sağlıklı (PH, n=17), sistemik olarak sağlıklı ancak PD'li (PD, n=17) ve Tip 2 DM ve periodontitisli (PD+T2DM, n=17). Bölgeye özgü sondalama cebi derinliği, klinik ataşman seviyesi, plak indeksi ve gingival indeksi kaydedildikten daha sonra, hastalık durumunu gösteren bölgelerden internal bevel insizyon kullanılarak subgingival plak ve gingival dokusu biyopsisi örnekleri alınmıştır. *T. denticola*, qPCR kullanılarak ve NLRC4 ekspresyonu ise, immünohistokimya methodu ile değerlendirilmiştir.

Bulgular: PH kontrolleriyle karşılaştırıldığında, PD-T2DM grubunda NLRC4 ekspresyonu daha anlamlı olmak üzere ($p<0.05$), PD ve PD+T2DM grubunda NLRC4 inflamasom yoğunluğu ve ekspresyonu anlamlı derecede yüksek saptandı. Her üç grupta da NLRC4 ekspresyonu (ortalama yüzde, yoğunluk) ve *T. denticola* seviyeleri anlamlı bir pozitif korelasyon gösterdi ($p<0.05$).

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Sonuç: Periodontitisteki subgingival plak, *T. denticola* seviyeleri, hem T2DM varlığında hem de yokluğunda NLRC4 ekspresyonu ile önemli ölçüde ilişkilidir. NLRC4 aktivasyonu muhtemelen diabetes mellitus ile modifiye periodontitiste hiperenflamatuvar bir durumun oluşmasında rol oynamaktadır.

Anahtar Kelimeler: NLRC4 enflamasom, *Treponema denticola*, Tip 2 diabetes mellitus, immünohistokimya, qPCR, periplazmik flagella

Introduction

Treponema denticola (*T. denticola*), a well-known periodontopathic bacteria, is a member of the family Spirochaetaceae. Periplasmic Flagella in spirochete species is essential for motility and adhesion (1). The injectosomes or type III secretion system (T3SS) delivers effector proteins from the cytoplasm of the bacterial cell to host cell mediating invasion and bacterial adhesion (1,2). This secretion system allows bacterial flagellin to enter the host cell activating inflammasomes via pattern recognition receptors (PRR) (3-5). These inflammasomes are multi-protein signaling platforms that activate the inflammatory cascade in response to pathogen invasion (6-8).

Inflammasome sensors are cytosolic PRRs that recognize intracellular bacterial components. The NLR, apoptosis inhibitory proteins (NAIPs) sense various intracellular bacterial ligands, specifically flagellin and T3SS components. Following ligand binding, NAIPs undergo conformational changes in their structure and become activated. The ligand-NAIP then activates a single NLRC4 monomer, which activates another monomer, resulting in oligomers. Caspase-1 is activated by the ligand-NAIP-NLRC4 inflammasome complex either in an ASC (the adaptor molecule apoptosis-associated speck-like protein containing a CARD) dependent or independent manner, releasing mature interleukin (IL)-1 and IL-18 (9,10).

Hyperglycemia promotes cellular senescence and the senescence associated secretory phenotype (SASP), which are key players in the process of inflammageing, that is linked to diabetic complications (11). Inflammasomes are associated with inflammageing and are activated by DAMPs such as hyperglycemia. Lately, Ebersole et al. (12), Song et al. (13), Furman et al. (14) and Mejias et al. (15), implicated NLRC4 in inflammageing. The activation of NLRC4 by hyperglycemia through IRF8 signaling, which results in the senescence of macrophage cells and the SASP phenotype in gingival tissues, was shown by Zhang et al. (16) in 2019. In addition, hyperglycemia increased the serum levels of SASP factors which further increased the load of senescent cells in gingival tissues. The crucial role of NLRC4 in establishing sterile inflammation and consequently diabetic complications has been highlighted in recent times. However, NLRC4 expression in diabetes modified periodontitis has not been studied till date.

Gram-negative bacteria stimulate the macrophages and turn on the NLRC4 inflammasome via functional bacterial T3SS/Type 4 Secretory System (T4SS) activating caspase-1 (17,18). Numerous studies have suggested that the presence of flagellated bacterial load influences NLRC4 expression favoring disease progression (4,19). Therefore, in this study, we hypothesized a heightened expression of NLRC4 along with a collective increase in the levels of *T. denticola*, in cases with greater destruction of the periodontium. Furthermore, a higher expression of NLRC4 in the DM cohort, as a part of the hyper-inflammatory trait, was also hypothesized.

The present cross-sectional analytical study sought to elucidate NLRC4 expression in gingival tissue samples and assess its relationship with *T. denticola* levels in T2DM with periodontitis.

Materials and Methods

Participants and Ethical Clearance

Fifty-one patients between the ages of 20 and 70 years were enlisted from the Outpatient Clinic of Periodontics at SRM Dental College and Hospital in Chennai. After obtaining written informed consent, they were categorized as follows: Periodontally healthy (PH, group I), systemically healthy with periodontitis (PD, group II), and Type 2 Diabetes Mellitus with Periodontitis (PD+T2DM, group III). The Institutional Scientific and Ethical Review Board approved the study proposal (SRMDC/IRB/2015/MDS/ no: 505).

Eligibility Criteria

Inclusion criteria for group I (PH) included having clinically healthy periodontium with no signs of gingival inflammation, ≤3 mm of probing pocket depth (PPD) with clinically no loss of attachment (CAL), more than 20 teeth present, radiographically no evidence of bone loss, and no previous history of periodontitis.

Inclusion criteria for group II (PD) included subjects with stage III/IV generalized periodontitis (according to 2017 EFP/AAP classification) (20) with clinical signs of gingival inflammation, gingival index >1, PPD ≥6 mm, CAL ≥5 mm with the evidence of bone loss on the radiograph in more than 30% of the teeth extending till the 1/2 or apical 1/3rd of the root.

Inclusion criteria for group III (PD+T2DM) included subjects with stage III/IV, grade B generalized periodontitis (T2DM under treatment with oral hypoglycemic agents for a minimum of one year and HbA1c values <7%) with clinical signs of gingival inflammation, gingival index >1, PPD ≥6 mm, CAL ≥5 mm, with the evidence of bone loss on the radiograph in more than 30% of the teeth extending till the 1/2 or apical 1/3rd of the root.

Pregnant and lactating women, smokers, individuals taking medications that could influence the periodontium, those suffering from medical problems and individuals who underwent periodontal therapy within the last six months were excluded. Patients with T2DM under insulin therapy and HbA1C level >7% were excluded in group III.

Clinical Parameters Recorded

Gingival index (21), plaque index (22), full mouth and tooth specific PPD and CAL (six sites on each tooth) were recorded.

Subgingival Plaque Collection

The deepest probing site was identified and then using a sterile curette (Hu-friedy), plaque samples were taken from the subgingival pockets and transferred to a sterile Eppendorf tube containing DNA lysis buffer solution. The samples were stored at a temperature of -20°C and later transported to the laboratory.

Gingival Tissue Collection

Tissue samples from the gingiva were collected for the PH group, either during crown lengthening procedure or during extraction of teeth for orthodontic purposes. For the PD and the PD+T2DM groups, gingival tissue samples were procured using an internal bevel incision starting from 1 mm apical to the crest of the marginal gingiva to the crest of the alveolar bone collected during extraction or just prior to subgingival debridement. The samples were placed in a sterile container with neutral buffered 10% formalin (pH 7.0) for fixation and later subjected to immunohistochemistry for NLRC4 inflammasome expression.

qPCR Analysis

The extraction and purification of plaque sample DNA was carried out as per manufacturer recommendation (cat#L6876, cat#NA2110, Sigma Aldrich, USA). To quantify *T. denticola*, PCR amplification of the 16S *rRNA* gene hypervariable regions V1 to V6 was carried out in 2.5 ng of total DNA with the following primers: Forward AGTTTGATCCTGGCTCAG and reverse TAGATACCCTGGTAGTCC. The PCR amplified products were quantified with Qubit fluorometer to find the concentration in each sample. All PCR amplicons were then diluted to obtain 3 ng concentration in all samples, which was used as a template in the next round of PCR with primers specific to *T. denticola*. The primers within the *16SrRNA* gene were identified and used for RT-PCR analysis: *T. denticola* forward primer TAATACCGAATGTGCTCATTTACAT, Reverse primer TCAAAGAAGCATTCCCTCTTCTTCTTA.

The SYBR green RT-master mix (cat#RR820A, Takara Clontech, Japan) was mixed with 10 μM of each of the aforementioned primers in a 20 μL reaction before samples were examined using rotor-gene Q real time PCR apparatus (Qiagen, Germany). The amplification of each sample was carried out for 25 cycles. Using six standards 106, 105, 104, 103, 102, and 101, the copy number of amplicons was determined in each sample and the quantification of bacteria was reported as copy numbers.

Immunohistochemistry for NLRC4

Gingival tissues were processed into paraffin embedded tissue blocks and then sectioned to approximately 3-4 μm using a microtome (Leica microsystem). The samples were fixed, sectioned, and immobilized on slides. Heat induced epitope retrieval (HIER) was performed on the paraffin embedded tissues to receive the primary antibody (CARD12/NLRC4, BT-AP07268, Bio Assay Technology) followed by the addition of secondary antibody and finally DAB chromogen was added to the slides. Hematoxylin was added for counter-staining, and the slides were finally air dried, cleared in xylene and cover slip mounted with DPX.

Expression of NLRC4

Each sample had a positive and negative control. The mounted sections were then viewed for NLRC4 positivity under a light microscope at 40x magnification for more representative areas. Positivity was based on the brown stain of diaminobenzidine. The total number of cells that stained and not stained were counted in each sample manually by two experienced oral pathologists using a grid from eight different fields at 40x magnification. The proportion of stained cells and the intensity of staining were assessed in each field.

Quantification

Each field was examined and the total cells were counted. Marker expression was analyzed by calculating the mean of eight fields for the respective sample. The percentage of stained cells in each field was calculated according to the method outlined by Tobón-Arroyave et al. (23) Percentage of stained cells was reported as follows: 0 indicated no positively stained cells; 1 indicated that <25% of cells were positively stained; 2 indicated that 25-50% cells were positively stained; 3 indicated that more than 50% of cells were positively stained.

In order to determine the staining-intensity-distribution score (SID score), the proportion score of the number of stained cells was multiplied by the intensity of staining. The degree of staining was assessed as follows: Negative staining (0), light staining (1), moderate staining (2), and severe staining (3). The mean of eight fields was calculated to obtain the SID score for each sample.

Statistical Analysis

Statistical Package for Social Science (SPSS) for Windows, version 17.0. (SPSS Inc, Released 2008, Chicago, USA) was used. The Kolmogorov-Smirnov test for normality revealed non-normal distribution of data. The Kruskal-Wallis test was used for comparison between the groups, and then the Mann-Whitney U test was applied for multiple pairwise comparisons. The correlations analysis was carried out using Kendall Tau-b correlation coefficient. The statistical significance was fixed at $p<0.05$.

Results

NLRC4 expression was found in all three groups' epithelium and connective tissue (Figure 1a-c). The expression of NLRC4 was higher in both periodontally diseased groups when compared to the PH group, with the highest expression observed in the PD+T2DM group. On intergroup comparison, a significant ($p<0.05$) statistical difference was observed between groups II, III, and group I (Table 1). The immunostaining intensity was lowest in the PH group, followed by a higher intensity in the PD group and the highest intensity in the PD+T2DM group (Table 1). The staining intensity differed significantly between the PH and both the periodontally diseased groups (II and III) ($p<0.05$) (Table 2). When compared to the PH group, the SID scores were similarly higher in the periodontally diseased groups, with a statistically significant difference ($p<0.05$) (Table 2). However, there was no significant difference in NLRC4 expression (mean%, staining intensity, and SID scores) between groups II and III ($p>0.05$) (Table 2).

When compared to the PH group, qPCR analysis of the subgingival plaque sample revealed higher levels of *T. denticola* in groups II and III. Compared to the PH group, the difference in microbe levels reached statistical significance ($p<0.05$), but no significant difference in microbe levels was observed between the periodontally diseased groups ($p=0.823$, $p=0.293$, $p=0.47$, $p=0.06$, $p=0.12$) (shown in Table 2). There was a significant and strong positive correlation between *T. denticola* levels and mean NLRC4 percentage and staining intensity in all three groups ($p<0.05$) (Table 3).

The scatter plot (Figure 2a-c) demonstrates the relationship between the independent variable *T. denticola* levels and the mean dependent variable NLRC4 %. Linear regression model for the line of best fit shows a positive relationship between the variables. The co-efficient of determination (R2) revealed that *T. denticola* level predicted 65 percent of the variance in NLRC4 expression in PH and PD, and 77 percent of the variance in group PD+T2DM.

Discussion

Innate immune responses against pathogens and danger-associated molecular patterns (PAMPs and DAMPs) are coordinated by the NLR family of cytosolic PRRs (24,25). Among the canonical inflammasomes, the most widely characterized and investigated in periodontal disease is NLRP3. The NLRC4 inflammasome has recently been associated with a number of chronic inflammatory diseases, but it has received little attention in the periodontium. It is activated by intracellular penetration of flagellin proteins or the inner rod like component of T3SS/T4SS of bacteria like Salmonella typhi and Pseudomonas aeruginosa (9,10).

Table 1. Descriptive statistics of clinical, microbiological (*T. denticola*) and immunological (NLRC4) parameters

Clinical parameters	Mean ± standard deviation			Kruskal-Wallis test
	PH	PD	(PD+T2DM)	p-value
Tooth specific PPD (mm)	2.53 ± 0.51	8.41 ± 1.69	7.59 ± 1.37	<0.01*
Tooth specific CAL (mm)	0.00 ± 0.00	7.76 ± 2.19	6.94 ± 2.07	<0.01*
Site-specific Pl	1.18 ± 0.52	1.59 ± 0.50	1.65 ± 0.49	<0.05*
Site-specific GI	-	1.76 ± 0.562	1.65 ± 0.493	<0.01*
***T. denticola* (copy numbers)**	911405.2 ± 2505967.8	6042806.6 ± 11988233.4	6356269.8 ± 8042771.3	<0.01*
NLRC4 mean %	9.27 ± 6.09	25.33 ± 12.28	29.20 ± 12.04	<0.01*
NLRC4 intensity	1.24 ± 0.43	1.76 ± 0.66	2.24 ± 0.75	<0.01*
SID score	1.35 ± 0.78	3.00 ± 2.20	3.94 ± 2.04	<0.01*

*p<0.05, statistically significant, PPD: Probing pocket depth, CAL: Clinical attachment level, PI: Plaque index, GI: Gingival index, SID score: Staining Intensity Distribution score, PH: Periodontally healthy, PD: Periodontitis, PD+T2DM: Periodontitis with type 2 DM

Additionally, elevated DAMPs in DM also independently upregulate NLRC4 expression (11).

Flagellated bacteria like *T. denticola* with Type III secretion system activate NLRC4 inflammasomes. Hence, the current study sought to correlate NLRC4 expression and subgingival levels of *T. denticola* in periodontitis with and without T2DM. We hypothesized that a potential increase of NLRC4 in diabetics would enable their further activation by an increase in the titre of *T. denticola*, resulting in the aggravated periodontal destruction seen in patients with T2DM.

Table 2. Intergroup pairwise comparisons of *Treponema denticola* and mean NLRC4 %, intensity, staining intensity distribution between the groups

Parameters	Pair	p-value
Treponema denticola	PH vs PD	**<0.01***
	PH vs PD+T2DM	**<0.01***
	PD vs PD+T2DM	0.823
Mean NLRC4 %	PH vs PD	**<0.01***
	PH vs PD+T2DM	**<0.01***
	PD vs PD+T2DM	0.293
NLRC4 intensity	PH vs PD	**0.047**
	PH vs PD+T2DM	**<0.01***
	PD vs PD+T2DM	0.063
Staining intensity distribution (SID)	PH vs PD	**0.007***
	PH vs PD+T2DM	**<0.01***
	PD vs PD+T2DM	0.122

*p<0.05, Statistically significant, PH: Periodontally healthy, PD: Periodontitis, PD+T2DM: Periodontitis with type 2 DM

In the present study, sites with periodontal tissue destruction demonstrated significantly higher *T. denticola* levels (PD & PD+T2DM) than the periodontally healthy (PH) sites. However, in the comparison of the *T. denticola* levels between the periodontitis groups II and III, there was no statistically significant difference between the *T. denticola* levels in these groups. A similar finding was reported by Zhou et al. (26) in 2013 wherein *T. denticola* levels in the subgingival samples showed no difference statistically in both the chronic PD and chronic PD+T2DM group. On the contrary, Li et al. (27), in 2012 reported that *T. denticola* levels were higher and at statistically significant levels in the subgingival samples from patients with chronic PD+T2DM when compared to generalized chronic PD.

The NLRC4 staining intensity distribution describes the level of inflammasome activation. The periodontally diseased groups (II and III) had a higher mean staining intensity distribution when compared to the PH group. Similarly, in their preliminary study, Parameswaran et al. (28) found a higher percentage of NLRC4 expression in the epithelium of diseased gingival tissue samples when compared to periodontally healthy sites. Rocha et al. (29), based on their research in a murine model of periodontal disease, suggested that NLRC4 prevented bone resorption and had a defensive function in an inflammatory environment. They demonstrated an increase in bone resorption in NLRC4/Card12/Ipaf knockout mice (Nlrc4-KO). Furthermore, an *in vitro* experiment showed that macrophage-derived

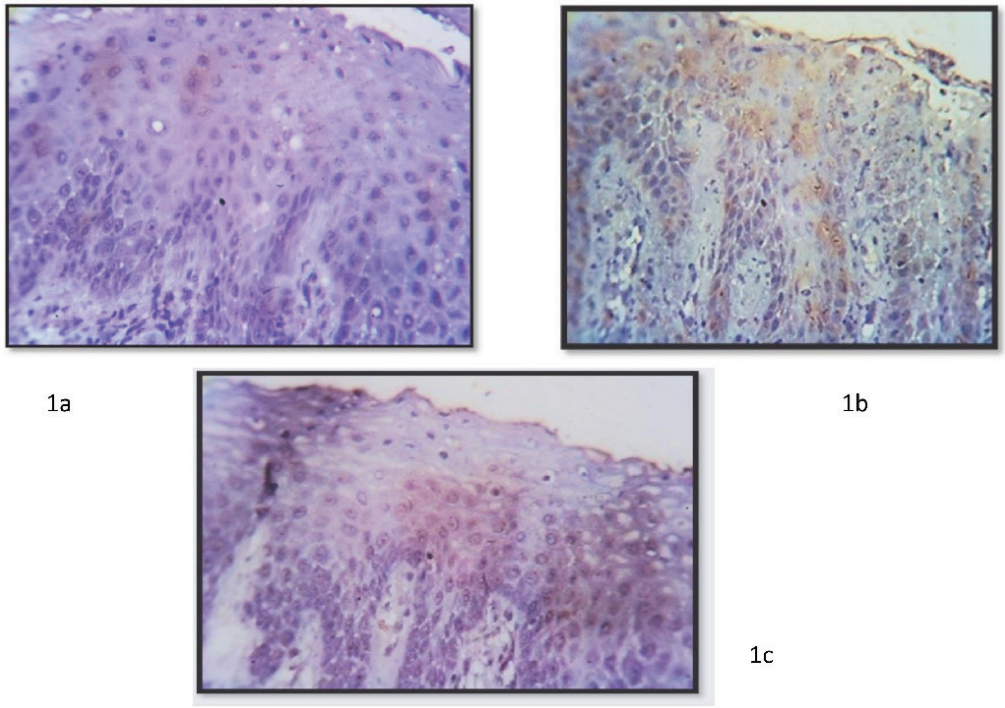

Figure 1a-c. Showing positive staining for NLRC4 in the epithelial layer in group I, II and III under 40x magnification

osteoclasts from the bone marrow of NLRC4-deficient mice exhibited increased bone resorbing characteristics and upregulated *MMP-9* gene expression (29).

An initial understanding that Type 3SS/T4SS is essential for NLRC4 inflammasome activation is gradually being modified in the light of the presence of "Sterile Inflammation", which highlights the possibility of NLRC4 receptor upregulation and activation by DAMPs in chronic diseases such as Diabetes Mellitus (16,30). This could imply that the diabetic state induces upregulated NLRC4 in the peripheral tissues, which could be activated both by the bacterial flagellum and by phosphorylation of Ser-533. These changes are perhaps consequential to the hyper-inflammatory state seen in diabetics.

In this study, there was no discernible difference in NLRC4 expression between the PD and PD+T2DM groups. This may be because diabetics under control produce fewer DAMPs and hence have reduced NLRC4 receptor expression/activation. Furthermore, based on diabetic mouse models and *in vitro* experiments by Zhang et al. (16), metformin, the most commonly used drug for T2DM, has the ability to reduce SASP and macrophage senescence by possibly dephosphorylating NLRC4 in the presence of high glucose concentrations.

Beausejour et al. (31) in 1997, demonstrated *in vitro* that *T. denticola* derived protease enzymes mediated the cleavage of IL-1β precursor with its subsequent activation. They further observed that the addition of protease inhibitors did not completely prevent activation, implying the involvement of other enzymes and mechanisms. With the breakthrough discovery of inflammasomes in 2002, the intracellular activation of IL-1β and IL-18

Table 3. Correlation between the levels of *Treponema denticola* and NLRC4 expression (mean % and intensity) for all 3 groups

Group	Microbial agent		NLRC4 Mean%	NLRC4 Intensity
Group I (PH)	*Treponema denticola*	Correlation Coefficient	0.803	0.605
		P-value	<0.01*	<0.01*
Group II (PD)		Correlation Coefficient	0.794	0.748
		P-value	<0.01*	<0.01*
Group III (PD+DM)		Correlation Coefficient	0.868	0.710
		P-value	<0.01*	<0.01*

*p<0.05, statistically significant, PH: Periodontally healthy, PD: Periodontitis, DM: Diabetes mellitus

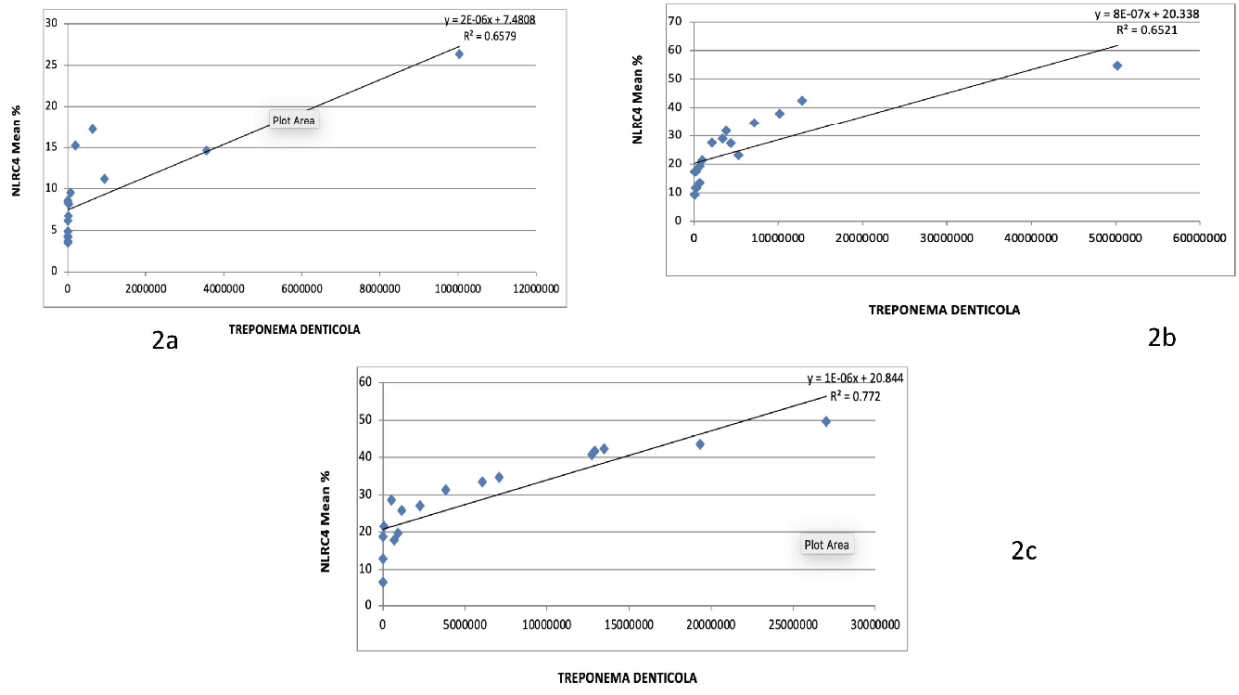

Figure 2a-c. Scatter plot showing correlation between *Treponema denticola* and mean NLRC4 % in group I, II and III

via caspases and their role in propagating inflammation was elucidated with NLRC4 inflammasomes being progressively deciphered as playing a vital role in its activation (6). The study highlights that higher titre of *T. denticola* in periodontal pockets is associated with higher levels of activated NLRC4 levels in chronic periodontitis with and without T2DM. Furthermore, NLRC4 expression on the cells lining the periodontal pocket probably contributes to the hyper-inflammatory trait observed in Diabetes Mellitus patients.

Future research that longitudinally monitors NLRC4 levels is needed to fully understand the regulatory mechanisms controlling the NLRC4 inflammasome activation. In addition, gaining new knowledge on the interaction of bacteria and NLRs may help create possible diagnostic and therapeutic approaches by shedding light on the immune-evading mechanisms used by bacteria to thwart human defence.

Conclusion

The present study showed a significantly higher expression of NLRC4 in periodontitis sites, in the presence and absence of T2DM, when compared to periodontal health, with a significant positive correlation with the Treponema denticola levels.

Ethics

Ethics Committee Approval: The Institutional Scientific and Ethical Review Board approved the study proposal (SRMDC/IRB/2015/MDS/no: 505).

Informed Consent: Informed consent from all patients were obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.P., D.A., D.V., S.V., V.K.N., Concept: S.P., D.A., D.V., S.V., V.K.N., Design: S.P., D.A., D.V., S.V., V.K.N., Data Collection or Processing: S.P., D.A., D.V., S.V., Analysis or Interpretation: S.P., D.A., D.V., S.V., Literature Search: S.P., D.A., D.V., Writing: S.P., D.A., D.V.

Conflict of Interest: No conflict of interest was declared by the authors.

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