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DIFFERENTIAL EXPRESSION OF INFLAMMASOME REGULATORY TRANSCIPTS IN PERIODONTAL DISEASE

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Running Title: Inflammasomes in Periodontal Disease

Summary Sentence: Inflammasome regulators may be dysregulated in periodontal disease.

ABSTRACT

Background: The inflammasome modulates the release of key pro-inflammatory cytokines associated with periodontal disease pathogenesis. The aim of this study was to evaluate the expression of proteins that regulate the inflammasome, namely Pyrin domain-only proteins (POPs), Caspase recruitment domain (CARD)-only proteins (COPs), and tripartite motif-containing (TRIM) proteins, in periodontal diseases.

Methods: A total of 68 participants (34 males and 34 females) were divided into 4 groups, including periodontal health (H), gingivitis (G), chronic periodontitis (CP) and aggressive periodontitis (AgP) based on clinical parameters. Gingival tissue samples were obtained from all participants for reverse transcription polymerase chain reaction (RT-PCR)-based gene expression analyses of molecules that regulate the inflammasome, including Apoptosis-associated speck-like protein containing a CARD (ASC), Caspase-1, Interleukin-1β (IL-1β), Interleukin-18 (IL-18), Nucleotide-binding domain, leucine rich family (NLR) Pyrin Domain Containing 3 (NLRP3), NLR family Pyrin Domain Containing 2 (NLRP2), (Absent in Melanoma 2) AIM2, POP1, POP2, CARD16, CARD18, TRIM16 and TRIM20 by (RT-PCR).

Results: NLRP3 and IL-1β were upregulated in the G, CP and AgP groups compared with group H ($p<0.05$). AIM2 was downregulated in the CP group compared with the H, G, and AgP groups ($p<0.05$). TRIM20, TRIM16 and CARD18 were downregulated in the G, CP and AgP groups compared with the H group ($p<0.05$). POP1 and POP2 were downregulated in the CP and AgP, and AgP and G groups, respectively ($p<0.05$).

Conclusion: Active periodontal disease may result in downregulation of inflammasome regulators that may increase the activity of NLRP3 and IL-1β in periodontal disease.

Keywords: Periodontal disease, inflammasomes, Pyrin Domain, Caspase Activation and Recruitment Domains, Tripartite Motif Proteins.

INTRODUCTION

Periodontal disease is a chronic inflammatory disease that is initiated by a microbial dysbiosis. In response, proinflammatory cytokines including Interleukin-1 (IL-1) family members, IL-1β and IL-18, are released by host tissues leading to periodontal tissue inflammation[1]. These molecules are produced as inactive precursors, pro-IL-1β and -IL-18, in response to pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs)[2]. DAMPs and PAMPs are detected by pattern recognition receptors (PRRs), including nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)[2]. The conversion of pro-IL-1β and pro-IL-18 into their biologically active forms[3] occurs due to the activation of the inflammasome complex, which consists of a NLR, an adaptor protein, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain CARD (ASC) and caspase-1[4].

The inflammasome complex is a signalling centre that regulates innate immune processes triggered in response to infections and foreign substances[5]. Several different types of inflammasomes have been identified, including NLRP2 (NLR family, pyrin domain containing 2), NLRP3 and AIM2 (Absent in melanoma 2)[6]. The inflammasome is regulated by different proteins and processes, including pyrin domain (PYD)-only proteins (POPs) and CARD-only proteins (COPs), tripartite motif family proteins (TRIMs), autophagy and interferons[7]. POP1 and POP2 inhibit inflammasome activation by interacting with ASC and NLRP3, and NLRP2 and AIM2, respectively [8]. COPs, including CARD16 and CARD18[9], are upregulated by TNF-α and lipopolysaccharide (LPS)[10] and inhibit the inflammasome through caspase-1 activity. TRIM20 and TRIM16 also inhibit inflammasome activation by interacting with both NLRPs and caspase-1[7].

The relationship between inflammasomes and periodontal disease has been evaluated in the literature. Notably, the gingival expression of NLRP2 and NLRP3 inflammasomes were increased in different types of periodontal disease, including Chronic Periodontitis (CP), Aggressive Periodontitis (AgP) and Gingivitis (G) compared to healthy controls [11]. In addition, AIM2 expression was significantly elevated in the gingiva of periodontitis patients [12]. Salivary NLRP3 levels were also significantly elevated in periodontitis patients compared to healthy controls[13]. Therefore, current data support a role for inflammasomes in the pathogenesis of periodontal disease. Mechanistically, dysregulation of the inflammasome may result in uncontrolled IL-1β production that, in turn, promotes periodontal tissue destruction. Thus, evaluation of inflammasome regulators and their differential expression in different types of periodontal disease may provide significant insight into the mechanisms involved in disease pathogenesis.

We hypothesize that periodontal disease may exhibit dysregulation of the inflammasome regulators POPs, COPs and TRIMs, which may increase NLRP3 inflammasome activity that, in turn, elevates IL-1β expression. Therefore, the aim of this study was to evaluate the expression of the inflammasome regulators, including POP1, POP2, CARD16, CARD18, TRIM16 and TRIM20 in different types of periodontal disease.

MATERIALS AND MEHTODS

Participants

A total of 68 participants (34 male and 34 female) between 18 to 43 years of age presented for periodontal treatment between July and October 2018 at Malatya Sehit Mehmet Kilinc Oral and Dental Health Hospital, Turkey. Ethics committee approval was obtained from the Ege University, Faculty of Medicine, Clinical Studies Ethics Committee (E.197492/320).

Permission was also obtained from the Republic of Turkey, Ministry of Health (92852811-771). Written informed consent was obtained from all participants.

Participants were divided into four groups; periodontally healthy participants (H) (n=10, 5 males and 5 females), untreated patients with gingivitis (G) (n=19, 10 females and 9 males), untreated patients with chronic periodontitis (CP) (n=19, 9 females and 10 males) and untreated patients with aggressive periodontitis (AgP) (n=20, 10 females and 10 males).

Eligibility Criteria

Inclusion criteria for the H group included the absence of radiographic bone loss, no history of periodontal disease, having ≥20 teeth, and no sites with clinical attachment level (CAL) >1 mm or probing depth (PD) >3mm. Inclusion criteria for the G group included ≥20 teeth without alveolar bone loss, Gingival Index (GI)>2, and no PD >3mm. Inclusion criteria for the CP group included having ≥20 teeth, more than 6 sites in 2 different quadrants with PD ≥ 6 mm and CAL ≥5 mm, ≥ 50% radiographic alveolar bone loss, the presence of consistent amounts of plaque biofilm/calculus deposits commensurate with the severity of periodontal tissue breakdown. Inclusion criteria for the AgP group included having ≥16 teeth, 8 or more teeth with PD ≥6 mm and CAL ≥5 mm, a pattern of severe tissue destruction characterized by interproximal attachment loss affecting ≥3 permanent teeth other than first molars and incisors (bone loss ≥30% of root length) and relatively low levels of plaque biofilm and calculus in affected patients. Generalized AgP patients were otherwise clinically healthy but had familial aggregation without previous history of treated periodontitis.

Exclusion criteria for all groups were as follows: systemic or immune-system related disease, use of immunosuppressants, use of prophylactic antibiotics prior to treatment, use of a regular medicament affecting periodontal health, smoking or previous smoker within 5 years, pregnancy or lactating, and periodontal treatment within the last 6 months [19].

Clinical Parameters

All participants underwent a periodontal examination, including documentation of their plaque (PI)[14] and gingival index (GI)[15], probing depth (PD), clinical attachment level (CAL), and bleeding on probing (BOP) recorded from six sites per tooth (mesiobuccal, midbuccal, distobuccal, distolingual, midlingual, and mesiolingual) using a periodontal probe*. The BOP percentage (%) was determined by dividing the number of bleeding sites by the total number of sites examined [16].

Radiographic Parameters

A radiographic examination was also performed for all participants to confirm the presence or absence of periodontal disease. The radiographic and clinical criteria proposed by the International Workshop for the Classification of Periodontal Diseases and Conditions in 1999 were used for diagnosis[17].

Gingival Tissue Sampling

Gingival tissue samples were collected from the proximal sites of two neighboring teeth during gingivectomy, flap surgery or crown lengthening procedures for aesthetic purposes. Tissue samples were transferred to sterile tubes containing RNA stabilization solution† and stored at -80°C [11, 18]. Four measurements (mesiobuccal, distobuccal, mesiolingual, and distolingual) were obtained for the two adjacent teeth. Healthy gingival tissues were obtained from sites with PI <1, GI <1, and PD ≤ 3mm without BOP. For group G, samples were obtained from the most inflamed site (GI ≥2, %BOP=100). For groups AgP and CP, biopsies were selected from the areas exhibiting the greatest inflammation with the deepest sites; PD ≥7mm with a CAL ≥ 6mm, %BOP=100. To standardize for comparisons, all samples were obtained from single-rooted teeth.

RT-PCR Analysis

mRNA was isolated from tissue samples using an extraction kit[‡], according to the manufacturer's instructions. After spectrophotometric quantification of the total RNA, 20 µL were denatured and reverse transcribed to cDNA using a cDNA synthesis kit[§]. Relative quantification of genes was performed using a reverse transcription polymerase chain reaction (RT-PCR) kit [ǀ]. Specific primers and probes for inflammasome-related transcripts [18] included ASC (Hs00203118), Caspase-1 (Hs00354836), IL-1β (Hs00174097), IL-18 (Hs01038787), NLRP3 (Hs00918082), NLRP2 (Hs01546938), AIM2 (Hs00915710), POP1 (Hs00415401), POP2 (Hs03037798), CARD16 (Hs00430993), CARD18 (Hs01043258), TRIM16 (Hs00414879) and TRIM20 (Hs00165145), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905) used as the housekeeping control. An RT- PCR system[#] was used for the detection and analysis of the PCR products. CT values were obtained for all genes. To assess differences, the fold-change in expression of the target mRNA was normalized to that of the housekeeping control mRNA using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

The sample size required for the study was calculated using a software**. It was calculated based on a previous [11] study in the literature. It was estimated that 17 patients for each group (total of 68 individuals) would be adequate to find a significant difference in NLRP3 levels (0.45 effect size at an $\alpha = 0.05$ significance level, actual power = 0.8694, non-centrality parameter = 13.77, critical F = 2.75, numerator df = 3, dominator df = 64). Data were expressed as mean ± standard deviation. The data exhibited a normal distribution as determined by the Kolmogorov-Smirnov test. Comparisons of normally distributed data were analysed using both parametric (ANOVA and Tukey's multiple comparison) and non-parametric (Kruskal-Wallis)

statistical tests. Statistical analyses were conducted using a statistical package program [††]. A p value < 0.05 was considered statistically significant.

RESULTS

Participants

No differences were found between the study groups when evaluated by gender ($p>0.05$). Age was significantly higher in the CP group (37.05±2.99) compared to the groups H (29.60±4.16), G (30.84±4.57) and AgP (28.95±2.96) ($p<0.005$).

Clinical Parameters

PI scores ($p<0.005$) and GI scores ($p<0.05$) in the H group were significantly lower compared with the periodontal disease groups (G, AgP and CP). GI values in the G group were also lower compared with the CP and AgP groups ($p<0.05$). The PD values were similar between groups H and G but lower compared with the CP and AgP groups ($p<0.005$) (Table 1). The %BOP was significantly lower in the H group, compared with all periodontal disease groups (CP, AgP and G) ($p<0.005$). %BOP was also significantly higher in the CP and AgP group compared with the G group ($p<0.005$). The CAL results were similar between the H and G groups but lower compared with the CP and AgP groups ($p<0.001$) (Table 1).

Comparisons were also performed between sampling sites of the study groups for clinical parameters (Table 2). Healthy groups showed significantly lower PI scores ($p<0.005$) and GI scores ($p<0.001$) in the H compared with the periodontal disease groups (G, AgP and CP). Periodontitis groups (CP and AgP) showed significantly higher PD ($p<0.001$) and CAL values ($p<0.001$) compared with Groups H and G. All periodontal disease groups (CP, AgP and G) showed significantly higher %BOP compared with group H ($p<0.001$) (Table 2).

When comparing the evaluated genes with the clinical parameters, a significant positive correlation emerged between NLRP3 levels and sampling site PI (r=0.482; P=0.000), GI (r=0.833; P=0.000), PD (r=0.647; P=0.000) and CAL (r=0.538; P=0.000) in all participants (Table 3). NLRP2 levels were significantly and positively correlated with sampling site of PI (r=0.274; P = 0.024), GI (r=0.809; P=0.000), PD (r=0.445; P=0.000) and CAL (r=0.464; P=0.000) and BOP (r=0.311; P=0.010) in all participants. CASP1 and PD (r=0.450; P=0.000), GI (r=0.283; P=0.036) and PI (r=0.297; P=0.040) levels were positively correlated in all participants. IL-1β levels were positively correlated with all clinical parameters (PI: r=0.466; p=0.000)(GI: r=0.823; p=0.000)(PD: r=0.450; p=0.000)(BOP: r=0.451 ;p=0.000) (CAL: r=0.322 ;p=0.007) in all participants. IL-18 was significantly and positively correlated with PI (r=0.293; p=0.045) and GI (r=0.498; p=0.000) in all participants. POP2 was significantly and negatively correlated only with PD (r=-0.537; p=0.000) and CAL (r=-0.686; p=0.000) in all participants. CARD16 were negatively correlated with GI (r=-0.760; p=0.000), PD (r=-0.501; p=0.000), BOP (r=-0.270; p=0.032) and CAL (r=-0.414; p=0.001) in all groups. CARD18 levels were only negatively correlated with PD (r=-0.432; p=0.035) in all groups. TRIM16 were negatively correlated with PI (r=-0.346; p=0.021), PD (r=-0.315; p=0.038), BOP (r=-0.298; p=0.005) in all participants. TRIM20 levels were also negatively correlated with PI (r=-0.525; p=0.001) and GI (r=-0.818; p=0.000), PD (r=-0.696; p=0.000) and CAL (r=-0.588 ;p=0.000) in all participants (Table 3).

A correlation analysis between IL-1β and inflammasome-associated genes were also performed. Results showed that IL-1β levels were positively correlated with NLRP3 (r=0.810; P=0.000), NLRP2 (r=0.904; P=0.000), CASP1 (r=0.387; P=0.004) and IL-18 (r=0.363; P=0.008) levels; and negatively correlated with CARD16 (r=-0.881; P=0.000) and TRIM20 (r=0.912; P=0.000) levels in all participants (Table 4).

Radiographic Parameters

In H and G groups there was no alveolar bone loss (BL) (distance between CEJ and bone crest ≤3 mm at > 95% of proximal tooth sites) detected radiographically. CP patients were found to have ≥50% radiographic BL in at least two quadrants, which was proportional to the level of plaque present. AgP patients exhibited a destructive vertical BL pattern, which was not commensurate with the plaque present. In addition, in these patients BL was ≥30% of the root length and affected at least 3 permanent teeth besides first molars and incisors.

RT-PCR Analysis

NLRP3, NLRP2 and IL-1β expression levels were elevated in all periodontally diseased groups (G, CP, and AgP) compared with the periodontally healthy group (Group H) ($p<0.05$) (Fig. 1A, 1B and F). However, the expression levels of Caspase-1, ASC and IL-18 were similar between groups ($p>0.05$) (Fig. 1D, 1E and G). AIM2 expression was decreased in the CP group compared with the H, G, and AgP groups ($p<0.005$) (Fig. 1C).

POP1 levels were downregulated in the CP and AgP groups ($p<0.05$) compared with the G and H groups ($p<0.05$) (Fig.1H). The expression of POP2 was also detected at lower levels in the G and AgP groups compared with the H and CP groups ($p<0.005$) (Fig 1I).

CARD16 was not differentially expressed between groups, although a downregulation trend was detected in the periodontal disease groups ($p>0.05$) (Fig.1J). CARD18 expression was detected at lower levels in the G and CP groups ($p<0.005$), and in the AgP group ($p<0.05$) compared with healthy controls (Fig.1K).

TRIM20 and TRIM16 were significantly downregulated in the CP, AgP ($p<0.001$), and G groups ($p<0.01$) compared with the H Group (Fig. 1L and M).

DISCUSSION

IL-1β is involved in the pathogenesis of periodontal disease and its expression is regulated by the inflammasome complex, a signalling hub, which controls the host response to infections and foreign substances [11, 12, 18]. Dysregulation of the inflammasome may, therefore, lead to uncontrolled IL-1β production and periodontal disease pathogenesis. In the current study, the periodontally diseased groups (G, CP, and AgP) expressed increased levels of IL-1β and NLRP3 compared with the periodontally healthy group (H). In addition, a significant downregulation in POP1, POP2, CARD16, TRIM16 and TRIM20 was detected in the G, CP, and AgP groups compared with the H group. These data suggest that during periodontal disease, inflammasome regulator activity is diminished, which subsequently leads to increased levels and activity of NLRP3 and IL-1β.

NLRP3 inflammasome expression in periodontal disease has been evaluated both *in vitro* and in relation to clinical parameters [11, 13, 18-20]. Periodontal pathogens, including *P. gingivalis* [18], *Aggregatibacter actinomycetemcomitans* (*A.actinomycetemcomitans)* [21, 22] and *F.nucleatum* [23] can promote IL-1β expression by overactivation of the NLRP3 inflammasome *in vitro*. Clinical data supports these findings. Indeed, Isaza-Guzman et al.[11,13,18-20] reported an increase of IL-1β and NLRP3 levels in the saliva of periodontitis patients compared with healthy controls. Park et al.[7], Xue et al.[19] and Cheng et al.[20] found an overexpression of NLRP3 in the gingiva of periodontitis patients compared with healthy participants. Furthermore, Bostanci et al.[11] also detected a significant overexpression of inflammasome genes in the gingiva of periodontitis and gingivitis patients compared with the gingiva of healthy patients. Notably, there were differences between the periodontal disease groups. Consistently, the current study also found an overexpression of NLRP3 in both gingivitis and periodontitis groups compared with the healthy group ($p<0.05$) and no differences between periodontal disease groups ($p>0.05$) (Fig. 1A). A significant positive correlation was also detected between NLRP3 and periodontal parameters, including PI, GI, PD and CAL ($p=0.000$)(Table 3) and IL-

1β expression. In aggregate, these data indicate that NLRP3 plays a significant role during periodontal disease pathogenesis.

Similarly, the NLRP2 inflammasome was also evaluated in periodontal disease. *In vitro* studies reported conflicting results with regards to the activation of the NLRP2 inflammasome by periodontal pathogens. Some authors reported a downregulation in response to *P. gingivalis,* [11] whereas others did not find any effect after infection with *P. gingivalis* [24], or with supragingival and subgingival biofilms [25] or with *A. actinomycetemcomitans* [26] in several different cell types. Bostanci et al.[11] reported a significant overexpression in NLPR2 levels in the gingival tissues of AgP and G patients compared with periodontally healthy participants. Data from the current study is, therefore, consistent with the literature, as an increase in NLRP2 levels was detected in all periodontally diseased groups (Group G, AgP and CP)($p<0.05$). However, no differences were found between any of the disease groups ($p>0.05$) (Fig.1B). Thus, similar to NLRP3, the NLRP2 inflammasome may also be associated with tissue breakdown in the pathogenesis of periodontal disease.

The relationship between the AIM2 inflammasome and periodontal disease was also explored in the literature. Although AIM2 expression was reported to be upregulated in response to both supragingival and subgingival biofilms[25], *P. gingivalis*[18] and *A.actinomycetemcomitans* [12], some authors detected no changes in AIM2 levels in response to infection with *A.actinomycetemcomitans* [26] or subgingival biofilms [27]. Clinical studies also revealed controversial results. Park et al. [18] found a significant overexpression in the gingiva of CP patients compared with healthy participants. However, Xue et al.[14] reported no differences between CP and AgP periodontitis and healthy groups. Conversely, in the current study, no upregulation in AIM2 was detected in any of the periodontally diseased groups. Furthermore, a downregulation in AIM2 was found in the CP patients compared with the AgP

and H groups (Fig.1C). Therefore, chronic periodontitis may be associated with a downregulation in AIM2 that may negatively affect periodontal tissues.

POPs are additional regulators of inflammasome activation. Two well-described members of this family are POP1 and POP2. POP1 is expressed mainly in monocytes and macrophages and it interacts with ASC and sequesters it from the NLRs, which subsequently inhibit inflammasome activity [8, 28]. The current study is the first to evaluate the role of POP1 in periodontal disease, and results show that POP1 was significantly downregulated in the CP and AgP groups compared with the H and G groups ($p<0.05$) (Fig.1H). These data suggest that the downregulation of POP1 in periodontitis groups may lead to increased activity of NLRP3 and IL-1β expression, which are associated with periodontal disease. Thus, the dysregulation of POP1 may only be in periodontitis and not gingivitis. POP2 is reportedly expressed in primary peripheral blood leukocytes and monocytic cells and induced by LPS and TNF-α [29]. Initially it was understood to bind to PRRs including NLRP3, NLRP2, AIM2 to prevent inflammasome activation [8, 28]. The role of POP2 in periodontal disease has also not been previously evaluated and our current findings now show a downregulation in POP2 in the AgP and G groups compared with the H and CP groups ($p<0.05$) (Fig.1I). These data indicate that both proteins may be negatively affected in periodontal disease. The differences in POP2 between CP and AgP patients may originate from the differences between these two diseases, which needs to be confirmed with further studies. In addition, the mechanism behind the role of POP1 and POP2 needs to be further elucidated. The incomplete determination of the role of these inflammasome mediators is a limitation of the current study.

COP family members include CARD16 (COP/Pseudo-ICE), CARD17 (INCA), and CARD18 (ICEBERG), Caspase-12s and Nod2-s, and these are also inflammasome regulators [8]. CARD16 and CARD18 are known to be upregulated by LPS and TNF-α in THP-1 monocytes [8]. However, to date no study has evaluated CARD16 and CARD18 in periodontal disease

clinically. The current study showed a significant downregulation in CARD18 in all periodontally diseased groups compared with the periodontally healthy group ($p<0.05$) (Fig.1K). A downregulation trend was also found for CARD16 (Fig.1J). Thus, the downregulation of CARD18 in periodontal disease may also contribute to increased expression of IL-1β. Thus, Both CARD16 and CARD18 may be dysregulated in all types of periodontal disease ranging from gingivitis to periodontitis. However, further studies are required to elucidate this mechanism in the context of periodontal disease pathogenesis. Other genes such as COPS, CARD17, Caspase-12s and Nod2-s were not be evaluated in the current study; this is a limitation of the current study.

TRIM family proteins play roles in different physiological processes, including cell proliferation, DNA repair, and inflammasome-mediated IL-1β responses [30]. TRIM20, one of the TRIM family members, supresses caspase-1 activation and IL-1β production by interacting with NLRP3, CASP1 and NLRP1 [31]. TRIM16, another TRIM family member, is a novel pro-IL-1β binding protein found in macrophages, and it increases IL-1β production by interacting with pro-caspase-1 and NLRP1 to enhance innate immunity [7]. Knockdown of TRIM16 causes a reduction in IL-1β secretion in keratinocytes, although other inflammasome proteins may be responsible for pro-IL-1β maturation and secretion in this cell type [32]. In the current study, TRIM16 and TRIM20 were downregulated in all periodontally diseased groups ($p<0.05$) (Fig.1L and M). A negative correlation was found between IL-1β and TRIM20 levels (Table 4). Thus, the overexpression of NLRP3 and IL-1β in the periodontally diseased groups may occur due to the downregulation of TRIM 16 and TRIM20; thereby implicating these TRIMs in periodontal disease pathogenesis without disease type differences.

The expression of caspase-1 as an inflammasome-related component in periodontal disease was also evaluated. Although an upregulation of caspase-1 in response to periodontal bacterial challenge [18,21,22,25,33] was reported, others noted no changes [27,34] or a decrease in its

levels [25] in *in vitro* studies. Clinical data has shown that caspase-1 was higher in the gingiva of CP patients compared with healthy controls [18,20,35], however similar caspase-1 levels were reported in the saliva of CP and AgP patients, and periodontally healthy groups [13]. The current study also identified no differences in caspase-1 levels between the CP, AgP and G groups compared with the H group, although a tendency toward increased levels were seen in the diseased groups ($p>0.05$)(Fig.1D).

Another inflammasome component, ASC, has been reported as upregulated [12,25], downregulated [25] and unaffected [18,27,34,36] in response to periodontal pathogens in different cell types. Clinical results have shown that ASC expression is higher in the saliva [13] and gingiva [12,35] of periodontally diseased patients. In contrast, in the current study no differences were detected in ASC levels between any of the disease and control groups ($p>0.05$) (Fig.1E).

Inflammasomes also control the release of IL-18. However, the data are variable in terms of the triggers that regulate its release. For example, supragingival biofilm [25] and *A.actinomycetemcomitans* [22,33] initiate its release, but its levels are decreased [25] or unaffected following subgingival biofilm exposure [36] in different cell types. In the clinical setting, IL-18 was significantly higher in the gingiva of CP, AgP and G patients compared with healthy subjects [11]. In the current study, no differences in IL-18 levels were detected in any of the periodontally diseased groups compared with the H group (Fig.1G) ($p>0.05$), however a positive correlation was found between IL-18 and IL-1β levels (Table 4).

The data from the current study has shown that NLRP3 and NLRP2 inflammasomes did not differ between periodontal disease groups, however a downregulation was found for AIM2 inflammasome levels only in CP patients. In addition, although some inflammasome regulators, including TRIMs and COPs were similar between periodontitis and gingivitis groups, POP1 levels were differentially expressed between periodontitis and gingivitis patients

and POP2 levels were only dysregulated in AgP and G groups. Thus, inflammasomes and inflammasome regulator molecules may be dysregulated in different types of periodontal disease.

CONCLUSION

Inflammatory periodontal disease may cause a reduction in inflammasome regulators, including POPs, TRIMs and some COPs, which in turn promote the increased expression and activity of NLRP3 and IL-1β that trigger periodontal tissue breakdown.

FOOTNOTES

* Williams Periodontal Probe, Hu-Friedy, Chicago, USA

† RNAlater, Thermo Fisher, Waltham, USA

‡ RNeasy Plus Micro and Mini kits, Qiagen, Hilden, Germany

§ SuperScript III RT, Invitrogen, Carlsbad, USA

ǁ Quantitect, Qiagen, Valencia, USA

¶ Taqman Gene Expression Assay Probes, Thermo Fisher Scientific, Waltham, USA

ABI PRISM 7000, Applied Biosystems, Foster City, USA

** G*Power version 3.1.7 software, Franz Faul, Christian-Albrechts-University, Kiel, Germany

†† SPSS 20.0, IBM, Chicago, USA

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REFERENCES

1. Kinane DF, Lappin DF. Immune processes in periodontal disease: a review. *Ann Periodontol* 2002;7:62-71.
2. Bauernfeind F, Ablasser A, Bartok E, et al. Inflammasomes: current understanding and open questions. *Cell Mol Life Sci* 2011;68:765-783.
3. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 2002;10:417-426.
4. Schroder K, Tschopp J. The inflammasomes. *Cell* 2010;140:821-832.
5. Lamkanfi M, Dixit VM. In Retrospect: The inflammasome turns 15. *Nature* 2017;548:534-535.
6. Indramohan M, Stehlik C, Dorfleutner A. COPs and POPs Patrol Inflammasome Activation. *J Mol Biol* 2018;430:153-173.
7. Rathinam VA, Vanaja SK, Fitzgerald KA. Regulation of inflammasome signaling. *Nat Immunol* 2012;13:333-342.
8. Le HT, Harton JA. Pyrin- and CARD-only Proteins as Regulators of NLR Functions. *Front Immunol* 2013;4:275.
9. Kersse K, Vanden Berghe T, Lamkanfi M, Vandenabeele P. A phylogenetic and functional overview of inflammatory caspases and caspase-1-related CARD-only proteins. *Biochem Soc Trans* 2007;35:1508-1511.
10. Druilhe A, Srinivasula SM, Razmara M, Ahmad M, Alnemri ES. Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. *Cell Death Differ* 2001;8:649-657.
11. Bostanci N, Emingil G, Saygan B, et al. Expression and regulation of the NALP3 inflammasome complex in periodontal diseases. *Clin Exp Immunol* 2009;157:415-422.

12. Kim S, Park MH, Song YR, Na HS, Chung J. Aggregatibacter actinomycetemcomitans-Induced AIM2 Inflammasome Activation Is Suppressed by Xylitol in Differentiated THP-1 Macrophages. *J Periodontol* 2016;87:e116-126.

13. Isaza-Guzman DM, Medina-Piedrahita VM, Gutierrez-Henao C, Tobon-Arroyave SI. Salivary Levels of NLRP3 Inflammasome-Related Proteins as Potential Biomarkers of Periodontal Clinical Status. *J Periodontol* 2017;88:1329-1338.

14. Silness J, Loe H. Periodontal Disease in Pregnancy. Ii. Correlation between Oral Hygiene and Periodontal Condtion. *Acta Odontol Scand* 1964;22:121-135.

15. Loe H. The Gingival Index, the Plaque Index and the Retention Index Systems. *J Periodontol* 1967;38:Suppl:610-616.

16. Lang NP, Joss A, Orsanic T, Gusberti FA, Siegrist BE. Bleeding on probing. A predictor for the progression of periodontal disease? *J Clin Periodontol* 1986;13:590-596.

17. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* 1999;4:1-6.

18. Park E, Na HS, Song YR, Shin SY, Kim YM, Chung J. Activation of NLRP3 and AIM2 inflammasomes by Porphyromonas gingivalis infection. *Infect Immun* 2014;82:112-123.

19. Xue F, Shu R, Xie Y. The expression of NLRP3, NLRP1 and AIM2 in the gingival tissue of periodontitis patients: RT-PCR study and immunohistochemistry. *Arch Oral Biol* 2015;60:948-958.

20. Cheng R, Liu W, Zhang R, Feng Y, Bhowmick NA, Hu T. Porphyromonas gingivalis-Derived Lipopolysaccharide Combines Hypoxia to Induce Caspase-1 Activation in Periodontitis. *Front Cell Infect Microbiol* 2017;7:474.

21. Yamaguchi Y, Kurita-Ochiai T, Kobayashi R, Suzuki T, Ando T. Activation of the NLRP3 inflammasome in Porphyromonas gingivalis-accelerated atherosclerosis. *Pathog Dis* 2015;73.

22. Shenker BJ, Ojcius DM, Walker LP, Zekavat A, Scuron MD, Boesze-Battaglia K. Aggregatibacter actinomycetemcomitans cytolethal distending toxin activates the NLRP3 inflammasome in human macrophages, leading to the release of proinflammatory cytokines. *Infect Immun* 2015;83:1487-1496.

23. Bui FQ, Johnson L, Roberts J, et al. Fusobacterium nucleatum infection of gingival epithelial cells leads to NLRP3 inflammasome-dependent secretion of IL-1beta and the danger signals ASC and HMGB1. *Cell Microbiol* 2016;18:970-981.

24. Champaiboon C, Poolgesorn M, Wisitrasameewong W, Sa-Ard-Iam N, Rerkyen P, Mahanonda R. Differential inflammasome activation by Porphyromonas gingivalis and cholesterol crystals in human macrophages and coronary artery endothelial cells. *Atherosclerosis* 2014;235:38-44.

25. Bostanci N, Meier A, Guggenheim B, Belibasakis GN. Regulation of NLRP3 and AIM2 inflammasome gene expression levels in gingival fibroblasts by oral biofilms. *Cell Immunol* 2011;270:88-93.

26. Belibasakis GN, Johansson A. Aggregatibacter actinomycetemcomitans targets NLRP3 and NLRP6 inflammasome expression in human mononuclear leukocytes. *Cytokine* 2012;59:124-130.

27. Belibasakis GN, Guggenheim B, Bostanci N. Down-regulation of NLRP3 inflammasome in gingival fibroblasts by subgingival biofilms: involvement of Porphyromonas gingivalis. *Innate Immun* 2013;19:3-9.

28. Stehlik C, Krajewska M, Welsh K, Krajewski S, Godzik A, Reed JC. The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and pro-caspase-1 regulation. *Biochem J* 2003;373:101-113.

29. Atianand MK, Harton JA. Uncoupling of Pyrin-only protein 2 (POP2)-mediated dual regulation of NF-kappaB and the inflammasome. *J Biol Chem* 2011;286:40536-40547.

30. Ozato K, Shin DM, Chang TH, Morse HC, 3rd. TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 2008;8:849-860.

31. Kimura T, Jain A, Choi SW, Mandell MA, Johansen T, Deretic V. TRIM-directed selective autophagy regulates immune activation. *Autophagy* 2017;13:989-990.

32. Munding C, Keller M, Niklaus G, et al. The estrogen-responsive B box protein: a novel enhancer of interleukin-1beta secretion. *Cell Death Differ* 2006;13:1938-1949.

33. Zhao P, Liu J, Pan C, Pan Y. NLRP3 inflammasome is required for apoptosis of Aggregatibacter actinomycetemcomitans-infected human osteoblastic MG63 cells. *Acta Histochem* 2014;116:1119-1124.

34. Guo W, Wang P, Liu Z, Yang P, Ye P. The activation of pyrin domain-containing-3 inflammasome depends on lipopolysaccharide from Porphyromonas gingivalis and extracellular adenosine triphosphate in cultured oral epithelial cells. *BMC Oral Health* 2015;15:133.

35. Garcia-Hernandez AL, Munoz-Saavedra AE, Gonzalez-Alva P, et al. Upregulation of proteins of the NLRP3 inflammasome in patients with periodontitis and uncontrolled type 2 diabetes. *Oral Dis* 2019;25:596-608.

36. Guo W, Ye P, Yu H, Liu Z, Yang P, Hunter N. CD24 activates the NLRP3 inflammasome through c-Src kinase activity in a model of the lining epithelium of inflamed periodontal tissues. *Immun Inflamm Dis* 2014;2:239-253.

FIGURE LEGENDS

Figure 1. Inflammasome regulator expression levels in the clinical groups.

(A and B) NLRP3 and NLRP2 relative transcript levels in the AgP, G and CP groups compared with the H Group*$p<0.05$. (C) AIM2 was significantly lower in the CP group compared with the AgP, H and G groups *,$^{\theta}$,$^{\delta}$ $p<0.005$. (D and E) Similar levels of expression were found in the clinical groups for the caspase-1 (CASP1) and ASC transcripts. (F) IL-1β transcript levels were upregulated in groups G, CP and AgP compared with group H. *$p<0.05$ (G) No differences were found for IL-18 transcript levels between groups. (H) POP1 was downregulated in CP and AgP groups compared with the G and H groups *,$^{\theta}$ $p<0.05$. (I) POP2 expression was decreased in groups AgP and G compared with groups CP and H *,$^{\theta}$ $p<0.005$. (J) CARD16 expression was similar between groups $p>0.05$. (K) CARD18 downregulated in CP, G and AgP groups compared to the group H *$p<0.05$, $^{\theta}$ $p<0.005$. (L and M) TRIM16 and TRIM20 were downregulated in groups CP, AgP and G compared with Group H. *$p<0.001$, $^{\theta}$ $p<0.01$.

TABLES

Table 1. Periodontal Clinical Findings in the Study Groups

	Group H	**Group G**	**Group CP**	**Group AgP**
PI	0,05 ± 0,10*	1,77±0,46	2,36±0,29$^{\#}$	1,91±0,36
GI	0,00 ± 0,00$^{\theta}$	2,00±0,32$^{\Phi}$	2,44±0,35	2,38±0,27
PD (mm)	1,52 ± 0,34**	2,25±0,54**	6,77±0,45	7,0±0,48
BOP (%)	0,00 ± 0,00$^{\#\#}$	69,74±21,37$^{\theta\theta}$	98,68±5,73	97,50±76,95
CAL (mm)	0,00 ± 0,00$^{\Phi\Phi}$	0,00 ± 0,00$^{\Phi\Phi}$	7,61±1,31	7,96±1,32

Data was shown as mean ± standard deviation. $^{*, \#, \theta, \Phi}$ p<0,05 $^{**, \#\#, \theta\theta}$ p<0,005 $^{\Phi\Phi}$ p<0,001

* Compared with groups G, CP and AgP

$^{\#}$ Compared with group G and AgP

$^{\theta}$ Compared with groups G, CP and AgP

$^{\Phi}$ Compared with group CP and AgP

** Compared with group CP and AgP

$^{\#\#}$ Compared with groups G, CP and AgP

$^{\theta\theta}$ Compared with groups CP and AgP

$^{\Phi\Phi}$ Compared with groups CP and AgP

Table 2. Sampled site clinical parameters for the study population

	Group H	Group G	Group CP	Group AgP
PI	0,00 ± 0,00*****	2,34±0,23	2,56±0,27	2,08±0,38$^{\#}$
GI	0,00 ± 0,00$^{\theta}$	2,69±0,22	2,63±0,29	2,61±0,28
PD (mm)	1,50 ± 0,33$^{\Phi}$	2,36±0,35**	8,07±0,61	8,15±0,67
BOP (%)	0,00 ± 0,00$^{\#\#}$	100±0,00	100±0,00	100±0,00
CAL (mm)	0,00 ± 0,00$^{\theta\theta}$	0,00 ± 0,00$^{\Phi\Phi}$	7,61±1,92	8,05±1,04

Data shown as mean ± standard deviation, $^{\#}p<0,05$ $*p<0,005$ $^{\theta,\Phi,**,\#\#,\Phi\Phi}p<0,001$

* Compared with groups G, CP and AgP

$^{\#}$ Compared with group CP

$^{\theta}$ Compared with groups G, CP and AgP

$^{\Phi}$ Compared with groups CP and AgP

** Compared with groups CP and AgP

$^{\#\#}$ Compared with groups G, CP and AgP

$^{\theta\theta}$ Compared with groups CP and AgP

$^{\Phi\Phi}$ Compared with groups CP and AgP

Table 3. Correlation of inflammasome-associated genes with clinical parameters

	Healthy Group		Disease Groups		All participants	
Parameters	rho	P	rho	P	rho	P
PI and NLRP3	-	-	0,267	0,043*	0,482	0,000*
PI and NLRP2	-	-	0,110	0,410	0,274	0,024*
PI and AIM2	-	-	-0,156	0,330	-0,219	0,135
PI and CASP1	-	-	0,297	0,040*	0,331	0,014*
PI and ASC	-	-	-0,010	0,948	-0,003	0,983
PI and IL-1β	-	-	0,254	0,055*	0,466	0,000*
PI and IL-18	-	-	0,293	0,045*	0,152	0,276
PI and POP1	-	-	-0,060	0,704	-0,100	0,488
PI and POP2	-	-	-0,224	0,188	-0,191	0,221
PI and CARD16	-	-	-0,083	0,544*	-0,211	0,098
PI and CARD18	-	-	-0,128	0,612	-0,262	0,217
PI and TRIM16	-	-	-0,243	0,147	-0,346	0,021*
PI and TRIM20	-	-	-0,103	0,618	-0,525	0,001*
GI and NLRP3	-	-	0,788	0,000*	0,833	0,000*
GI and NLRP2	-	-	0,809	0,000*	0,760	0,000*
GI and AIM2	-	-	0,226	0,122	-0,115	0,438
GI and CASP1	-	-	0,226	0,122	0,283	0,036*
GI and ASC	-	-	0,082	0,590	0,071	0,615
GI and IL-1β	-	-	0,813	0,000*	0,823	0,000*
GI and IL-18	-	-	0,498	0,000*	0,317	0,021*
GI and POP1	-	-	-0,023	0,886	-0,064	0,659
GI and POP2	-	-	0,130	0,450	0,043	0,785
GI and CARD16	-	-	-0,857	0,000*	-0,760	0,000*
GI and CARD18	-	-	0,118	0,642	-0,151	0,481
GI and TRIM16	-	-	0,041	0,811	-0,169	0,272
GI and TRIM20	-	-	-0,819	0,000*	-0,818	0,000*
PD and NLRP3	0,952	0,000*	0,512	0,000*	0,647	0,000*
PD and NLRP2	0,946	0,000*	0,445	0,000*	0,522	0,000*
PD and AIM2	-0,210	0,652	0,366	0,019*	0,154	0,297
PD and CASP1	0,397	0,379	-0,107	0,468	0,450	0,000*
PD and ASC	-0,238	0,607	-0,013	0,931	-0,002	0,989
PD and IL-1β	0,946	0,000*	0,235	0,076	0,450	0,000*
PD and IL-18	0,939	0,005*	0,203	0,172	0,101	0,472
PD and POP1	-0,032	0,940	0,019	0,903	-0,038	0,792
PD and POP2	0,627	0,131	-0,761	0,000*	-0,537	0,000*
PD and CARD16	-0,816	0,025*	-0,473	0,000*	-0,501	0,000*
PD and CARD18	-0,125	0,813	-0,487	0,040*	-0,432	0,035*

PD and TRIM16	-0,302	0,510	-0,180	0,287	-0,315	0,038*
PD and TRIM20	-0,942	0,000*	-0,441	0,024*	-0,696	0,000*
BOP and NLRP3	-	-	-	-	0,495	0,000*
BOP and NLRP2	-	-	-	-	0,311	0,010*
BOP and AIM2	-	-	-	-	-0,173	0,240
BOP and CASP1	-	-	-	-	0,187	0,171
BOP and ASC	-	-	-	-	0,018	0,897
BOP and IL-1β	-	-	-	-	0,451	0,000*
BOP and IL-18	-	-	-	-	-0,133	0,344
BOP and POP1	-	-	-	-	-0,079	0,588
BOP and POP2	-	-	-	-	-0,070	0,654
BOP and CARD16	-	-	-	-	-0,270	0,032*
BOP and CARD18	-	-	-	-	-0,257	0,225
BOP and TRIM16	-	-	-	-	-0,298	0,05*
BOP and TRIM20	-	-	-	-	-0,609	0,000
CAL and NLRP3	-	-	0,459	0,000*	0,538	0,000*
CAL and NLRP2	-	-	0,464	0,000*	0,478	0,000*
CAL and AIM2	-	-	0,436	0,004*	0,287	0,048*
CAL and CASP1	-	-	-0,048	0,745	0,033	0,811
CAL and ASC	-	-	0,005	0,975	0,002	0,991
CAL and IL-1β	-	-	0,197	0,139	0,322	0,007*
CAL and IL-18	-	-	0,120	0,424	0,040	0,775
CAL and POP1	-	-	0,101	0,526	0,052	0,721
CAL and POP2	-	-	-0,798	0,000	-0,686	0,000*
CAL and CARD16	-	-	-0,426	0,001*	-0,414	0,001*
CAL and CARD18	-	-	-0,376	0,124	-0,372	0,074
CAL and TRIM16	-	-	-0,162	0,338	-0,267	0,079
CAL and TRIM20	-	-	-0,479	0,013*	-0,588	0,000*

Table 4. Correlation of IL-1β levels with inflammasome-associated genes

	Healthy Group		Disease Groups		All participants	
Parameters	rho	P	rho	P	rho	P
IL-1β and NLRP3	0,962	0,000*	0,785	0,000*	0,810	0,000*
IL-1β and NLRP2	1,000		0,899	0,000*	0,904	0,000*
IL-1β and AIM2	-0,324	0,478	0,003	0,985	-0,098	0,507
IL-1β and CASP1	0,548	0,203	0,359	0,012*	0,387	0,004*
IL-1β and ASC	-0,048	0,919	0,172	0,253	0,120	0,393
IL-1β and IL-18	0,877	0,022*	0,513	0,000*	0,363	0,008*
IL-1β and POP1	-0,350	0,395	0,083	0,599	-0,023	0,873
IL-1β and POP2	0,545	0,206	0,044	0,797	-0,010	0,948
IL-1β and CARD16	-0,840	0,018*	-0,920	0,000*	-0,881	0,000*
IL-1β and CARD18	-0,032	0,952	0,054	0,833	-0,175	0,412
IL-1β and TRIM16	-0,388	0,389	-0,014	0,936	-0,232	0,130
IL-1β and TRIM20	-0,959	0,000*	-0,872	0,000*	-0,912	0,000*