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ORIGINAL ARTICLE

Association between periodontitis and peripheral markers of innate immunity activation and inflammation

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Abstract

Background: Immune response leading to increased systemic inflammation is one of the mechanisms linking periodontitis to chronic inflammatory diseases. The aim of this study was to compare the expression of toll-like receptors 2 and 4 in monocytes and neutrophils (TLR2M, TLR2N, TLR4M, and TLR4N) and its endogenous ligands (cellular fibronectin [cFN] and heat shock protein 60 [HSP60]) in patients with and without periodontitis. Additionally, the relationship between cFN and HSP60 expression with innate immunity activation and systemic inflammatory response (interleukin 6 [IL-6]) was also evaluated.

Methods: A case-controlled study was designed in which 30 patients with periodontitis (cases) and 30 age- and sex-matched participants without periodontitis (controls) were included. Fasting blood samples were collected to determine: (1) expression of TLR2N, TLR2M, TLR4N, and TLR4M by flow cytometry; and (2) serum concentrations of cFN, HSP60, and IL-6 by ELISA technique.

Results: Expression of TLR2M (411.5 [314.2, 460.0] vs. 236.5 [204.0, 333.0] AFU), TLR2N (387.0 [332.0, 545.5] vs 230.0 [166.2, 277.7] AFU), TLR4M (2478.5 [1762.2, 2828.0] vs 1705.0 [1274.5, 1951.2] AFU), and TLR4N (2791.0 [2306.7, 3226.2] vs. 1866.0 [1547.5, 2687.2] AFU) as well as serum levels of cFN (301.1 [222.2, 410.9] vs. 156.4 [115.3, 194.0] ng/ml) and IL-6 (10.4 [6.5, 11.5] vs. 3.5 [2.6, 4.9] pg/ml) were significantly higher in periodontitis patients than those without periodontitis. A positive association was found between periodontitis and cFN (odds ratio [OR] = 1.028, $p < 0.001$), TLR2N (OR = 1.026, $p < 0.001$), TLR4M (OR = 1.001, $p = 0.002$), and IL-6 (OR = 1.774, $p < 0.001$).

José Dopico and João Botelho contributed equally as first authors.

Tomás Sobrino and Yago Leira contributed equally as senior authors.

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Conclusions: Periodontitis patients exhibited high expression of TLRs, cFN, and IL-6.

KEYWORDS
fibronectin, inflammation, innate immunity, periodontitis, toll-like receptor

1 | INTRODUCTION

Periodontitis is characterized by a microbially associated, host-mediated inflammation that results in periodontal attachment loss.[1] Unfavorable alterations in the composition of the microbiota lead to a dysbiotic state of dental plaque that causes increased inflammation in the periodontium,[2–5] which triggers activation of host-derived proteinases that lead to tissue destruction, apical migration of junctional epithelium, and progression of the biofilm in an apical direction.[6] Indeed, the histopathology of periodontitis lesions was characterized over 40 years ago.[7] In the early gingivitis lesion, the initial stages of disease, neutrophils and monocytes/macrophages dominate; therefore, they may play a central role in the initiation of disease.

It has been suggested that dysregulation in innate immunity may be of importance in the pathogenesis of periodontitis.[8] Among the immune mechanisms that may be involved, toll-like receptors (TLRs) dysregulation is one of the main candidates, since TLR activation has the potential to upregulate cytokine and chemokine production.[8,9] Both TLR-2 and TLR-4 are expressed in monocytes and neutrophils when exposed to lipopolysaccharides (LPS),[10] and higher TLR-2 and TLR-4 mRNA expression was found in chronic periodontitis patients.[11] Also, the TLR4 299Gly allele appeared to be associated with chronic periodontitis.[12]

TLRs recognize pathogen-associated molecular patterns (PAMPs), including proteins and peptides, polysaccharides and proteoglycan, nucleic acids, phospholipids, and small organic molecules. Products of well-documented periodontal pathogens such as *Porphyromonas gingivalis* LPS, *Actinomyces actinomycetemcomitans* LPS, and *P. gingivalis* fimbriae are recognized by TLRs.[9,13] Recognition of pathogen associated molecular patterns fails to explain all functions of TLRs. An increasing number of endogenous ligands derived from damaged or stressed host tissues or cells, capable of activating TLR signaling and inducing sterile inflammatory responses are being described.[13]

Both fibronectin (FN) and heat shock proteins (HSPs) (e.g., HSP60) can activate TLR signaling,[14–17] and their potential role in the pathogenesis of periodontitis is yet to be fully elucidated. Wu et al.[18] have reported increased levels of cellular fibronectin (cFN) in both periodontitis and peri-implantitis patients. HSPs play a main role in protein repair after cell damage, and its production reflect cellular stress.[19] It has been shown that oral microorganisms can induce production of HSPs.[20] Since a bulk of evidence has shown that periodontitis is associated with a low-grade chronic systemic inflammatory response that eventually could lead to the onset or progression of several noncommunicable chronic conditions,[21] the role of potential key markers related to innate immune activation during periodontitis is worth exploring. To date, there is a lack of human studies in the periodontal literature investigating the effect of cFN and HSP60 on the link between TLRs activation and subsequent systemic inflammatory response.

The aim of this study was, therefore, two-fold. First, to investigate whether there are differences in terms of expression of markers of innate immune system activation (TLR2 and 4 expressed in monocytes and neutrophils as well as two of its main endogenous ligands cFN and HSP60) and systemic inflammation (interleukin [IL]-6) in biological samples from periodontitis patients and controls without periodontitis. Second, the role of these two endogenous ligands in the association between innate immune response and systemic pro-inflammatory state was also assessed.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

Age- and sex-matched case-control study, in which 60 "self-perceived health" subjects who never smoked were recruited from the Faculty of Odontology of Santiago de Compostela (Spain) during the period comprised between May 2017 and September 2018. Cases consisted of 30 patients diagnosed with periodontitis[22] among referrals to the Periodontology Unit (University of Santiago de Compostela, Spain) for diagnosis and treatment of periodontitis. Thirty controls without periodontitis were defined as those with clinical periodontal health[23] or in some cases localized gingivitis with intact periodontium (full-mouth bleeding score [FMBS] between 10% and 30%) [24]. These participants were identified among friends of the patients with periodontitis ($n = 9$) or from a research registry of

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participants from previous studies carried out by our research group ($n = 21$).[25–27]

Exclusion criteria were as follows: (1) <18 years of age; (2) <15 teeth (excluding third molars and retained roots); (3) periodontal treatment in the last year; (4) smokers; (5) concomitant medical conditions (e.g., diabetes, cardiovascular diseases, hypertension, or hypercholesterolemia) or active infectious/inflammatory diseases (e.g., HIV, hepatitis, tuberculosis, rheumatoid arthritis, allergies, or asthma); (6) pregnant or lactating females; (7) malignancy; (8) treatment with systemic antibiotics, corticosteroids and/or immunosuppressive agents within 3 months prior to periodontal examination.

This research was performed in accordance with the Declaration of Helsinki of the World Medical Association (as revised in 2013) and approved by the Ethics Committee of the Servizo Galego de Saúde (ID:2016/079). Written informed consent was obtained from each participant or their relatives after full explanation of the periodontal examination and blood sample collection.

2.2 | Periodontal assessment

A full-mouth periodontal examination was performed in all participants by a calibrated periodontist (Y.L.).[25–27] The following parameters were measured in all teeth (except third molars): pocket depth (PD), clinical attachment level (CAL), full-mouth plaque score (FMPS), and FMBS.[28] Measurements were recorded at six sites per tooth (mesiobuccal, distobuccal, midbuccal, mesiolingual, distolingual, and midlingual) using a calibrated University of North Carolina periodontal probe.[*] Number of teeth present in the mouth of each individual was also recorded. Mild periodontitis was defined as ≥2 interproximal sites with CAL ≥3 mm and ≥2 interproximal sites with PPD ≥4 mm (not on the same tooth) or one site with PPD ≥5 mm. Moderate periodontitis was defined as ≥2 interproximal sites with CAL of ≥4 mm (not on the same tooth) or ≥2 interproximal sites with PPD of ≥5 mm, also not on the same tooth. Severe periodontitis was defined as the presence of ≥2 interproximal sites with CAL of ≥6 mm (not on the same tooth) and ≥1 interproximal sites with PPD of ≥5 mm. Total periodontitis was the sum of mild, moderate, and severe periodontitis.[22] For the purpose of this study, we grouped moderate and severe periodontitis cases. Therefore, according to periodontal status, three groups were defined: periodontally healthy (without periodontitis), mild and moderate-to-severe periodontitis.[22]

In addition, the periodontal inflamed surface area (PISA) was calculated for all participants. PISA reflects the surface area of bleeding pocket epithelium in mm^2. As previously described,[25,27] PISA was calculated using a spreadsheet software,[†] in the following steps: (i) mean CAL and gingival recession for each particular tooth is calculated; (ii) linear mean CAL and gingival recession is translated into the periodontal epithelial surface area (PESA) for each specific tooth[29] (the PESA for a particular tooth consists of the root surface area of that tooth measured in mm^2, which is covered with pocket epithelium); (iii) the PESA for a specific tooth is then multiplied by the proportion of sites around the tooth that was affected by bleeding on probing, resulting in the PISA for that particular tooth; and (iv) the sum of all individual PISAs around individual teeth is calculated, rendering the full-mouth PISA value in mm^2 for each participant.[30]

2.3 | Clinical and socio-demographic variables

Body mass index (BMI) was calculated for all participants using the formula weight/height^2 (kg/m^2). Age and sex were recorded from each subject. Education level was considered low when only primary studies were completed.

2.4 | Laboratory tests

Fasting blood samples were obtained in the morning. Briefly, 2 ml of venous blood was collected from the antecubital fossa by venepuncture using a 20-gauge needle with a 2 ml syringe. Blood samples were allowed to clot at room temperature and, after 1 h, serum was separated by centrifugation (15 min at 3000 × g) and 0.5 ml of extracted serum was immediately transferred to 1.5-ml aliquots. Each aliquot was stored at −80°C until required for analysis. Serum levels of cFN, HSP60, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) technique following the manufacturer's instructions IL-6 ELISA kit[‡] minimum assay sensitivity was 1.6 pg/ml and with an intra- and interassay coefficient of variation (CV) of <8% and <9%, respectively; cFN ELISA kit[§] minimum assay sensitivity was 7.5 ng/ml and with an intra-assay and interassay CV of < 8% and < 10%, respectively; and HSP60 ELISA kit[**] minimum assay sensitivity was 1.9 ng/ml and with an intra and interassay CV of <5.3% and <10%, respectively.

[*] UNC 15, Hu-Friedy, Chicago, IL, USA.

[†] Microsoft Office Excel, Microsoft Corporation, Redmond, WA, USA.
[‡] IL-6 ELISA kit, BioLegend, San Diego, CA, USA.
[§] cFN ELISA kit, Abbexa, Cambridge, UK.
[**] HSP60 ELISA kit, Assayro, USA.

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TLR2 and TLR4 expression analyses were performed by flow cytometry in blood samples, withdrawn from all periodontitis patients and control subjects, collected in EDTA-anticoagulated tubes. For the expression analysis of TLR2 and TLR4, monocytes, lymphocytes and neutrophils were separated by their forward and side scattering signal characteristics. Fluorescein isothiocyanate (FITC) anti-TLR2-conjugated monoclonal antibodies[††] and phycoerythrin (PE) anti-TLR4 conjugated monoclonal antibodies[‡‡] were used to quantify TLR expression. Samples were analyzed on a flow cytometer.[§§] Cell fluorescence was measured immediately after staining, and data were analyzed with a software.[***] Mean expression of TLR2 and TLR4 in monocytes and neutrophils (TLR2M, TLR2N, TLR4M, and TLR4N) was expressed as AFU (arbitrary fluorescence units).

Determinations were performed in an independent laboratory blinded to clinical data. Clinical investigators were unaware of the laboratory results until the study had ended. All biochemical analyses were done at the Clinical Neurosciences Research Laboratory (University Clinical Hospital of Santiago de Compostela).

2.5 | Statistical analysis

No formal sample size calculation was performed for this study. Nevertheless, a post hoc power analysis based on the results obtained from our study confirmed sufficient statistical power (>90%) to detect a mean difference of 751 AFU with a standard deviation of 203 AFU when compared TLR4M expression (study primary outcome) between periodontitis patients and controls. Mean values ± standard deviation (SD) and median [P25, P75] were calculated for normally and non-normally distributed continuous variables, respectively. Statistical tests used to compare continuous data were independent *t* test, Mann–Whitney *U* test or Kruskal–Wallis H test. Categorical variables were reported as percentages and compared by X2 test. Nonparametric correlation analyses between biomarkers and clinical periodontal parameters were performed using Spearman's rank correlation coefficient. Both logistic and linear regression models were created to test association between periodontitis and biomarkers. All statistical tests were carried out at a significance level of $\alpha = 0.05$, using statistics software.[†††]

[††] Fluorescein isothiocyanate (FITC) anti-TLR2-conjugated monoclonal antibodies, IMMUNOSTEP, Salamanca, Spain.
[‡‡] Phycoerythrin (PE) anti-TLR4-conjugated monoclonal antibodies, IMMUNOSTEP, Salamanca, Spain.
[§§] FACSAria flow cytometer, BD Biosciences, Franklin Lakes, NJ, USA.
[***] FACSDiva software, BD Biosciences, Franklin Lakes, NJ, USA.
[†††] IBM SPSS Statistics version 24.0 for Windows, IBM Corporation, Armonk, NY, USA.

3 | RESULTS

3.1 | Sample characteristics

Subjects with and without periodontitis were matched for age and sex (both $p = 1.000$). No differences were observed for BMI and education level ($p > 0.05$) (Table 1). As expected, periodontitis patients exhibit worse periodontal conditions in terms of high levels of gingival/periodontal inflammation and periodontal attachment and tooth loss when compared to those without periodontitis (all $p < 0.001$) (Table 1). However, plaque accumulation was similar in both groups ($p = 0.288$). Out of 30 periodontitis patients, 12 had mild periodontitis and 18 were classified as moderate-to-severe periodontal cases.

3.2 | Biomarkers

Expression levels of TLR2 and TLR4 in both monocytes and neutrophils as well as TLR endogenous ligand (cFN) were higher in periodontal patients compared to controls (all $p < 0.01$) (Table 2). No statistically significant differences were found for HSP60 ($p = 0.836$). Similarly, elevated serum concentrations of pro-inflammatory cytokine (IL-6) were found in periodontitis cases in comparison to controls (Table 2). Expression of TLR2 and TLR4 in both neutrophils and monocytes was associated with severity of periodontitis (Figure 1A and B).

3.3 | Correlation analysis

Positive correlations were observed for most of the clinical periodontal parameters and TLRs, with the exception of PD and CAL that did not correlate with TLR4M (Table 3). Dental plaque accumulation only correlated with cFN ($r = 0.266$, $p = 0.040$). No correlations were found between HSP60 and clinical periodontal variables (all $p > 0.05$). Whilst cFN was moderately correlated to TLR4M ($r = 0.404$, $p = 0.001$), TLR2N ($r = 0.515$, $p < 0.001$), and TLR2M ($r = 0.592$, $p < 0.001$); no correlation was observed between TLR4N and cFN ($r = 0.241$, $p = 0.064$). HSP60 did not correlated with any of the TLRs (data not shown). Moderate positive correlations between TLRs and IL-6 were found (TLR2N: $r = 0.449$, $p < 0.001$; TLR2M: $r = 0.600$, $p < 0.001$; TLR4N: 0.415, $p = 0.001$; TLR4M: $r = 0.421$, $p = 0.001$).

3.4 | Regression analysis

Diagnosis of periodontitis was statistically significant associated with expression of cFN (odds ratio [OR] = 1.028, 95%

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TABLE 1 Characteristics of participants according to periodontal status

Variable	Periodontitis ($N = 30$)	No periodontitis ($N = 30$)	*p*-Value
Age (years)	47.9 ± 7.5	47.9 ± 6.3	1.000
Males, *n* (%)	23 (76.7)	23 (76.3)	1.000
Low education level, *n* (%)	9 (30.0)	6 (20.0)	0.371
BMI (kg/m2)	25.6 ± 4.0	23.9 ± 4.3	0.124
Clinical periodontal parameters			
FMPS (%)	21.8 ± 3.7	20.7 ± 3.9	0.228
FMBS (%)	46.6 ± 26.7	20.8 ± 6.9	**<0.001**
PD (mm)	3.3 ± 0.7	2.4 ± 0.5	**<0.001**
PD6, *n*	7.2 ± 6.4	0.1 ± 0.2	**<0.001**
CAL (mm)	3.6 ± 0.7	2.7 ± 0.6	**<0.001**
CAL5, *n*	12.2 ± 7.6	0.2 ± 0.4	**<0.001**
PISA (mm^2)	547.6 [195.3, 629.4]	23.7 [16.8, 43.2]	**<0.001**
Teeth present, *n*	25.3 ± 1.5	26.5 ± 1.4	**0.003**

Abbreviations: BMI, body mass index; CAL, clinical attachment level; CAL5, number of periodontal pockets with CAL ≥5 mm; FMBS, full-mouth gingival bleeding score; FMPS, full-mouth plaque score; PISA, periodontal inflamed surface area; PD, pocket depth; PD6, number of periodontal pockets with PD ≥6 mm.
Significant results are reported in bold ($p < 0.05$).

TABLE 2 Biochemical parameters of study sample according to periodontal status

Biomarker	Periodontitis ($N = 30$)	No periodontitis ($N = 30$)	*p*-Value
Innate immunity			
TLR2M (AFU)	411.5 [314.2, 460.0]	236.5 [204.0, 333.0]	**<0.001**
TLR2N (AFU)	387.0 [332.0, 545.5]	230.0 [166.2, 277.7]	**<0.001**
TLR4M (AFU)	2478.5 [1762.2, 2828.0]	1705.0 [1274.5, 1951.2]	**0.003**
TLR4N (AFU)	2791.0 [2306.7, 3226.2]	1866.0 [1547.5, 2687.2]	**<0.001**
Endogenous TLR ligands			
cFN (ng/ml)	301.1 [222.2, 410.9]	156.4 [115.3, 194.0]	**<0.001**
HSP60 (ng/ml)	21.0 [14.5, 39.2]	22.3 [17.0, 25.6]	0.836
Systemic inflammation			
IL-6 (pg/mL)	10.4 [6.5, 11.5]	3.5 [2.6, 4.9]	**<0.001**

Abbreviations: TLRM, toll-like receptor expression (monocytes) (AFU: Active Fluorescent Units); TLRN, toll-like receptor expression (neutrophils) (AFU: Active Fluorescent Units); cFN, cellular fibronectin; HSP60, heat shock protein 60; IL-6, interleukin 6.
Significant results are reported in bold ($p < 0.05$).

confidence interval [CI]: 1.013–1.043 $p < 0.001$), TLR2N (OR = 1.026, 95% CI: 1.013–1.039 $p < 0.001$), TLR2M (OR = 1.017, 95% CI: 1.009–1.026; $p < 0.001$), TLR4N (OR = 1.001, 95% CI: 1.000–1.001; $p = 0.005$), TLR4M (OR = 1.001, 95% CI: 1.001–1.002; $p = 0.002$), and IL-6 (OR = 1.774, 95% CI: 1.330–2.288 $p < 0.001$). In addition, a positive association was found between cFN and TLR2N (β coefficient = 0.4, 95% CI: 0.2–0.6; $p = 0.002$), TLR2M (β coefficient = 0.6, 95% CI: 0.4–0.8; $p < 0.001$), and TLR4M (β coefficient = 2.5, 95% CI: 0.7–4.3; $p = 0.007$). Furthermore, a relationship was observed between IL-6 and TLR2N (β coefficient = 7.3, 95% CI: 2.6–12.0; $p = 0.003$), TLR2M (β coefficient = 9.4, 95% CI: 5.1–13.7; $p < 0.001$), and TLR4M (β coefficient = 44.6, 95% CI: 8.2–80.9; $p = 0.017$).

4 | DISCUSSION

The present case-control study with a cross-sectional biochemical design has investigated the expression of TLR2 and TLR4 in peripheral mononuclear blood cells, together with their potential endogenous ligands (HSP60 and cFN) and the pro-inflammatory cytokine (IL-6) in periodontitis.

Our findings indicated that periodontitis patients showed higher expression of TLR2 and TLR4 compared to subjects without periodontitis. These results are in line with those obtained by Sun et al.[11] and Buduneli et al.,[31] who observed that chronic periodontitis subjects had elevated expression TLR2 and TLR4 compared to individuals without periodontitis in gingival tissue and

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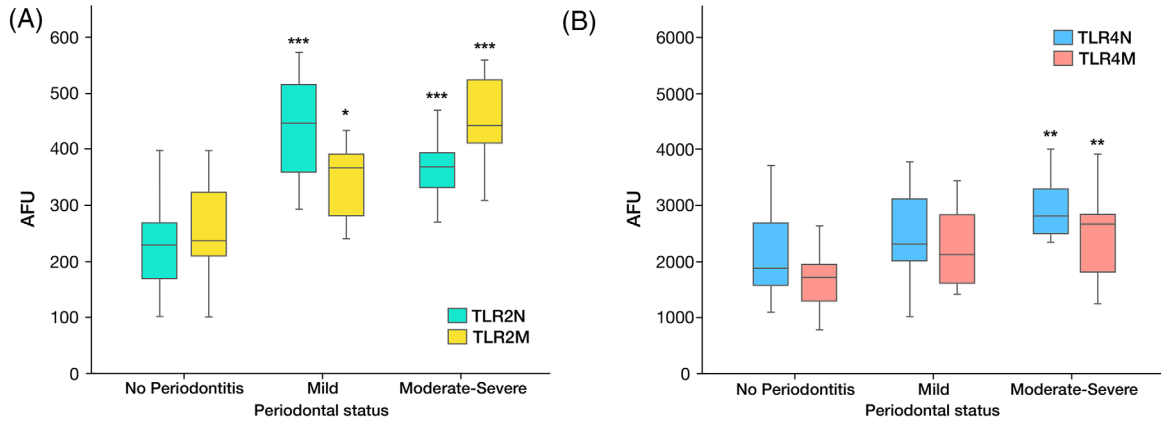

FIGURE 1 Expression of innate immunity markers according to different grades of periodontitis severity. (**A**) TLR2 expression in neutrophils (TLR2N) and monocytes (TLR2M). (**B**) TLR4 expression in neutrophils (TLR4N) and monocytes (TLR4M). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison with no periodontitis. No statistically significant differences were observed for any TLR when moderate-to-severe periodontitis group was compared with mild periodontitis

TABLE 3 Correlations between clinical periodontal parameters and biomarkers ($N = 60$)

Biomarker	PD (mm)	CAL (mm)	FMBS (%)	FMPS (%)	PD6	CAL5	PISA (mm^2)
TLR2M (AFU)	0.560	0.526	0.511	0.256	0.589	0.609	0.648
p-value	**<0.001**	**<0.001**	**<0.001**	0.052	**<0.001**	**<0.001**	**<0.001**
TLR2N (AFU)	0.359	0.318	0.354	0.202	0.438	0.538	0.518
p-value	**0.005**	**0.013**	**0.006**	0.122	**<0.001**	**<0.001**	**<0.001**
TLR4M (AFU)	0.151	0.178	0.336	0.007	0.354	0.422	0.460
p-value	0.250	0.174	**0.009**	0.960	**0.005**	**0.001**	**<0.001**
TLR4N (AFU)	0.374	0.367	0.362	0.142	0.397	0.251	0.455
p -value	**0.003**	**0.004**	**0.004**	0.280	**0.002**	0.053	**<0.001**
cFN (ng/ml)	0.676	0.638	0.616	0.266	0.760	0.697	0.761
p-value	**<0.001**	**<0.001**	**<0.001**	**0.040**	**<0.001**	**<0.001**	**<0.001**
HSP60 (ng/ml)	0.217	0.151	0.085	−0.086	0.183	0.122	−0.039
p-value	0.096	0.249	0.518	0.514	0.161	0.355	0.765
IL-6 (pg/ml)	0.500	0.497	0.589	0.226	0.648	0.628	0.655
p-value	**<0.001**	**<0.001**	**<0.001**	0.082	**<0.001**	**<0.001**	**<0.001**

Abbreviations: CAL, clinical attachment level; CAL5, number of periodontal pockets with CAL ≥5 mm; cFN, cellular fibronectin; FMPS, full-mouth plaque score; PD, pocket depth; PD6, number of periodontal pockets with PD ≥6 mm; HSP60, heat shock protein 60; IL-6, interleukin 6FMBS, full-mouth gingival bleeding score; TLRM, toll-like receptor expression (monocytes) (AFU: Active Fluorescent Units); TLRN, toll-like receptor expression (neutrophils) (AFU: Active Fluorescent Units).

Significant results are reported in bold ($p < 0.05$).

plasma samples, respectively. In the latter study, authors also found a positive correlation between bleeding on probing and TLR4.[31] Similarly, in our study, not only gingival bleeding but also other clinical parameters of periodontal inflammation/destruction were correlated with increased expression of TLR4 in both monocytes and neutrophils. Moreover, in our investigation, as periodontitis progressed, expression of TLRs tended to increase. In this sense, the key role of TLR4 activation on periodontitis pathogenesis is supported by a meta-analysis that included data from seven case-controlled studies showing that Caucasians who had one functional the TLR4 Asp299Gly functional polymorphism were 1.4 times more likely to develop chronic periodontitis than those without this polymorphism.[12]

On the other hand, TLRs recognize damage-associated molecular patterns from endogenous molecules, which are released as a consequence of tissue damage. These molecules, also known as endogenous ligands, are able to activate TLRs promoting the expression of pro-inflammatory cytokines. In this regard, cFn and HSP60 have been suggested as endogenous ligands for TLR2

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and/or TLR4 activation in inflammatory diseases. This is why we have analyzed the role of HSP60 and cFN as potential endogenous ligands for TLR2 and TLR4 in periodontitis. In this regard, we found a correlation between cFN levels and TLR2N, TLR2M, and TLR4M. Additionally, the correlation between TLR2 and TLR4 expression with the concentrations of IL-6 (a biomarker of systemic inflammation) was evaluated. A positive correlation was found between TLR2 and TLR4 expression (in both neutrophils and monocytes) and IL-6. Further regression analysis confirmed that cFN was associated with expression of TLR2N/TLR2M/TLR4M and that overexpression of these TLRs is related to elevated peripheral levels of IL-6. So, we hypothesized that TLR may mediate the increase in systemic inflammation through molecular mechanisms activated by cFn, which acts as endogenous ligand. This might be similar what occurs locally in the periodontally affected gingival tissues, where FN fragments produced by gingipains from *P. gingivalis* contribute to maintain as well as amplify the inflammatory response.[32] However, previous studies showed that gingival crevicular fluid and periodontal tissues samples from periodontitis patients had low levels of cFN compared to the ones from subjects without periodontitis.[18,33] This could be explained by the vasodilatory effect produced by periodontal inflammation that might increase the net rate of cFN removal from the gingival tissues and elevate cFN in the peripheral blood, as it can be seen in our studies where concentrations of systemic cFN were higher in the periodontitis group compared to the nonperiodontitis group. Nevertheless, further studies are needed to elucidate the endogenous ligands and their underlying molecular mechanisms that activate TLR leading to a low-grade chronic systemic pro-inflammatory state in periodontitis individuals.

Previous reports have shown that serum levels of HSP60 are elevated in periodontitis patients compared to controls and positively correlated to the severity of periodontitis.[34] In addition, an increased humoral response to HSP60 family in periodontitis was shown.[35] In contrast to these findings, the present study failed to confirm higher levels of circulating HSP60 in periodontitis group than controls. There are many potential reasons that could explain these discrepancies. For example, periodontitis was not classified in the same manner and this could impact the results. Also, our primary study outcome was not HSP60 levels; hence, the present study may be underpowered to show significant differences between for this biomarker.

Regarding why we have chosen IL-6 as a marker of systemic inflammation, IL-6 is a primary stimulant for the hepatic acute-phase response, with which other known proinflammatory risk factors (primarily C-reactive protein [CRP] and fibrinogen) are associated. Indeed, IL-6 is the only known cytokine capable of inducing all acute-phase proteins involved in the systemic inflammatory response. As such, the induction of CRP by IL-6 may be a key step in the systemic inflammatory response.[36] Given this, it is plausible that other proinflammatory molecules are actually surrogates for circulating IL-6 activity. On the other hand, CRP itself is unlikely to provide an effective target for intervention whereas IL-6 inhibition therapy has been proven to be not have an anti-inflammatory effect but also antithrombotic which may be relevant for atherosclerosis.[37] Therefore, there is a tendency to move upstream in the inflammatory cascade to identify novel therapeutic targets to tackle inflammatory diseases such as cardiovascular conditions.[38]

We have to be cautious when interpreting the present results. A number of limitations should be highlighted. Due to the cross-sectional nature of our investigation, we cannot assess causality between periodontitis and the biological processes investigated in the present study. No formal sample size calculation was performed for this study and could be considered small. However, a post hoc power analysis based on the results obtained from our study confirmed sufficient statistical power (>90%) to detect a mean difference of 751 AFU with a standard deviation of 203 AFU when compared TLR4M expression (study primary outcome) between groups. Systemic condition was confirmed by means of a questionnaire (self-reported). Undiagnosed chronic conditions such as hypertension, diabetes, or dyslipidemia could be present in some of the participants and may have an impact in our results. Additional objective measurements of systemic health status (clinical and biochemical data) for instance arterial blood pressure (systolic and diastolic), glycated hemoglobin/blood glucose, lipid fractions, or complete blood count should be included in future studies to completely rule out this possibility.

5 | CONCLUSIONS

Results from the present study showed high expression of systemic markers of innate immunity activation and inflammation in periodontitis patients. Experimental studies are needed to unravel the exact mechanisms behind these findings.

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AUTHOR CONTRIBUTIONS

Yago Leira and Tomás Sobrino have been involved in conception and study design. Yago Leira and Clara Domínguez have been involved in data collection. Yago Leira, Alberto Ouro, Marta Aramburu-Nuñez, Antía Custodia, Teresa Blanco, María Vázquez-Reza, Daniel Romaus-Sanjurjo, Juan Blanco, Rogelio Leira, and Tomás Sobrino have analyzed and interpreted the data. José Dopico, João Botelho, Vanessa Machado, and Yago Leira have drafted the manuscript. All authors have been involved in revising the manuscript critically and have given final approval of the version to be published.

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