



ORIGINAL ARTICLE

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

José Dopico¹ | João Botelho² | Alberto Ouro³ | Clara Domínguez⁴ |
Vanessa Machado² | Marta Aramburu-Núñez³ | Antía Custodia³ | Teresa Blanco⁵ |
María Vázquez-Reza¹ | Daniel Romaus-Sanjurjo³ | Juan Blanco¹ | Rogelio Leira⁴ |
Tomás Sobrino³ | Yago Leira^{1,3}

¹Faculty of Odontology and Medicine, Periodontology Unit, University of Santiago de Compostela, Santiago de Compostela, Spain

²Periodontology Department and Evidence-Based Hub, Clinical Research Unit, Centro de Investigação Interdisciplinar Egas Moniz, Instituto Universitário Egas Moniz and Cooperative de Ensino Superior, Caparica, Portugal

³NeuroAging Group, Clinical Neurosciences Research Laboratories (LINC), Health Research Institute of Santiago de Compostela (IDIS), Santiago de Compostela, Spain

⁴Neurology Department, University Clinical Hospital, Santiago de Compostela, Spain

⁵Allergy Department, University Hospital Puerta del Hierro, Madrid, Spain

Correspondence

Yago Leira, Clinical Neurosciences Research Laboratory, University Clinical Hospital of Santiago, Travesía da Choupana S/N, Santiago de Compostela (Spain).

Email: yagoleira@gmail.com

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.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 American Academy of Periodontology.



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 periodontitis²² 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 health²³ 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



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 mm². 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 tooth²⁹ (the PESA for a particular tooth consists of the root surface area of that tooth measured in mm², 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 mm² for each participant.³⁰

2.3 | Clinical and socio-demographic variables

Body mass index (BMI) was calculated for all participants using the formula weight/height² (kg/m²). 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 $\times$ 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.

[†] 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, Assaypro, USA.

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



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 $\pm$ 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 χ^2 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.

^{§§} FACS Aria 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 correlate 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: $r = 0.415$, $p = 0.001$; 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%

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/m ²)	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

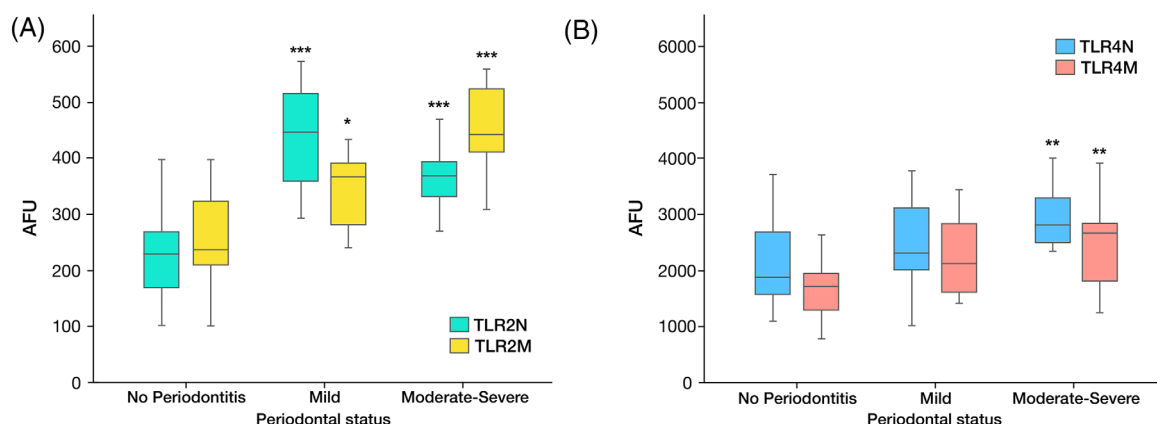


FIGURE 1 Expression of innate immunity markers according to different grades of periodontitis severity. (A) TLR2 expression in neutrophils (TLR2N) and monocytes (TLR2M). (B) TLR4 expression in neutrophils (TLR4N) and monocytes (TLR4M). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison with 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
<i>p</i> -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
<i>p</i> -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
<i>p</i> -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
<i>p</i> -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
<i>p</i> -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
<i>p</i> -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
<i>p</i> -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 6; FMBS, 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



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.

ACKNOWLEDGMENTS

This study was partially supported by grants from the Xunta de Galicia (TS: IN607A2018/3, TS: IN607D 2020/09, AC: IN606A-2021/015; DRS: IN606B-2021/010), Science Ministry of Spain (TS: RTI2018-102165-B-I00), and Institute of Health Carlos III (RL: PI15/01578). Furthermore, this study was also supported by grants from the INTERREG Atlantic Area (TS: EAPA_791/2018_NEUROATLANTIC project), INTERREG V A España



Portugal (POCTEP) (TS: 0624_2IQBIONEURO_6_E), and the European Regional Development Fund (ERDF). Moreover, MA-N (IFI18/00008), DRS (CD21/00166), and TS (CPII17/00027) are recipients of research contracts from the i-PFIS, Sara Borrell, and Miguel Servet Programs, respectively, from the Instituto de Salud Carlos III. YL is supported by a research contract in the context of the INTERREG Atlantic Area European Funding Programme (EAPA_791/2018_ NEUROATLANTIC project). AC is supported by a fellowship from the Xunta de Galicia (IN606A-2021/015). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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-Núñez, 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.

ORCID

José Dopico  <https://orcid.org/0000-0002-2255-523X>

João Botelho  <https://orcid.org/0000-0002-1019-8263>

Alberto Ouro  <https://orcid.org/0000-0003-4359-1704>

Tomás Sobrino  <https://orcid.org/0000-0002-9760-8690>

Yago Leira  <https://orcid.org/0000-0001-5027-7276>

REFERENCES

1. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: framework and proposal of a new classification and case definition. *J Periodontol*. 2018;89:159-172.
2. Marsh PD. Microbial ecology of dental plaque and its significance in health and disease. *Adv Dent Res*. 1994;8:263-271.
3. Kolenbrander PE, Palmer RJ, Rickard AH, Jakubovics NS, Chalmers NI, Diaz PI. Bacterial interactions and successions during plaque development. *Periodontol 2000*. 2006;42:47-79.
4. Kolenbrander PE, Palmer RJ, Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol*. 2010;8:471-480.
5. Marsh PD, Devine DA. How is the development of dental biofilms influenced by the host?: host influence on biofilm development. *J Clin Periodontol*. 2011;38:28-35.
6. Armitage GC. Development of a classification system for periodontal diseases and conditions. *Ann Periodontol*. 1999;4:1-6.
7. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest*. 1976;34:235-249.
8. Mahanonda R, Pichyangkul S. Toll-like receptors and their role in periodontal health and disease. *Periodontol 2000*. 2007;43:41-55.
9. Suárez LJ. Oral dysbiosis and autoimmunity: from local periodontal responses to an imbalanced systemic immunity. A review. *Front Immunol*. 2020;11:591255.
10. Sabroe I, Jones EC, Usher LR, Dower SK. Toll-Like Receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol*. 2002;168:4701-4710.
11. Sun Y, Guo Q-M, Liu D-L, Zhang M-Z, Shu R. In vivo expression of Toll-like receptor 2, Toll-like receptor 4, CSF2 and LY64 in Chinese chronic periodontitis patients. *Oral Dis*. 2010;16:343-350.
12. Ozturk A, Vieira AR. TLR4 as a risk factor for periodontal disease: a reappraisal. *J Clin Periodontol*. 2009;36:279-286.
13. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med*. 2010;14:2592-2603.
14. Erridge C. Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J Leukoc Biol*. 2010;87:989-999.
15. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol*. 2000;164:558-561.
16. Okamura Y, Watari M, Jerud ES, et al. The extra domain A of fibronectin activates Toll-like receptor 4. *J Biol Chem*. 2001;276:10229-10233.
17. Tsan M-F, Gao B. Endogenous ligands of Toll-like receptors. *J Leukoc Biol*. 2004;76:514-519.
18. Wu Y-Y, Cao H-H, Kang N, Gong P, Ou G-M. Expression of cellular fibronectin mRNA in adult periodontitis and peri-implantitis: a real-time polymerase chain reaction study. *Int J Oral Sci*. 2013;5:212-216.
19. Indumathy P, Arun K, Sai Pashanth P. Heat shock protein (HSP60) in periodontal disease: a review. *Int J Cur Res Rev*. 2014;6:18-21.
20. Goulhen F, Grenier D, Mayrand D. Oral microbial heat-shock proteins and their potential contributions to infections. *Crit Rev Oral Biol Med*. 2003;14:399-412.
21. Moutsopoulos NM, Madianos PN. Low-grade inflammation in chronic infectious diseases: paradigm of periodontal infections. *Ann NY Acad Sci*. 2006;1088:251-264.
22. Eke PI, Page RC, Wei L, Thornton-Evans G, Genco RJ. Update of the case definitions for population-based surveillance of periodontitis. *J Periodontol*. 2012;83:1449-1454.
23. Lang NP, Bartold PM. Periodontal health. *J Clin Periodontol*. 2018;45:9-16.
24. Trombelli L, Farina R, Silva CO, Tatakis DN. Plaque-induced gingivitis: case definition and diagnostic considerations. *J Clin Periodontol*. 2018;20:44-67.
25. Leira Y, Ameijera P, Domínguez C, Leira R, Blanco J. High serum procalcitonin levels in patients with periodontitis and chronic migraine. *J Periodontol*. 2018;89:1069-1074.
26. Ameijera P, Leira Y, Domínguez C, Leira R, Blanco J. Association between periodontitis and chronic migraine: a case-control study. *Odontology*. 2019;107:90-95.



27. Leira Y, Ameijeira P, Dominguez C, et al. Periodontal inflammation is related to increased serum calcitonin gene-related peptide levels in patients with chronic migraine. *J Periodontol*. 2019;90:1088-1095.
28. Ainamo J, Bay I. Problems and proposals for recording gingivitis and plaque. *Int Dent J*. 1975;25:229-235.
29. Hujoel PP, White BA, García RI, Listgarten MA. The den-
toringival epithelial surface area revisited. *J Periodontol Res*. 2001;36:48-55.
30. Nesse W, Abbas F, van der Ploeg I, Spijkervet FKL, Dijkstra PU, Vissink A. Periodontal inflamed surface area: quantifying inflammatory burden. *J Clin Periodontol*. 2008;35:668-673.
31. Buduneli N, Özçaka Ö, Nalbantsoy A. Salivary and plasma levels of Toll-like receptor 2 and Toll-like receptor 4 in chronic periodontitis. *J Periodontol*. 2011;82:878-884.
32. Feghali K, Grenier D. Priming effect of fibronectin fragments on the macrophage inflammatory response: potential contribution to periodontitis. *Inflammation*. 2012;35:1696-1705.
33. Lopatin DE, Caffesse ER, FredL Bye, Caffesse RG. Concentrations of fibronectin in the sera and crevicular fluid in various stages of periodontal disease. *J Clin Periodontol*. 1989;16:359-364.
34. Rizzo M, Cappello F, Marfil R, et al. Heat-shock protein 60 kDa and atherogenic dyslipidemia in patients with untreated mild periodontitis: a pilot study. *Cell Stress Chaperones*. 2012;17:399-407.
35. Tabeta K, Yamazaki K, Hotokezaka H, Yoshie H, Hara K. Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. *Clin Exp Immunol*. 2000;120:285-293.
36. Castell JV, Gomez-Lechon MJ, David M, et al. Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *FEBS Lett*. 1989;242:237-239.
37. Ridker PM, Devalaraja M, Baeres FMM, et al. Inhibition with ziltivekimab in patients at high atherosclerotic risk (RESCUE): a double-blind, randomised, placebo-controlled, phase 2 trial. *Lancet*. 2021;297:2060-2069.
38. Ridker PM. From C-reactive protein to interleukin-6 to interleukin-1: moving upstream to identify novel targets for atheroprotection. *Cir Res*. 2016;118:145-156.

How to cite this article: Dopico J, Botelho J, Ouro A, et al. Association between periodontitis and peripheral markers of innate immunity activation and inflammation. *J Periodontol*. 2023;94:11–19. <https://doi.org/10.1002/JPER.22-0216>