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Published in:
Journal of Clinical Periodontology

DOI:
10.1111/jcpe.13534

Publication date:
2021

Received: 24 February 2021 | Accepted: 25 July 2021

DOI: 10.1111/jcpe.13534

ORIGINAL ARTICLE CLINICAL PERIODONTOLOGY

Journal of Clinical Periodontology WILEY

Levels of myeloid-related proteins in saliva for screening and monitoring of periodontal disease

Ronaldo Lira-Junior[1] | Susan M. Bissett[2] | Philip M. Preshaw[3] | John J. Taylor[2] | Elisabeth A. Boström[1]

[1]Division of Oral Diagnostics and Rehabilitation, Department of Dental Medicine, Karolinska Institutet, Solna, Sweden

[2]School of Dental Sciences and Translational and Clinical Research Institute, Newcastle University, Newcastle upon Tyne, UK

[3]School of Dentistry, University of Dundee, Dundee, UK

Correspondence
Elisabeth A. Boström, Division of Oral Diagnostics and Rehabilitation, Department of Dental Medicine Karolinska Institutet, Huddinge, Sweden.
Email: elisabeth.bostrom@ki.se

Funding information
Stiftelsen Patentmedelsfonden för Odontologisk Profylaxforskning; Stockholms Läns Landsting, Grant/Award Number: SOF-project; the Engineering and Physical Sciences Research Council (EPSRC) UK funded by the Technology Strategy Board (Innovate UK), Grant/Award Number: EP/K502315/1

Abstract

Aim: To evaluate the salivary levels of myeloid-related markers in relation to periodontal disease and their potential screening capability, as well as the effects of periodontal treatment on these markers in periodontitis patients.

Materials and methods: Participants with a healthy periodontium ($n = 60$) and with gingivitis ($n = 63$) and periodontitis ($n = 72$) were recruited. Periodontitis patients received non-surgical treatment and were re-examined after 3 and 6 months. Unstimulated saliva was collected at baseline and at 1, 3, and 6 months after therapy for the periodontitis patients. Levels of colony-stimulating factor-1 (CSF-1), interleukin-34 (IL-34), S100A8/A9, S100A12, hepatocyte growth factor (HGF), IL-1β, and matrix metalloproteinase-8 (MMP-8) were analysed by immunoassays.

Results: CSF-1, S100A8/A9, S100A12, IL-1β, MMP-8, and HGF were significantly elevated in saliva from periodontitis and gingivitis patients in comparison to healthy individuals, whereas IL-34 was significantly lower in periodontitis compared to both healthy individuals and gingivitis patients. IL-34 increased significantly 3 months after treatment, while IL-1β and MMP-8 decreased 1 month after therapy. Additionally, periodontitis patients clustered in high and low levels of S100A8/A9, whereby those with high levels had more bleeding, deeper pockets, and higher S100A12.

Conclusions: Salivary levels of myeloid-related markers are altered in periodontitis and are partially modulated by periodontal treatment. Measuring S100A8/A9 in saliva may identify distinct groups of periodontitis patients.

KEYWORDS
myeloid cells, periodontal disease, saliva

Clinical Relevance

Scientific rationale for study: Myeloid cells are pivotal to the inflammatory process in periodontitis. Thus, measuring molecules that reflect their functions in saliva has the potential to improve screening and monitoring of periodontal inflammation.

Principal findings: Periodontitis patients showed altered levels of several myeloid-related markers in saliva, and periodontitis treatment led to a significant increase in IL-34 and a reduction in IL-

1β and MMP-8. S100A8/A9 levels in saliva identified two clusters of periodontitis patients with differences in clinical and inflammatory parameters.
Practical implications: Measuring myeloid-related markers in saliva can be useful to assess periodontal diseases. Evaluation of S100A8/A9 in saliva can inform identification of distinct groups of periodontitis patients, potentially helping patient monitoring and treatment.

1 | INTRODUCTION

Periodontitis is a highly prevalent chronic disease, which is characterized by a microbiota-induced inflammation of the tooth-supporting tissues and may eventually lead to tooth loss and contribute to the systemic inflammatory burden. Immune cells infiltrate the periodontal tissues and produce high concentrations of cytokines and proteolytic enzymes such as matrix metalloproteinases (MMPs), which mediate tissue damage leading to loss of function and clinical disease (Preshaw & Taylor, 2011). Among the immune cells, myeloid cells play a significant role in the pathogenesis of periodontitis, where they produce enzymes that undermine the integrity of the oral mucosa (Bjornfot Holmstrom et al., 2017) and mediate alveolar bone resorption (Lam et al., 2014). Therefore, molecules reflecting myeloid cell functions are potential biomarkers for the screening of periodontitis and in the assessment of response to therapy.

Colony-stimulating factor-1 (CSF-1) and interleukin-34 (IL-34) are growth factors regulating the survival, proliferation, and differentiation of mononuclear phagocytes, which are synthesized by different cell types (Pixley & Stanley, 2004), including gingival fibroblasts (Bostrom & Lundberg, 2013; Clark et al., 2020). Both CSF-1 and IL-34 regulate the differentiation of mononuclear phagocytes to osteoclasts in concert with the receptor activator of nuclear factor kappa-B ligand (Pixley & Stanley, 2004; Bostrom & Lundberg, 2013). Blockade of the CSF-1 receptor, which is shared by CSF-1 and IL-34, has been shown to reduce alveolar bone loss in a periodontitis mouse model, highlighting their role in periodontal pathology (Kimura et al., 2014). Additionally, our group has reported dysregulated levels of CSF-1 and IL-34 in saliva from patients with periodontitis (Lira-Junior, Akerman, et al., 2017; Martinez et al., 2017) as well as increased expression of CSF-1 in periodontitis-affected gingival tissue (Clark et al., 2020).

S100A8/A9, also known as calprotectin, and S100A12 are members of the S100 calcium-binding family of proteins, which are predominantly expressed by myeloid cells, although they are also inducible in other cell types (Perera et al., 2010). They function as danger signals to activate both immune and endothelial cells (Foell et al., 2007; Perera et al., 2010). S100A8/A9 and S100A12 are overexpressed at sites of inflammation in chronic inflammatory diseases (Foell et al., 2007), including periodontitis (Kido et al., 1999; Lira-Junior et al., 2020). Furthermore, these proteins are increased in saliva from periodontitis patients and could potentially be used as biomarkers of periodontitis (Haririan et al., 2016; Holmstrom et al., 2019; Lira-Junior et al., 2020). However, the assessment of these myeloid-related proteins in saliva to monitor periodontal inflammation longitudinally needs investigation, as well as their relationship to more well-established markers of periodontal inflammation, such as hepatocyte growth factor (HGF), IL-1β, and MMP-8 (Jaedicke et al., 2016).

Therefore, we set out to investigate the levels of myeloid-related markers in saliva in relation to periodontal disease and their potential to screen periodontitis. Furthermore, we aimed to evaluate the effects of non-surgical periodontal treatment on the salivary levels of these markers in individuals with periodontitis. These markers might be useful to screen periodontitis in settings where a clinical examination is not feasible and to stratify periodontitis patients with potential implications for therapy.

2 | MATERIALS AND METHODS

2.1 | Study participants

This study included 195 participants, among which 60 were periodontally healthy, 63 had gingivitis, and 72 had periodontitis. Individuals were recruited at the Newcastle upon Tyne Dental Hospital, following ethical approval (UK National Research Ethics Service North East Newcastle and North Tyneside 1 committee, ref: 12/NE/0396). All participants provided written informed consent and the study procedures were in accordance with the principles outlined in the Declaration of Helsinki.

The included participants were adults, between 18 and 65 years of age, non-smokers, and with a minimum of 20 natural teeth excluding third molars. Healthy participants had no sites with inter-proximal attachment loss, probing pocket depth (PPD) of ≤3 mm in all sites, bleeding on probing (BOP) ≤ 10%, and had <10% of sites with modified gingival index (mGI) ≥ 2; gingivitis participants had no sites with inter-proximal attachment loss or PPD > 4 mm, and had BOP ≥10% and >30% of the sites with mGI ≥3; patients with periodontitis had inter-proximal PPD ≥5 mm at ≥8 teeth and BOP ≥30%. Participants with periodontitis were diagnosed with moderate to severe periodontitis according to the classification system in use at the time (Armitage, 1999), corresponding to periodontitis stage 3 or 4, and grade B according to the new classification (Tonetti et al., 2018). Participants were excluded if they had infectious or systemic diseases, were undergoing treatment with antibiotics or immunosuppressants, were pregnant, or had smoked cigarettes within the last 2 years (Taylor et al., 2019).

2.2 | Periodontal examination and treatment

A full-mouth periodontal examination was performed by one of two calibrated clinical examiners who examined the same participants over

the course of the study, including BOP, PPDs, and clinical attachment loss (CAL) at six sites per tooth with a UNC-15 periodontal probe. mGI was also recorded (Lobene et al., 1986). Periodontal inflamed surface area and periodontal epithelial surface area were calculated as described previously (Nesse et al., 2008). Body mass index (BMI) was also calculated. Periodontitis patients received non-surgical periodontal therapy, which comprised root surface debridement and oral hygiene instruction. Patients were re-examined 3 and 6 months after therapy and provided with appropriate prophylaxis and oral hygiene instruction according to clinical need. A total of 65 patients returned to all follow-up visits.

2.3 | Saliva collection and biomarkers analysis

Unstimulated saliva samples were collected by expectoration into a plastic tube and centrifuged at 1500*g* for 15 min at 4°C. Aliquots were stored at −80°C until analysis. Saliva samples were collected at baseline from all participants, and at 1, 3, and 6 months after therapy for periodontitis patients. CSF-1, IL-34, S100A8/A9, S100A12, HGF, IL-1β, and MMP-8 levels in saliva were measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Apart from S100A proteins, all other assays were Quantikine assays. The assays had been previously validated for saliva in our respective labs (Jaedicke et al., 2012; Martinez et al., 2017; Holmstrom et al., 2019). Readings were taken using a microplate spectrophotometer (SpectraMAX 340, Sunnyvale, CA) at 450 nm with wavelength correction set to 540 nm to subtract background. Data regarding MMP-8 and IL-1β levels have been partially reported previously (Taylor et al., 2019).

Saliva samples were randomized and blindly analysed. The code key and the clinical status were revealed only after the laboratory analysis was completed. IL-34 was detected in 86% of the samples, whereas the other markers were detected in >95% of the samples. Samples below the detection limit were set as half of the lowest detected value. The sensitivity for each assay used in the study was as follows: CSF-1: 11.2 pg/ml; S100A8/A9: 20.4 pg/ml; S100A12: 7.8 pg/ml; IL-34: 3.06 pg/ml; HGF: 24.1 pg/ml; IL-1β: 1 pg/ml; and MMP-8: 0.058 ng/ml.

2.4 | Statistical analysis

Data analyses were performed using Statistical Package for Social Sciences, version 25 (SPSS, IBM Corporation, Armonk, NY) and GraphPad Prism 8 (GraphPad Software Inc., La Jolla, CA). Differences among the groups were calculated by Mann–Whitney, Kruskal–Wallis (with Dunn–Bonferroni post hoc), or Chi-square tests whenever appropriate. Intra-group differences after treatment were determined by the Friedman test with Dunn–Bonferroni post hoc test. Spearman's rank coefficient was used to assess the correlations between biomarkers levels in saliva and clinical parameters. The diagnostic capability of each marker was evaluated by calculating the areas under the curve of receiver-operating characteristics (AUC-ROC), sensitivity and specificity. Statistical significance was set at a p-value ≤ .05.

3 | RESULTS

3.1 | Clinical characteristics of the study participants

This study enrolled 60 periodontally healthy participants and 63 gingivitis and 72 periodontitis patients. The clinical characteristics of the three study groups are presented in Table 1. Periodontitis patients were significantly older than both healthy and gingivitis participants ($p < .01$), while no significant difference was seen between healthy and gingivitis subjects. Periodontitis patients were also more frequently male and former smokers ($p = .007$ and $p < .001$, respectively). All periodontal parameters were significantly worse in periodontitis patients than in both healthy and gingivitis participants. With the exception of the number of teeth, gingivitis patients also showed worse periodontal parameters than healthy subjects.

3.2 | Salivary levels of myeloid-related markers in periodontal disease

We then investigated the levels of several myeloid-related markers in saliva and their relation to periodontal parameters and to each other. Salivary levels of CSF-1, S100A8/A9, S100A12, IL-1β, MMP-8, and HGF were significantly elevated in both periodontitis and gingivitis groups compared to control groups (Figure 1a–g). Additionally, S100A8/A9, IL-1β, MMP-8, and HGF levels in saliva were significantly increased in periodontitis in comparison with gingivitis. On the other hand, salivary levels of IL-34 were significantly lower in the periodontitis group compared to both healthy and gingivitis groups (Figure 1b). While CSF-1 was increased in disease, IL-34 was increased in health. As both factors signal through the same receptor, CSF-1R, we looked at the ratio between CSF-1 and IL-34 and found it was significantly higher in periodontitis in comparison with both healthy and gingivitis ($p < .001$ and $p = .001$, respectively; Figure S1a). Levels of CSF-1, S100A8/A9, S100A12, IL-1β, MMP-8, and HGF showed significant positive correlations with all periodontal parameters. IL-34 levels correlated negatively with the percentage of PPD ≥5 mm (Figure 1h). Overall, there was no difference in any of the markers between men and women (data not shown).

We analysed the diagnostic performance of these markers to distinguish between periodontitis and health and gingivitis alone, as well as between periodontitis and non-periodontitis. Apart from IL-34, all markers showed good ability to discriminate between periodontitis and health (AUC > 0.75; Figure S2a). All markers also showed distinct ability to differentiate between periodontitis and gingivitis with AUC ranging from 0.615 to 0.770, with MMP-8 (AUC 0.759, 95% confidence interval [CI] 0.68–0.84) and HGF (AUC 0.770, 95% CI 0.69–0.85) displaying the highest values (Figure S2b). Regarding the ability to distinguish between periodontitis and non-periodontitis patients, MMP-8 and HGF displayed the highest areas, with AUC of, respectively, 0.834 and 0.838 (95% CI 0.78–0.89; Figure S2c), while the AUC for the other markers were as follows: CSF-1 (0.687, 95% CI 0.61–0.76); IL-34 (0.670,

TABLE 1 Clinical characteristics of the study groups at baseline

	Healthy ($n = 60$)	Gingivitis ($n = 63$)	Periodontitis ($n = 72$)	p-Value
Age (years)	35.7 (±12.5)	38.2 (±11.5)	46.3 (±8.0)[a,b]	<.001
Gender (M/F)	17/43	20/43	38/34	.007
Smoking (no/former)	54/6	54/9	44/28	<.001
BMI (kg/m^2)	24.6 (±5.7)	26.2 (±5.4)	27.0 (±5.7)[a]	.007
No. of teeth	26.8 (±1.5)	26.6 (±1.8)	26.0 (±1.9)[a]	.023
Mean mGI	0.3 (±0.2)	2.4 (±0.3)[a]	2.8 (±0.4)[a,b]	<.001
BOP (%)	2.7 (±3.0)	28.0 (±11.7)[a]	55.1 (±17.5)[a,b]	<.001
Mean PPD (mm)	1.7 (±0.4)	2.1 (±0.3)[a]	3.8 (±0.8)[a,b]	<.001
Mean CAL (mm)	—	—	4.5 (±0.9)	—
PPD ≥ 5 mm (%)	—	—	33.2 (±15.9)	—
PESA	849.7 (±183.6)	1040.6 (±206.9)[a]	2188.5 (±565.7)[a,b]	<.001
PISA	30.3 (±34.9)	311.8 (±148.7)[a]	1394.7 (±660.3)[a,b]	<.001

Note: Continuous variables are presented as the mean±standard deviation. Categorical variables are presented as natural frequencies. p-Values were computed by Kruskal–Wallis (with Dunn–Bonferroni post hoc) or Chi-square tests.

Abbreviations: BMI, body mass index; BOP, bleeding on probing; CAL, clinical attachment loss; mGI, modified gingival index; PESA, periodontal epithelial surface area; PISA, periodontal inflamed surface area; PPD, probing pocket depth.

[a]Significant difference in comparison with healthy.

[b]Significant difference in comparison with gingivitis.

95% CI 0.59–0.75); S100A8/A9 (0.738, 95% CI 0.66–0.81); S100A12 (0.693, 95% CI 0.62–0.77); and IL-1β (0.783, 95% CI 0.72–0.85). The sensitivity and specificity of each marker to discriminate periodontitis from non-periodontitis are presented in Table S1.

3.3 | Effects of periodontal therapy on clinical and salivary parameters

Next, we assessed the effects of periodontal therapy on periodontal parameters and salivary markers. Periodontitis patients received non-surgical periodontal treatment and were reassessed at 3 and 6 months after therapy. Out of the 72 periodontitis patients who started the study, 65 completed it. All periodontal parameters improved significantly 3 and 6 months after treatment in comparison with the baseline ($p < .001$). No further improvement was seen at 6 months compared with 3 months (Table 2). At the end of the follow-up, average reductions of 37.8% and 0.9 mm were seen in mean BOP and PPD values, respectively, whereas the mean CAL improved by 0.8 mm.

Considering the salivary markers, the levels of CSF-1 and HGF did not change significantly after treatment ($p = .067$ and $p = .089$, respectively; Figure 2a,g). IL-34 increased significantly 3 months after treatment compared to the baseline ($p = .042$; Figure 2b). No significant change was found in the levels of S100A8/A9 and S100A12 after non-surgical therapy (Figure 2c,d). On the contrary, IL-1β and MMP-8 levels decreased significantly 1 month after therapy in comparison with the baseline ($p = .001$ and $p = .010$; Figure 2e,f). Additionally, lowered level of IL-1β was seen 3 months after treatment, but this did not reach statistical significance ($p = .075$). The CSF-1/IL-34 ratio decreased significantly 3 months after treatment in comparison with baseline ($p = .002$; Figure S1b).

3.4 | Salivary levels of S100A8/A9 stratifies periodontitis patients

We observed two distinct clusters of periodontitis patients according to their levels of S100A8/A9 in saliva at baseline (Figure 3a). One cluster containing 34 patients presented low levels of S100A8/A9 (3080.2 ± 2453.5 ng/ml, "low" group). The second cluster, with 38 patients, presented on average 13 times higher S100A8/A9 in saliva (40262.5 ± 22662.5 ng/ml, "high" group; $p < .001$). Patients with high levels of S100A8/A9 were slightly younger than patients with low levels (44.2 ± 8.3 vs. 48.6 ± 7.0; $p = .043$). They also had more teeth, higher BOP, deeper pockets, and greater epithelial and inflamed surface areas ($p < .05$; Table 3). Additionally, patients with high levels of S100A8/A9 also had higher levels of S100A12 ($p < .001$; Figure 3d). No significant difference was found between the groups in the levels of the other markers (Figure 3; Figure S1c). Moreover, patients in the "high" group showed higher BOP, deeper pockets, and greater epithelial and inflamed surface areas 3 months after treatment. At the end of follow-up, patients in the "high" group had higher gingival index scores and greater epithelial surface area than the "low" group ($p < .05$; Table 3).

Regarding longitudinal changes in the levels of biomarkers, a distinct response pattern to therapy was observed in the groups. S100A8/A9 levels increased significantly 3 and 6 months after treatment in the "low" group ($p = .031$ and $p = .004$, respectively), while a significant decrease was found 1 month after treatment in the "high"

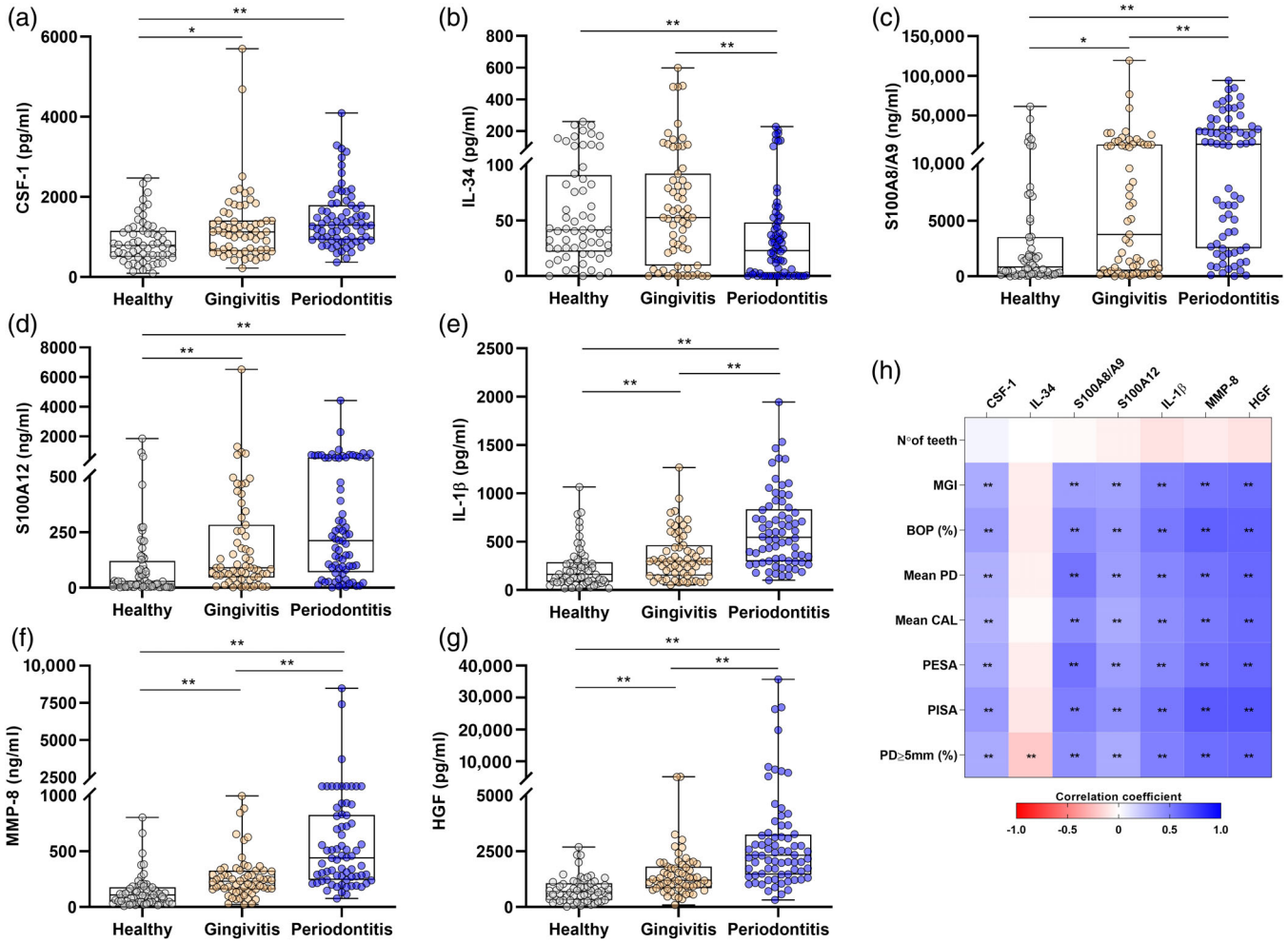

FIGURE 1 Salivary levels of myeloid-related markers in relation to periodontal disease and their correlations to clinical parameters. Salivary levels of (a) Colony-stimulating factor-1 (CSF-1), (b) interleukin (IL)-34, (c) S100A8/A9, (d) S100A12, (e) hepatocyte growth factor (HGF), (f) IL-1β, and (g) matrix metalloproteinase (MMP)-8 in healthy ($n = 60$) and gingivitis ($n = 63$) and periodontitis patients ($n = 72$). (h) Correlation heat map of salivary markers and periodontal parameters. Spearman correlation was used. Groups were compared using Kruskal–Wallis tests with Dunn's post hoc test. *$p < .05$, **$p < .001$. BOP, bleeding on probing; CAL, clinical attachment loss; MGI, mean gingival index; PESA, periodontal epithelial surface area; PISA, periodontal inflamed surface area; PPD, probing depth [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Clinical parameters of participants with periodontitis at baseline (M0) and 3 (M3) and 6 (M6) months post-treatment

	M0 ($n = 72$)	M3 ($n = 67$)	M6 ($n = 67$)	*p*-Value
No· of teeth	26.0 (±1.9)	25.9 (±2.0)	25.9 (±2.0)	.202
Mean mGI	2.8 (±0.4)	1.3 (±0.6)[a]	1.2 (±0.7)[a]	<.001
BOP (%)	55.1 (±17.5)	18.7 (±15.3)[a]	17.3 (±12.9)[a]	<.001
Mean PPD (mm)	3.8 (±0.8)	2.9 (±0.7)[a]	2.9 (±0.7)[a]	<.001
Mean CAL (mm)	4.5 (±0.9)	3.7 (±0.9)[a]	3.7 (±0.9)[a]	<.001
PPD ≥ 5 mm (%)	33.2 (±15.9)	14.8 (±12.9)[a]	14.7 (±12.4)[a]	<.001
PESA	2188.5 (±565.7)	1584.7 (±482.4)[a]	1571.4 (±509.1)[a]	<.001
PISA	1394.7 (±660.3)	400.8 (±433.6)[a]	377.4 (±411.7)[a]	<.001

Note: Continuous variables are presented as mean±standard deviation. *p*-Values were computed by Friedman tests with Dunn–Bonferroni post hoc test.
Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; mGI, modified gingival index; PESA, periodontal epithelial surface area; PISA, periodontal inflamed surface area; PPD, probing depth.
[a]Significant difference in comparison with M0.

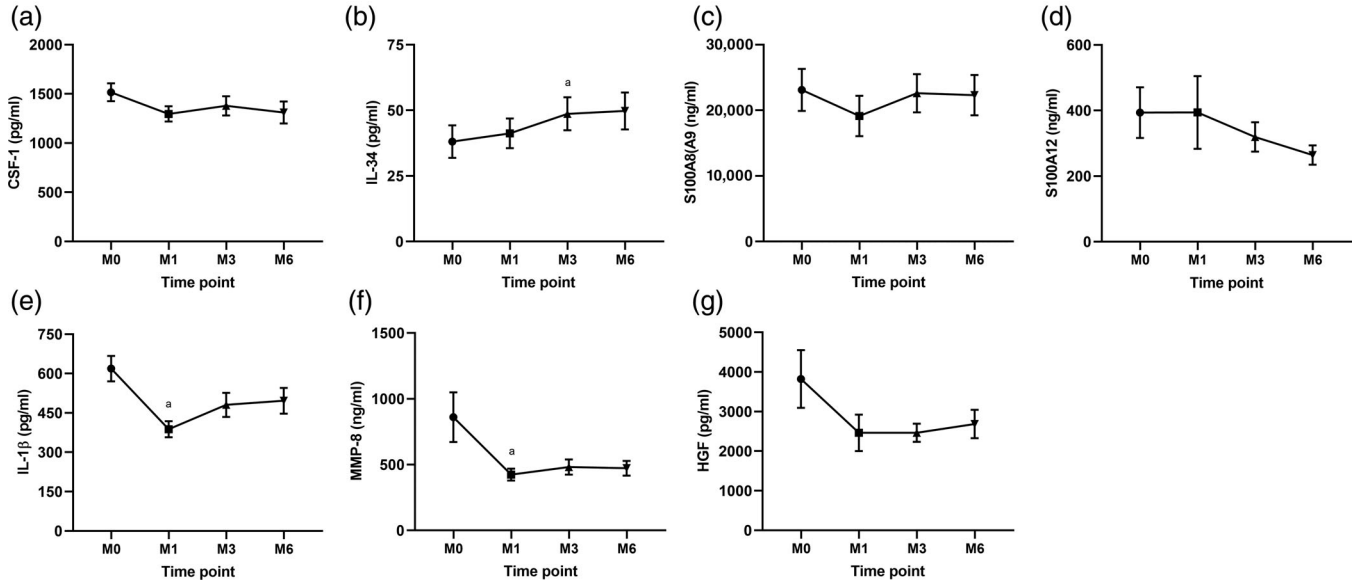

FIGURE 2 Effect of periodontal treatment on salivary levels of myeloid-related markers in periodontitis patients. Salivary levels of (a) Colony-stimulating factor-1 (CSF-1), (b) interleukin-34 (IL)-34, (c) S100A8/A9, (d) S100A12, (e) hepatocyte growth factor (HGF), (f) IL-1β, and (g) matrix metalloproteinase (MMP)-8 in periodontitis patients ($n = 66$) at baseline (M0) and 1 (M1), 3 (M3), and 6 months (M6) after non-surgical periodontal treatment. Groups were compared using Friedman tests with Dunn's post hoc test. [a]Significantly different in comparison with M0. Data are presented as mean±standard error of mean

group in comparison with the baseline ($p = .034$; Figure 3h). There was no significant decrease at 6 months in the "high" group ($p = .060$). S100A8/A9 levels remained significantly higher in the "high" group than in the "low" group at 1 and 3 months after therapy ($p < .001$ for both). IL-1β levels decreased significantly 1 and 3 months after treatment compared to baseline in the "low" group ($p = .001$ and $p = .026$, respectively; Figure 3l). Similarly, MMP-8 and HGF decreased significantly 1 month after treatment compared to baseline in the "low" group ($p = .001$ and $p = .022$, respectively; Figure 3m,n). Despite the lack of statistical significance, it is also interesting to note some suggestion of patterns of change, such as lower MMP-8 levels in the "low" group ($p = .066$; Figure 3m) and decreased CSF-1/IL-34 ratio in both "low" and "high" groups 3 months after therapy ($p = .063$ and $p = .096$, respectively; Figure S1d), as well as an increase in IL-34 levels in the "high" group ($p = .077$; Figure 3j), all of which warrant further investigation in larger cohorts.

4 | DISCUSSION

The use of saliva for screening and monitoring diseases is constantly evolving. Its use in periodontal disease has the potential to transform periodontal care and provide information on early disease detection, risk stratification, and response to therapy. In that regard, identification and thorough investigation of markers related to pathogenic processes are of utmost relevance. Myeloid cells play a significant role in the inflammation-driven tissue destruction in periodontitis (Lam et al., 2014; Bjornfot Holmstrom et al., 2017). Therefore, markers reflecting their functions are putative candidates to screen and monitor periodontal inflammation and to provide clues to disease pathogenesis. While MMP-8 has been widely studied in this context (Sorsa et al., 2016), the potential of other salivary markers to screen and monitor periodontal inflammation are to date less studied. This study found that salivary levels of the myeloid-related markers CSF-1, S100A8/A9, S100A12, HGF, IL-1β, and MMP-8 were increased, while IL-34 was decreased in periodontitis. IL-1β and MMP-8 levels decreased significantly 1 month, whereas IL-34 increased 3 months after therapy. Additionally, we found S100A8/A9 levels in saliva stratified periodontitis patients into two clusters with distinct clinical and inflammatory characteristics.

We found increased salivary levels of CSF-1, S100A8/A9, and S100A12 in both gingivitis and periodontitis in comparison with healthy participants. These markers also showed significant positive correlations with clinical periodontal parameters. On the other hand, IL-34 levels were significantly lower in periodontitis than in both healthy and gingivitis, and correlated negatively to the percentage of sites with PPD ≥5 mm. As both CSF-1 and IL-34 share a receptor, CSF-1R, and their levels are differentially modulated in response to periodontitis, we looked at their ratio and found it also to be higher in periodontitis. These results are partially in agreement with previous findings of altered levels of these markers in saliva in periodontal disease (Haririan et al., 2016; Lira-Junior, Akerman, et al., 2017; Martinez et al., 2017; Lira-Junior et al., 2020) and add more evidence to the role of myeloid cells in periodontal pathogenesis. In contrast to a previous study from our group (Martinez et al., 2017), here we found that CSF-1 and IL-34 levels were dysregulated in gingivitis in comparison with health and periodontitis, respectively. We believe this could be at least partially explained by the differences in the clinical

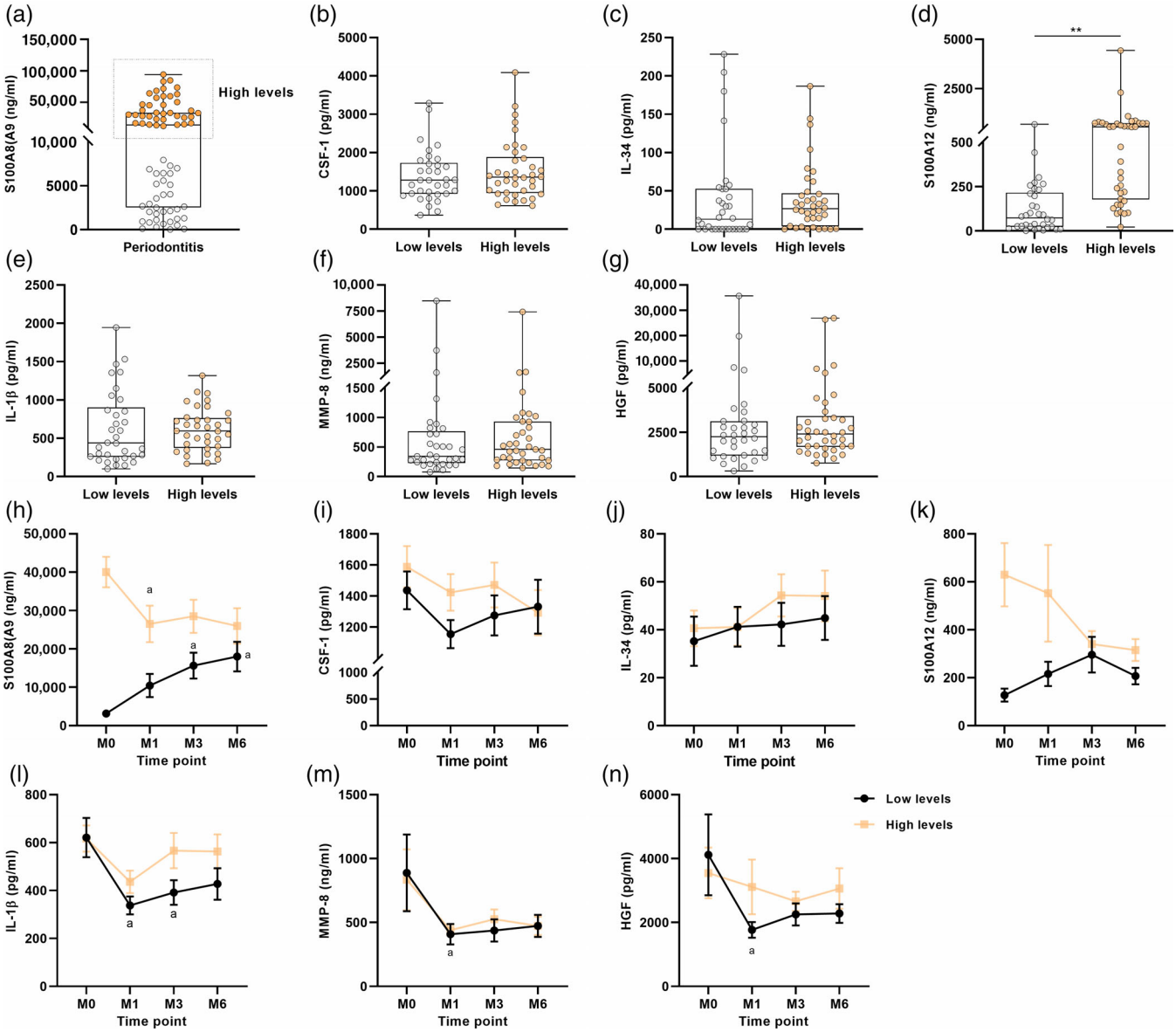

FIGURE 3 Salivary levels of myeloid-related markers according to baseline levels of S100A8/A9 and their modulation by periodontal treatment. (a) Salivary levels of S100A8/A9 in periodontitis patients ($n = 72$) displaying two groups with distinct expression. (b) Colony-stimulating factor-1 (CSF-1), (c) interleukin-34 (IL)-34, (d) S100A12, (e) hepatocyte growth factor (HGF), (f) IL-1β, and (g) matrix metalloproteinase (MMP)-8 levels in periodontitis patients with low ($n = 34$) and high ($n = 38$) levels of S100A8/A9 at baseline. (h) S100A8/A9, (i) CSF-1, (j) IL-34, (k) S100A12, (l) HGF, (m) IL-1β, and (n) MMP-8 at baseline (M0) and 1 (M1), 3 (M3), and 6 months (M6) after non-surgical periodontal treatment in patients with low and high S100A8/A9 levels at baseline. Groups were compared using Mann–Whitney tests. $^{*}p < .05$, $^{**}p < .001$. Intra-group comparisons were done with Friedman tests with Dunn's post hoc test. [a]Significantly different in comparison with M0. Data are presented as mean±standard error of mean [Colour figure can be viewed at wileyonlinelibrary.com]

inflammation between the cohorts, as the gingivitis group in the previous study had a mean BOP of 10% whereas the mean here was 28%.

It is noteworthy to report that these myeloid-related markers correlated significantly to the more well-known markers of periodontal inflammation such as IL-1β and MMP-8 (Jaedicke et al., 2016). This is in line with other studies pointing out that inflammatory cytokines correlate to each other in saliva (Lira-Junior, Akerman, et al., 2017; Martinez et al., 2017; Medara et al., 2020), forming an inflammatory network. Since these markers are mainly expressed by myeloid cells, it is possible that their levels reflect ongoing changes in these cells in tissue during disease. Along with the correlation to myeloid-related markers, HGF, IL-1β and MMP-8 also displayed higher levels in saliva from both periodontitis and gingivitis than healthy participants. Moreover, similarly to S100A8/A9 and the CSF-1/IL-34 ratio, they were also higher in periodontitis than in gingivitis. This is in concordance with previous literature, which has consistently shown increased levels of these markers in periodontitis (reviewed in Sorsa et al., 2016; Jaedicke et al., 2016).

TABLE 3 Clinical response to treatment of participants with periodontitis according to their baseline levels of S100A8/A9

	Low levels ($n = 34$)				High levels ($n = 38$)			
	M0	M3	M6	*p*-Value	M0	M3	M6	*p*-Value
No. of teeth	25.6 (±1.7)	25.5 (±1.8)	25.5 (±1.9)	.513	26.4 (±2.0)*	26.3 (±2.2)	26.3 (±2.1)	.368
Mean mGI	2.8 (±0.3)	1.2 (±0.6)[a]	0.9 (±0.6)[a]	<.001	2.8 (±0.5)	1.4 (±0.7)[a]	1.5 (±0.7)[a,*]	<.001
BOP (%)	50.5 (±13.9)	13.3 (±8.6)[a]	13.6 (±8.8)[a]	<.001	59.3 (±19.3)*	23.4 (±18.2)[a,*]	20.7 (±15.0)[a]	<.001
Mean PPD (mm)	3.5 (±0.7)	2.7 (±0.5)[a]	2.7 (±0.5)[a]	<.001	4.0 (±0.8)*	3.0 (±0.8)[a,*]	3.1 (±0.7)[a]	<.001
Mean CAL (mm)	4.6 (±0.8)	3.7 (±0.9)[a]	3.7 (±0.9)[a]	<.001	4.3 (±1.0)	3.7 (±0.9)[a]	3.7 (±0.9)[a]	<.001
PPD ≥ 5 mm (%)	29.0 (±15.2)	11.2 (±9.2)[a]	12.4 (±9.0)[a]	<.001	36.9 (±15.9)*	18.0 (±14.8)[a,*]	16.8 (±14.8)[a]	<.001
PESA	1996.7 (±550.8)	1421.6 (±383.9)[a]	1425.7 (±399.4)[a]	<.001	2360.1 (±528.7)*	1725.1 (±518.34)[a,*]	1704.7 (±565.1)[a,*]	<.001
PISA	1193.1 (±566.0)	256.4 (±210.9)[a]	262.9 (±214.9)[a]	<.001	1575.2 (±693.0)*	525.2 (±531.2)[a,*]	482.1 (±513.1)[a]	<.001

Note: Continuous variables are presented as mean±standard deviation. Comparisons were made with $n = 65$ (patients who completed all examinations). *p*-Values were computed by Friedman tests with Dunn–Bonferroni post hoc test.

Abbreviations: BOP, bleeding on probing; CAL, clinical attachment loss; mGI, mean gingival index; PESA, periodontal epithelial surface area; PISA, periodontal inflamed surface area; PPD, probing depth.

[a]Significant difference in comparison with M0.

*Significant difference compared to the same time point in "low" levels (Mann–Whitney test).

We also assessed the ability of these myeloid-related markers to differentiate between the different periodontal conditions. While most markers exhibited good ability to discriminate between periodontitis and health, this ability decreased when analysing gingivitis alone or in combination with health. This is expected, as some of these markers also showed altered levels in gingivitis and had previously been appreciated for IL-1β and MMP-8, for example (Ebersole et al., 2015). Despite that, CSF-1, IL-34, and the S100A proteins exhibited moderate performance in distinguishing periodontitis patients from gingivitis/healthy participants, with AUC varying from 0.67 to 0.74, whereas HGF, IL-1β, and MMP-8 performed well in differentiating periodontitis from gingivitis/health. It is important to fully validate these biomarkers and analyse their performance in differentiating between the different periodontal conditions, as those that reliably reflect the periodontal condition could be used, among others, in settings where a full-mouth examination is not feasible or in large-scale population screenings, after which individuals can be referred to a dentist.

Furthermore, we assessed the effect of periodontal intervention on the levels of these markers and found that IL-1β and MMP-8 decreased significantly at 1 month, while the CSF-1/IL-34 ratio decreased at a later timepoint, that is, 3 months after periodontal treatment. IL-34, on the other hand, increased significantly 3 months post therapy. These results are partially in agreement with previous studies demonstrating the effects of root surface debridement in reducing the salivary levels of IL-1β, MMP-8 (Kinney et al., 2011; Sexton et al., 2011), and CSF-1/IL-34 ratio in periodontitis (Martinez et al., 2017). A previous small exploratory study, however, did not find a significant effect of periodontal therapy on IL-34 levels (Martinez et al., 2017). It is important to mention that we had reported a significant decrease in MMP-8 levels 6 months after treatment (Taylor et al., 2019), and now we extend this finding by showing that this decrease happened 1 month after treatment and it was maintained until the end of follow-up without further significant change.

We observed two distinct clusters of periodontitis patients based on their levels of S100A8/A9, where patients with "high" levels exhibited 13 times higher mean S100A8/A9 than patients with "low" levels. These clusters presented different clinical and inflammatory features, such as slightly younger age, more bleeding and inflamed surface, deeper pockets, and increased S100A12 in patients with high S100A8/A9. High variability in S100A8/A9 levels and patient clustering have been previously reported in gingival crevicular fluid (Que et al., 2004; Farina et al., 2012) and saliva (Lira-Junior, Ozturk, et al., 2017). Our previous study found participant clustering based on the levels of S100A8/A9, where the group with "high S100A8/A9" was older and showed increased levels of CSF-1, MMP-8, and macrophage migration inhibitory factor in saliva (Lira-Junior, Ozturk, et al., 2017). However, this pattern observed in the previous study included participants with different periodontal conditions, while in the current study only periodontitis patients were included in the analysis, which can account at least partially for the contradictory results. This will need to be addressed in further studies of independent patient cohorts.

Interestingly, S100A8/A9 levels followed opposite trends after treatment in the two clusters of patients: decreasing in patients with "high levels" at baseline, and increasing in those with "low levels", reaching similar levels 6 months after treatment. This was accompanied by differences in the periodontal condition after treatment, where patients with "high levels" had worse gingival inflammation and greater epithelial surface area at the end of follow-up in comparison with those with "low levels". Furthermore, HGF, IL-1β and MMP-8 decreased significantly in patients with "low levels". In concordance with our findings, Que et al. (2004) found, in a study of experimental gingivitis, that the amount of S100A8/A9 in GCF identified two groups of participants with different response patterns. Additionally, Kaner et al. (2011) reported elevated levels of S100A8/A9 in GCF in sites that were found to progress, suggesting that it could be used to predict disease activity. S100A8/A9 is mainly expressed by myeloid cells and comprises approximately 45% of the cytosolic protein content in neutrophils (Edgeworth et al., 1991). Thus, given its short half-life in circulation, it is tempting to speculate S100A8/A9 as a good marker of ongoing inflammatory activity in the periodontal tissues. However, whether the levels of S100A8/A9 indicate different susceptibility profiles to periodontitis and are related to risk of future periodontal breakdown deserve investigation.

Our findings should be interpreted in the context of the limitations of the clinical study. This was an observational study in which only periodontitis patients were monitored longitudinally. Randomized clinical trials assessing the effect of periodontal treatment on the levels of these biomarkers are warranted. Also, monitoring of both periodontally healthy and gingivitis participants would have been of great value to better understand the variations in biomarker concentrations and whether they can predict progression from gingivitis to periodontitis. Longer follow-ups in larger cohorts are also necessary to evaluate whether these markers can predict disease activity. Further, an evaluation of the salivary microbial profile could increase our understanding of the distinct S100A8/A9 groups and their relationship to disease progression and response to treatment (Kinney et al., 2011). As periodontitis stems from an intricate, dysregulated interaction between the host response and the microbial challenge, clinical studies assessing a wide range of markers reflecting different pathogenic processes will be critical in identifying useful markers that could be translated into clinical practice and improve periodontal care (Kinney et al., 2011; Salminen et al., 2014). Nevertheless, some strengths of our study should also be highlighted, such as the well-characterized clinical groups, the exclusion of potential confounders, and the blinded evaluation of the biomarkers. Likewise, the analysis of markers related to myeloid cells is relevant given the critical role of these cells in the pathogenesis of periodontitis.

In conclusion, the salivary levels of myeloid-related markers are altered in periodontitis and could be used to distinguish periodontitis from non-periodontitis patients. Treatment of periodontitis had a significant impact on the levels of IL-34, IL-1β, MMP-8, and the CSF-1/IL-34 ratio. Furthermore, we showed that measuring S100A8/A9 in saliva could identify distinct groups of periodontitis patients with different clinical and inflammatory features at baseline and after

treatment. Assessment of biomarkers in saliva is already a reality, and gives insights into disease pathogenesis, stratification, and monitoring.

ACKNOWLEDGEMENTS

This project was supported in part by a grant (to John J. Taylor and Philip M. Preshaw) from the Engineering and Physical Sciences Research Council (EPSRC) UK, funded by the Technology Strategy Board (Innovate UK), grant reference EP/K502315/1, as well as by grants (to Elisabeth A. Boström) from Stockholm County Council and Swedish Patent Revenue Fund for Research in Preventive Odontology.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding this work.

AUTHOR CONTRIBUTIONS

Ronaldo Lira-Junior, Philip M. Preshaw, John J. Taylor, and Elisabeth A. Boström contributed to the conception of the study; Susan M. Bissett, Philip M. Preshaw, and John J. Taylor contributed to the clinical part of the study and sample collection; Ronaldo Lira-Junior, John J. Taylor, and Elisabeth A. Boström contributed to laboratory analyses and drafted the manuscript. All authors critically revised and gave final approval of the manuscript.

ETHICS STATEMENT

This study was approved by the UK National Research Ethics Service North East Newcastle and North Tyneside 1 committee (ref: 12/NE/0396), and the study procedures were conducted in accordance with the principles outlined in the Declaration of Helsinki. All participants provided written informed consent.

DATA AVAILABILITY STATEMENT

Data are available upon reasonable request to the corresponding author.

ORCID

Philip M. Preshaw https://orcid.org/0000-0002-7153-4865
Elisabeth A. Boström https://orcid.org/0000-0003-1788-7501

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How to cite this article: Lira-Junior, R., Bissett, S. M., Preshaw, P. M., Taylor, J. J., & Boström, E. A. (2021). Levels of myeloid-related proteins in saliva for screening and monitoring of periodontal disease. *Journal of Clinical Periodontology*, 1–11. https://doi.org/10.1111/jcpe.13534