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MMP-12 and S100s in saliva reflect different aspects of periodontal inflammation

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ABSTRACT

Matrix metalloproteinase (MMP)-12, S100A8/A9, and S100A12 are involved in innate immune responses. We addressed whether different aspects of oral health and non-disease-related covariates influence their levels in saliva. 436 participants were clinically examined, completed a health questionnaire, and provided stimulated saliva. Salivary levels of MMP-12, S100A8/A9, and S100A12 were determined by enzyme-linked immunosorbent assays. Lower MMP-12 levels were observed in individuals 40–64 years old (yo) compared to < 40 yo, and higher S100A8/A9 levels were found in individuals > 64 yo compared to 40–64 yo. Smokers exhibited lower MMP-12 and S100A12 levels compared to non-smokers. All three proteins were elevated in individuals with bleeding on probing (BOP) > 20% compared to those with BOP ≤ 20%, and the S100A8/A9 levels were higher in individuals having ≥ 10% gingival pocket depths (PPD) ≥ 4 mm compared to the ones with shallow pockets < 4 mm. The extent of alveolar bone loss or presence of manifest caries did not alter any of the markers. MMP-12, S100A8/A9, and S100A12 levels were higher in participants with high periodontal inflammatory burden. All three proteins correlated positively to BOP, PPD, and to several inflammatory mediators. The explanatory variables for MMP-12 in saliva were age, smoking, presence of any tumor, and percentage of PPD ≥ 4 mm. The determinant of salivary S100A8/A9 was percentage of BOP, while S100A12 levels were associated with percentage of BOP and presence of any tumor. Taken together, MMP-12 and the S100/calgranulin levels in saliva reflect different aspects of periodontal inflammation. Smoking and age should be taken into account in further investigation of these proteins as biomarker candidates of periodontal disease.

1. Introduction

Saliva is noninvasive to access and is emerging as an attractive fluid for biomarker analysis in oral diseases such as periodontal disease, caries, and oral cancer as well as systemic diseases. Whole saliva is a complex biofluid that contains components from the salivary glands, oral mucosa cells, gingival crevicular fluid, plaque, and blood. Around 27% of whole-saliva proteins are also found in plasma, and approximately 40% of the circulating candidate biomarkers of cancer and cardiovascular disease can also be found in whole saliva [1,2].

Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in extracellular matrix (ECM) remodeling, organogenesis, and inflammatory reactions. MMP-12 (macrophage metalloelastase) is produced and released by monocyte-derived cells and macrophages upon stimulation with bacterial components or cytokines, and exerts the ability to degrade ECM components [3,4]. MMP-12 has been associated with chronic inflammatory and tissue destructive diseases such as Crohn's disease and rheumatoid arthritis (RA) [5,6]. MMP-12 has also

Abbreviations: MMP, matrix metalloproteinase; ECM, extracellular matrix; RA, rheumatoid arthritis; PIBI, periodontal inflammatory burden index; BOP, bleeding on probing; PPD, probing pocket depth; MCL, manifest caries lesions; DMFT, decayed, missing and filled teeth; IL, interleukin; CSF, colony stimulating factor; TIMP, tissue inhibitor of metalloproteinases; GCF, gingival cervicular fluid

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been implicated in aggressive periodontitis where its levels decrease in gingival crevicular fluid after periodontal treatment [7]. We have recently found an increase in MMP-12 tissue expression in chronic periodontitis, and identified monocyte-derived $CD68^+CD14^+CD64^+$ cells as a source of MMP-12 [8]. The MMP pathway has been implicated in periodontal pathogenesis and several biomarker candidates including MMP-8 and MMP-14 have been proposed [9,10]. Considering the functional properties of MMP-12 and its involvement in tissue destructive diseases, it is relevant to assess the potential of MMP-12 as a biomarker of oral diseases.

Calgranulins are a subgroup of molecules within the broader family of S100 calcium-binding proteins comprising S100A8 (calgranulin A), S100A9 (calgranulin B), and S100A12 (calgranulin C). S100A8 and S100A9 preferentially form a heterodimer, S100A8/A9 (calprotectin). These proteins are mainly expressed by neutrophils and monocytes/macrophages [11], and act as danger signals that activate immune and endothelial cells [12]. S100A8/A9 and S100A12 are implicated in RA and are used clinically to assess inflammatory bowel disease activity [13–16]. Increased S100A8/A9 levels in saliva and serum from periodontitis patients have been reported [17,18]. Similarly, increased levels of S100A12 have been measured in gingival crevicular fluid and serum from periodontitis patients [19]. Regarding caries, an increase in the transcript levels of S100A8, S100A9, and S100A12 has been found in carious pulpal tissue [20], and therefore their levels in saliva might be altered due to the carious process.

The S100/calgranulins have the ability to bind zinc and inhibit MMP activity by sequestration of zinc from their active sites [21,22]. S100A8/A9 has further been shown to up-regulate MMP-3, -9 and -13 expressions in murine macrophages [23], and to increase the expression of MMP-2 and MMP-12 in gastric cancer cells [24]. Periodontitis is a complex inflammatory condition and therefore, the analysis of multiple markers that reflect different biological processes has been suggested [10]. To the best of our knowledge, a comprehensive evaluation of MMP-12 or S100/calgranulins levels in saliva and their association to oral diseases in a large population are yet to be performed. In this study, we therefore aimed to evaluate MMP-12, S100A8/A9, and S100A12 levels in saliva in relation to different aspects of oral health, as well as to assess the influence of non-disease-related covariates on the levels of these proteins.

2. Material and methods

2.1. Study participants and saliva sampling

This study included 436 participants from a cohort previously described [25]. General description and clinical parameters of the cohort are presented in Table 1. All the participants signed an informed consent prior to enrolment, which was approved by the regional ethics committee at the Lund University, Sweden (Dnr. 2013/125). Participants in Skåne county of Sweden were randomly selected and invited to fill out a health questionnaire and those that completed the form were subjected to a clinical oral examination by dentists at the Department of Oral Diagnostics, Faculty of Odontology, Malmö University. Stimulated saliva samples were collected during 5 min of chewing on 0.5 g paraffin. Salivary flow rates were measured and the saliva was immediately frozen at −20 °C until processing when the samples were centrifuged and supernatants were aliquoted and stored at −80 °C until analysis.

2.2. Measures of oral health

The clinical oral examination included recordings of plaque index, bleeding on probing (BOP), probing pocket depth (PPD), number of manifest caries lesions (MCL), and number of decayed, missing and filled teeth (DMFT). Radiographic examination was performed for evaluation of alveolar bone loss. To investigate the association between oral health measures and analyzed protein levels in saliva, the cohort was divided and assessed within the following groups: BOP ≤ 20% or > 20%; PPD < 4 mm, 1–9% or ≥ 10% of the sites having PPD ≥ 4 mm; loss of supporting bone tissue < 1/3 of the root length (PD- group), ≥ 1/3 of the root length in < 30% of the sites (PD group), and horizontal bone loss ≥1/3 of the root length in > 30% of the sites (PD+ group).

Table 1
Demographic characteristics of the study population.

Variable	Whole cohort (n = 436)
Age (years)	48.43 (± 16.89)
Sex (female/male)	221/215
Smoking (n, %)	74 (17.00%)
Heart disease (n, %)	32 (7.34%)
Hypertension (n, %)	71 (16.28%)
Diabetes (n, %)	15 (3.44%)
Bowel diseases (n, %)	30 (6.88%)
Muscle and joint diseases (n, %)	94 (21.56%)
Tumor (n, %)	16 (3.67%)
Mental illness (n, %)	24 (5.50%)
Manifest caries lesion (n)	0.69 (± 1.48)
DMFT (n)	14.38 (± 8.09)
%-chance of avoiding new caries	63.54 (± 21.63)
Plaque index (%)	21.59 (± 21.50)
Bleeding on probing (%)	27.91 (± 20.00)
Probing pocket depth ≥ 4 mm (%)	7.50 (± 10.43)
PIBI (n)	7.87 (± 11.12)
Missing teeth (n)	2.49 (± 4.32)
Salivary flow (ml/min)	1.56 (± 0.75)

Data are presented as mean ± standard deviation or frequencies. DMFT: decayed, missing and filled teeth. PIBI: periodontal inflammatory burden index.

Periodontal inflammatory burden index (PIBI) [26] was also calculated and a modified version incorporating BOP percentage and bone loss was employed, resulting in the following formula: [PPD 4–5 mm + (2 * PPD ≥ 6 mm) + BOP] * PD group. The numbers 1, 2 and 3 were attributed to the PD-, PD and PD + groups, respectively. The data was presented in tertiles of the index. The %-chance of avoiding caries, which represents the caries risk, was evaluated using the Cariogram [27].

2.3. Enzyme-linked immunosorbent assays (ELISA)

Salivary levels of MMP-12 (Abnova, Neihu District, Taipei, Taiwan), S100A8/A9, and S100A12 (R&D Systems, Minneapolis, MN, USA) were analyzed by ELISA according to the manufacturer's instructions. Kits were validated for saliva by performing spike and recovery tests. The intra-assay coefficients of variance were 9.86%, 4.51%, and 9.41% for MMP-12, S100A8/A9, and S100A12, respectively. The inter-assay coefficients were 12.65%, 8.51%, and 15.86% for MMP-12, S100A8/A9, and S100A12, respectively. MMP-12 was detected in all except five samples and both S100A8/A9 and S100A12 were detected in all samples.

2.4. Statistical analysis

Data were analyzed with Statistical package for Social Sciences (SPSS) version 23 (SPSS inc., Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$. Significant differences in MMP-12, S100A8/A9, and S100A12 levels when comparing two groups were analyzed by Mann-Whitney U test and when three groups were compared we applied the Kruskal-Wallis with Dunn-Bonferroni post-hoc test. The correlations between the MMP-12, S100A8/A9 and S100A12 as well as other inflammatory markers analyzed previously in this cohort [28,29] were analyzed with Pearson's correlation test. We also performed a multivariate linear regression analysis with each biomarker, MMP-12, S100A8/A9, and S100A12 as a dependent variable. Demographic characteristics, BOP, % of PPD ≥ 4 mm, number of

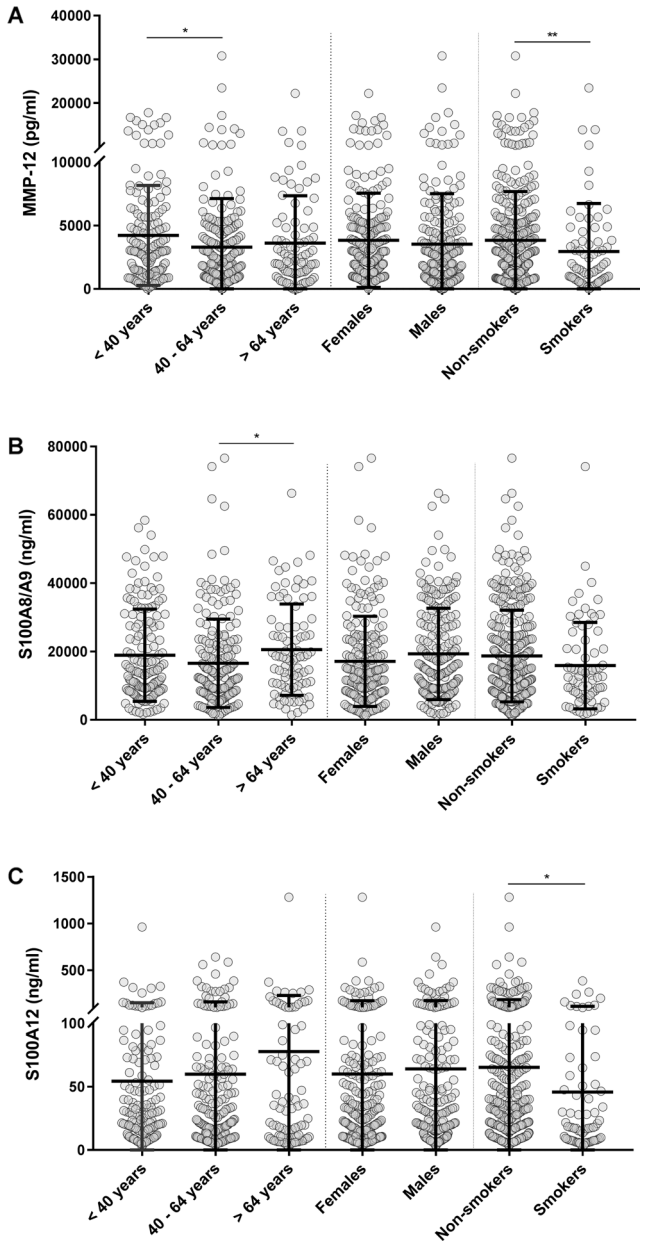

Fig. 1. Salivary levels of MMP-12, S100A8/A9, and S100A12 according to age, sex, and smoking status. (A) MMP-12 levels in saliva from individuals < 40 years (n = 152), 40–64 years (n = 197), and > 64 years old (n = 87), from female (n = 221) and male participants (n = 215), and from non-smokers (n = 362) and smokers (n = 74). (B) S100A8/A9 levels in saliva from individuals < 40 years (n = 152), 40–64 years (n = 197), and > 64 years old (n = 87), from female (n = 221) and male participants (n = 215), and from non-smokers (n = 362) and smokers (n = 74). (C) S100A12 levels in saliva from individuals < 40 years (n = 152), 40–64 years (n = 197), and > 64 years old (n = 87), from female (n = 221) and male participants (n = 215), and from non-smokers (n = 362) and smokers (n = 74). Lines represent mean (± standard-deviation). P-value was determined by Mann-Whitney or Kruskal-Wallis with Dunn-Bonferroni post-hoc tests. Two samples were missing for S100A12 and S100A8/A9. *$P < 0.05$, **$P < 0.01$.

MCL, and systemic conditions were included as independent variables.

3. Results

This study investigated the association between oral diseases and the salivary levels of MMP-12, S100A8/A9, and S100A12, as well as the influence of non-disease-related covariates on their levels in a large cohort of Swedish adults (n = 436). The cohort description is presented in Table 1.

3.1. Influence of age, sex, and smoking on the levels of MMP-12, S100A8/A9, and S100A12 in saliva

We assessed the influence of age, sex, and smoking on the levels of MMP-12, S100A8/A9, and S100A12 in saliva. Therefore, we compared the levels in three age categories (< 40 years old (yo), 40–64 yo, and > 64 yo). Mean levels of MMP-12 were 0.78-fold lower in participants aged 40–64 yo compared to participants < 40 yo ($P = 0.022$; Fig. 1A). Levels of S100A8/A9 were 1.24-fold higher in individuals > 64 yo compared to the ones aged 40–64 yo ($P = 0.021$; Fig. 1B), while no significant differences were observed compared to the participants < 40 yo. No significant differences between the three age categories were observed for S100A12 (Fig. 1C).

Regarding sex, no difference was observed for MMP-12, S100A8/A9, or S100A12 between males and females (Fig. 1A–C). Mean levels of MMP-12 and S100A12 in smokers were, respectively, 0.77- and 0.70-fold lower than in non-smokers ($P = 0.003$, $P = 0.035$; Fig. 1A and C). For S100A8/A9, the decrease in smokers did not reach statistical significance ($P = 0.067$; Fig. 1B).

3.2. Salivary MMP-12, S100A8/A9, and S100A12 levels in relation to periodontal parameters

Next, the levels of MMP-12, S100A8/A9, and S100A12 in saliva were evaluated in relation to periodontal parameters. Mean levels of MMP-12, S100A8/A9, and S100A12 were, respectively, 1.29-, 1.37-, and 1.97-fold higher in participants with BOP > 20% compared to participants with BOP ≤ 20% ($P = 0.021$, $P < 0.0001$, $P < 0.0001$; Table 2). Mean levels of MMP-12 were equal in individuals having 1–9% and ≥10% of PPD ≥ 4 mm to the ones with PPD < 4 mm (Table 2). S100A8/A9 levels were 1.35-fold higher in participants with ≥10% of PPD ≥ 4 mm in comparison to participants with PPD < 4 mm ($P = 0.006$; Table 2). There was no significant difference in the S100A12 levels according to the categories of PPD ≥ 4 mm (Table 2). No significant differences in the levels of the three markers were observed when the extent of alveolar bone loss was compared (PD-, PD, and PD + groups) (Table 2).

Mean levels of MMP-12, S100A8/A9, and S100A12 were 1.56-, 1.64-, and 2.70-fold higher in tertile 3 of the modified PIBI in comparison with tertile 1 ($P = 0.008$, $P < 0.001$, $P < 0.001$; Fig. 2A–C). S100A8/A9 and S100A12 levels were 1.38- and 1.47-fold higher in tertile 2 than in tertile 1 ($P < 0.001$, $P = 0.017$; Fig. 2B and C). In addition, S100A12 mean levels were 1.84-fold higher in tertile 3 than in tertile 2 ($P = 0.019$; Fig. 2C).

3.3. Effects of caries lesions on MMP-12, S100A8/A9, and S100A12 levels in saliva

We investigated the effect of MCL on the levels of MMP-12, S100A8/A9, and S100A12 in saliva. For that purpose, the cohort was stratified into participants having or not having MCL. No significant differences between the groups were observed for MMP-12, S100A8/A9, and S100A12 levels (Suppl. Fig. 1A–C).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.06.036.

3.4. Correlations between clinical characteristics and MMP-12, S100A8/A9, and S100A12 levels in saliva

Correlations between clinical characteristics and the analyzed proteins are depicted in Fig. 3. Age correlated significantly only with MMP-12 levels. Plaque index correlated significantly with S100A8/A9. The percentage of BOP and PPD ≥ 4 mm, PIBI, as well as the modified PIBI

Table 2
Salivary levels of MMP-12, S100A8/A9, and S100A12 in relation to periodontal parameters.

	MMP-12 (pg/ml)	S100A8/A9 (ng/ml)	S100A12 (ng/ml)
Bleeding on probing (BOP)			
BOP ≤ 20% (n = 185)	3133.2 (± 3142.1)	14988.9 (± 10902.7)	39.8 (± 58.7)
BOP > 20% (n = 251)	4070.2 (± 4257.4)*	20576.4 (± 14420.2)**	78.5 (± 137.9)**
Probing pocket depth (PPD)			
PPD < 4 mm (n = 97)	3664.0 (± 3606.6)	15554.1 (± 12355.7)	51.9 (± 77.8)
1–9% PPD ≥ 4 mm (n = 228)	3484.1 (± 3441.1)	17890.1 (± 12880.9)	58.5 (± 99.1)
≥ 10% PPD ≥ 4 mm (n = 111)	4072.1 (± 4749.9)	21135.6 (± 14519.1)[a]	78.2 (± 156.5)
Bone loss			
PD-group (n = 302)	3732.1 (± 3676.3)	17987.0 (± 13090.4)	56.7 (± 95.0)
PD group (n = 89)	3324.8 (± 3121.8)	18520.9 (± 11819.1)	75.6 (± 154.3)
PD + group (n = 45)	4260.6 (± 5967.1)	19638.5 (± 17265.6)	72.5 (± 125.9)

P-value was determined by Mann-Whitney or Kruskal-Wallis with Dunn-Bonferroni post-hoc tests. Two samples were missing for S100A12 and S100A8/A9.
* P < 0.05.
** P < 0.01.
[a] Significantly different from PPD < 4 mm (p < 0.01).

correlated significantly with the three markers. MMP-12 and S100A8/A9 levels correlated with the number of MCL, but only MMP-12 correlated with DMFT. We observed significant negative correlations of MMP-12, S100A8/A9, and S100A12 levels with the %-chance of avoiding new caries. The levels of MMP-12, S100A8/A9, and S100A12 correlated significantly with each other. We also assessed the correlations between the levels of MMP-12, S100A8/A9, S100A12, and other molecules previously evaluated in this cohort, and found significant positive correlations for all three markers with IL-1β, IL-6, IL-8, colony stimulating factor (CSF)-1, MMP-8, and lysozyme, but not with tissue inhibitor of metalloproteinases (TIMP)-1 [28,29].

3.5. Predictors of MMP-12, S100A8/A9, and S100A12 levels in saliva

Next, we sought to investigate the variables associated with the levels of MMP-12, S100A8/A9, and S100A12 in saliva. Therefore, we performed a linear regression analysis (Table 3). We found that age, smoking, self-reported presence of any tumor, and the percentage of PPD ≥ 4 mm were significantly associated with MMP-12 levels in saliva. These four variables together accounted for 27.6% of the variance in the salivary levels of MMP-12. Regarding S100A8/A9, only the percentage of BOP remained associated with its levels. BOP accounted for 22.9% of the variance in S100A8/A9 levels. The percentage of BOP and self-reported presence of any tumor were associated with the levels of S100A12, and both variables together accounted for 22.6% of its variance.

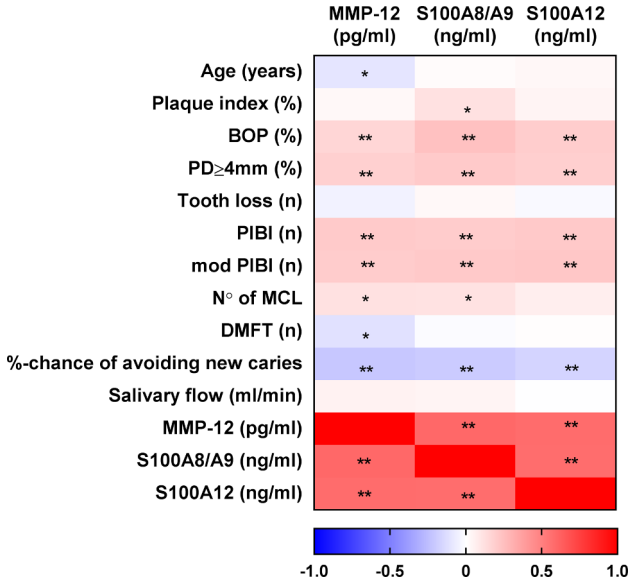

Fig. 3. Correlation heat map of clinical characteristics and MMP-12, S100A8/A9, and S100A12 levels in saliva. Correlation heat map of clinical characteristics and MMP-12, S100A8/A9, and S100A12 levels. Pearson correlation was used. *$P < 0.05$, **$P < 0.01$.

4. Discussion

To the best of our knowledge, this is the first study that comprehensively investigates the possible influence of different aspects of oral health, as well as of non-disease variables, on the levels of MMP-12, S100A8/A9, and S100A12 in saliva. Our results revealed that MMP-12 levels were associated with the percentage of gingival pockets ≥ 4 mm, while the levels of both S100/calgranulin proteins were associated with the degree of gingival inflammation. These different associations might

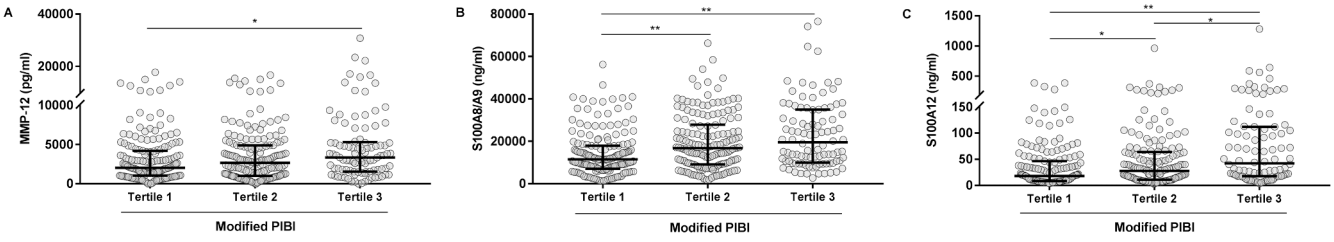

Fig. 2. Salivary levels of MMP-12, S100A8/A9, and S100A12 according to the modified periodontal inflammatory burden index. **(A)** MMP-12 levels in saliva from individuals belonging to tertile 1 (n = 175), tertile 2 (n = 168), and tertile 3 (n = 105) of the modified periodontal inflammatory burden index (PIBI). **(B)** S100A8/A9 levels in saliva from individuals belonging to tertile 1 (n = 175), tertile 2 (n = 168), and tertile 3 (n = 105) of the modified PIBI. **(C)** S100A12 levels in saliva from individuals belonging to tertile 1 (n = 175), tertile 2 (n = 168), and tertile 3 (n = 105) of the modified PIBI. PIBI tertile 1 ranges from 0 to 23, tertile 2 from 23 to 52.8, and tertile 3 from 52.8 to 597. Lines represent mean (± standard-deviation). P-value was determined by Kruskal-Wallis with Dunn-Bonferroni post-hoc tests. Two samples were missing for S100A12 and S100A8/A9. *$P < 0.05$, **$P < 0.01$.

Table 3
Linear regression analysis of the association of demographic variables, oral and systemic conditions with MMP-12, S100A8/A9, and S100A12 levels in saliva (n = 436).

	Coefficient (β)	95% CI	p-value
MMP-12 (pg/ml)[a]			
Age (years)	−44.05	−66.73 to −21.37	0.00015
Smoking	−1280.48	−2239.73 to −321.23	0.009
Presence of tumor	2625.14	700.86–4549.43	0.008
Probing pocket depth ≥4 mm (%)	83.43	46.03–120.82	0.000015
S100A8/A9 (ng/ml)[b]			
Bleeding on probing (%)	153.37	91.06–215.68	0.000002
S100A12 (ng/ml)[c]			
Bleeding on probing (%)	1.06	0.53–1.59	0.0001
Presence of tumor	76.07	20.72–131.43	0.007

[a] $R^2 = 0.276$.
[b] $R^2 = 0.229$.
[c] $R^2 = 0.226$.

be a result of the cell population dynamics during periodontal inflammation. Age and smoking were also predictors of MMP-12 levels. Self-reported presence of any tumor was a predictor of both MMP-12 and S100A12. Our results indicate that different salivary markers might reflect different aspects of periodontal inflammation and that their levels are also influenced by environmental and endogenous factors.

Age has a clear impact on the immune system and investigations of changes with age in the context of oral diseases are needed [30]. In this study we found that MMP-12 levels in saliva were influenced by age with significantly lower levels in individuals 40–64 yo compared to individuals below 40 yo, and age remained as a negative determinant of MMP-12 in a multivariate model. In contrast to our findings in saliva, the MMP12 gene expression was higher in gingival tissue from older compared to younger individuals [31]. This might be explained by differences in mRNA and protein as mRNA only partly explain protein variance, which is also affected by translation and degradation processes. We found lower levels of S100A8/A9 in participants 40–64 yo compared to participants over 64 yo, however, age did not remain associated with S100A8/A9 levels in a multivariate model. In agreement with our finding, age had no significant influence on the salivary levels of S100A8/A9 [17]. We did not find any association between age and salivary S100A12 levels. Nevertheless, age might be important to take into consideration when analyzing the levels of inflammatory markers in different diseases.

Smoking is a risk factor for periodontal disease and its influence on the levels of salivary markers is relevant to evaluate. We found lower salivary levels of MMP-12 and S100A12 in smokers, but only MMP-12 remained associated with smoking in a multivariate model. Similarly, the levels of IL-8, MMP-8/TIMP-1 ratio, and CSF-1 were lower in smokers in this cohort [28,29]. In line with this, lower levels of several pro-inflammatory markers were observed in gingival cervicular fluid (GCF) from smokers [32]. In agreement with our findings, no relation has been found between smoking and salivary levels of S100A8/A9 [17]. It is plausible to speculate that smoking might impair the immune responses leading to decreased MMP-12, and as a consequence reduced pathogen defense [33]. Another explanation could be that smokers have lower GCF volume compared to non-smokers, with reduced flow into saliva and therefore a decrease in salivary proteins is measured [34].

We investigated the association between MMP-12 and S100/calgranulins with the clinical parameters BOP, PPD, and the extent of alveolar bone loss as measures of periodontal disease and MCL as a measure of dental caries. The cut-off at 20% of sites with BOP was applied based on the knowledge that patients with a mean BOP ≤ 20% have a significantly lower risk for further attachment loss [35]. Higher levels of the three markers were detected in individuals with BOP > 20%, whereas only the levels of S100A8/A9 were higher in participants with ≥10% of PPD ≥ 4 mm. A modified PIBI was calculated, incorporating PPD, BOP and bone loss, three measures of the extent of periodontal disease. Individuals with a high modified PIBI had higher levels of MMP12, S100A8/A9 and S100A12. In a multivariate model, the percentage of PPD ≥ 4 mm was found as a predictor of MMP-12, and the percentage of bleeding as a predictor of both S100 proteins. In line with our results, the levels of MMP-12 in GCF were reported to correlate to the percentage of PPD > 4 mm in patients with localized aggressive periodontitis [7]. MMP-12 is mainly produced by monocyte-derived cells [36,37], but also by gingival fibroblasts in response to IL-1β or *P. gingivalis* lipopolysaccharide [38,39]. We have recently observed an altered phenotype of monocyte-derived cells in gingival tissue from periodontitis patients with higher MMP-12 production associated with MMP-12 mediated ECM degradation, suggesting that MMP-12 may play a role in connective tissue degradation in periodontitis [8].

S100A8/A9 and S100A12 are mainly expressed by neutrophils and monocytes, which infiltrate the gingiva at an early stage of the inflammatory response, and by epithelial cells under inflammatory conditions [11,12]. The short half-life of both proteins in blood might allow them to reflect ongoing inflammation; therefore, it is plausible to assume a stronger association with BOP than with PPD ≥ 4 mm, since BOP is an objective measure of gingival inflammation, while probing depth is a sequel of disease. Regarding the chemotactic function of the S100A8/A9 and S100A12, together with the ability of MMP-12 to activate chemokines and matrikines, they can all induce the recruitment of phagocytes, leading to exacerbation of inflammation in periodontal tissues [4,40]. Sites with BOP in periodontitis patients are characterized by a marked increase in the density of inflammatory cells in comparison to sites without bleeding [41]. In accordance with our results, increased S100A8/A9 levels in GCF has been found in bleeding sites as compared to sites without bleeding [42], potentially due to the increased infiltration of phagocytes with the ability to produce the S100 proteins. To the best of our knowledge, this is the first time an association has been shown between salivary S100A12 levels and BOP.

It is noteworthy to highlight that S100A8/A9 and S100A12 can play a role in host protection by counteracting tissue damage. This is due to their ability to inactivate MMPs by sequestration of Zn^{2+}-ion and antimicrobial activity [43]. Expression of S100A8/A9 in oral epithelial cells reduces *P. gingivalis* invasion [44]. On the other hand, S100A8/A9 induces the secretion of IL-6 and IL-8 in human gingival fibroblasts [45], and induces apoptosis and the expression of *IL6*, *IL8*, *TNFA*, and *COX2* in human periodontal ligament cells [46]. Taken together, it is conceivable to assume that the pro-inflammatory effects of S100A8/A9 surpass its ability to protect the host; while preventing microbial invasion into epithelial cells, S100A8/A9 stimulates pro-inflammatory responses in stromal cells and recruit phagocytes to the site of inflammation. For S100A12, the molecular functions in periodontal pathogenesis need to be further explored.

We observed no significant difference in the presence of manifest caries lesions on the levels of MMP-12 and the S100 proteins in saliva. Though, the number of MCL and the caries risk correlated with the levels of MMP-12 and S100A8/A9. Since MMP-12 is only able to degrade the type IV out of all collagens, and the dentin ECM is mainly composed of collagen type I, the impact of MMP-12 on the cavity progression may not be of relevance. Other MMPs, such as the collagenase MMP-8 is increased in saliva of patients with MCL [47]. Transcript levels of S100A8, S100A9, and S100A12 were higher in carious than in healthy pulpal tissue, and the protein levels of S100A8/A9 were elevated during carious disease [20]. On the other hand, decreased expression of S100A12 was found in inflamed compared to non-inflamed dental pulp [48]. The role of S100 proteins in the carious process and a possible spillover into saliva needs further investigation.

A limitation of this study is that we did not address the origin of MMP-12 and the S100/calgranulins in saliva. We speculate that the

increased GCF flow into saliva during inflammation influences the levels, and therefore reflect periodontal disease, however they may also be produced in the salivary glands, by the oral mucosa, as well as in caries lesions. Another limitation is the cross-sectional design of the study, which does not allow determining causality. A clinical study addressing the effects of periodontal treatment on the levels of MMP-12 and the S100 proteins is warranted. However, this large cohort allowed us to investigate the association between different aspects of the two most prevalent oral diseases, periodontal disease and caries, and these proteins in saliva. Nevertheless, the findings that MMP-12 and S100A12 levels are associated with self-reported presence of tumor identified them as potential candidates in the screening for cancer biomarkers. The fact that presence of tumors in this study was self-reported is a weakness and further studies with confirmed diagnosis are warranted.

In conclusion, salivary MMP-12 and the S100/calgranulin proteins S100A8/A9 and S100A12 reflect different aspects of periodontal disease, and their levels are differently affected by age and smoking, as well as by the presence of tumor.

Declaration of interest

The authors have no conflicts of interest to declare.

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