Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: a cross-sectional study


Background and Objective: Saliva has been proposed as a noninvasive diagnostic fluid that could be used in the diagnosis of oral and systemic diseases. The levels of salivary biomarkers, such as cytokines, could potentially be used as a surrogate to distinguish periodontally healthy individuals from subjects with periodontitis. Therefore, the goal of the present investigation was to determine if the levels of 10 different cytokines in saliva differed between a group of periodontally healthy individuals and a group of subjects with periodontitis. Correlations between the concentrations of these 10 cytokines and clinical parameters of periodontal disease were also examined.

Material and Methods: In this cross-sectional study, 74 subjects with chronic periodontitis and 44 periodontally healthy individuals were periodontally examined and had the levels of granulocyte–macrophage colony-stimulating factor, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interferon-γ and tumor necrosis factor-α measured in whole saliva using a multiplexed bead immunoassay (Luminex). Significance of statistical differences in the levels of salivary cytokines between groups was determined using nonparametric analysis of covariance, adjusting for age and smoking status. The Spearman rank correlation coefficient was used to explore associations between the mean levels of salivary cytokines and mean clinical parameters.

Results: There were no statistically significant differences between groups for any of the cytokines. There were weak, statistically significant positive associations between salivary interleukin-8 and pocket depth ($r_s = 0.2, p < 0.05$) and bleeding on probing ($r_s = 0.2, p < 0.05$), and weak negative correlations between salivary interleukin-10 and attachment level ($r_s = -0.2, p < 0.05$) and bleeding on probing ($r_s = -0.3, p < 0.001$).

Conclusion: Mean salivary levels of granulocyte–macrophage colony-stimulating factor, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interferon-γ and tumor necrosis factor-α could not discriminate between periodontal health and disease.
Several publications have recently addressed the potential diagnostic properties of saliva. It has been proposed that saliva could not only be used to help diagnose oral diseases, but as 'the body's mirror' it would also have application in the diagnosis of systemic conditions (1, 2). Currently, the clinical diagnosis of periodontal diseases involves primarily the assessment of clinical measures of tissue destruction and signs of tissue inflammation. Although clinicians and researchers have successfully relied on these parameters to determine the periodontal status of patients and research subjects, the time and expertise required for a full periodontal examination pose severe limitations to epidemiological surveys. Investigations designed to uncover risk factors and risk indicators of periodontal infections would also benefit from a faster screening of subjects, which could facilitate a larger sample size. The same issues are valid for studies of associations between systemic diseases and periodontal infections. Saliva could be used as a noninvasive diagnostic fluid to measure biomarkers released during disease initiation and progression (3–5). Characterization of specific salivary biomarkers associated with periodontal disease presence and severity could have a major impact in the diagnosis and monitoring of periodontal diseases.

As a result of their role in the immunopathology of periodontal infections, different cytokines have been measured in gingival crevicular fluid and their levels have been related to the disease status of sites and subjects. For the most part, these studies have found an increase in the levels of several gingival crevicular fluid cytokines, associated with a worsening in clinical parameters of periodontal disease (6–9). In addition, treatment and improvement of the periodontal condition has been associated with decreases in the gingival crevicular fluid levels of certain cytokines (10–12). These findings suggest that cytokines could be putative biomarkers of periodontal disease initiation and progression.

Several lines of evidence suggest that the primary source of cytokines in whole saliva is gingival crevicular fluid. Ruhl et al. (13) measured the levels of IL-1α, interleukin-6, interleukin-8, epidermal growth factor, nerve growth factor and albumin in parotid, submandibular/sublingual and whole saliva. They found that interleukin-1α, interleukin-6 and interleukin-8 were present in whole saliva at concentrations significantly higher than in major salivary gland secretions. The authors concluded that the inflammatory cytokines detected in whole saliva did not come from the secretions of major salivary glands, and proposed that gingival crevicular fluid was the probable source of these cytokines. In another study, the levels of transforming growth factor-β, interleukin-1α and tumor necrosis factor-α were statistically significantly higher in whole saliva compared with parotid saliva (14). Although not statistically significant, interleukin-8 and interleukin-6 also displayed a trend towards higher levels in whole saliva (14). Therefore, it is possible that whole saliva contains gingival crevicular fluid from all periodontal sites, providing an assessment of periodontal disease status. For instance, gingival crevicular fluid biomarkers that were positively associated with increased attachment loss, such as beta-glucuronidase (15), were further examined by the same group of investigators in saliva. They found that the salivary levels of beta-glucuronidase also had a strong, positive correlation with clinical parameters of periodontal disease (16).

Despite the recognized diagnostic potential of saliva, only a few reports have attempted to correlate the levels of cytokines in saliva with the periodontal condition of the subjects. Miller et al. (17) conducted a study to determine if salivary biomarkers specific for three aspects of periodontitis, namely inflammation, collagen degradation and bone turnover, correlated with clinical features of periodontal disease. They examined the relationship between clinical parameters of periodontal disease and the levels of interleukin-1β, matrix metalloproteinase (MMP)-8 and osteoprotegerin in whole saliva. They reported that the mean levels of interleukin-1β and MMP-8 in saliva were significantly higher in subjects with periodontitis than in periodontally healthy controls. The levels of MMP-8 and interleukin-1β correlated with periodontal indices, whereas, after adjustment for confounders, osteoprotegerin did not. Combined elevated salivary levels of MMP-8 and interleukin-1β increased the risk of experiencing periodontal disease 45-fold.

Ng et al. (18), in a cross-sectional study, evaluated the association between radiographic evidence of alveolar bone loss and the concentration of host-derived bone-resorptive factors (interleukin-1β, tumor necrosis factor-α, interleukin-6 and prostaglandin E2), as well as markers of bone turnover (pyridinoline cross-linked carboxyterminal telopeptide of type I collagen, osteocalcin and osteonectin) in stimulated whole saliva collected from 110 untreated dental patients. Variables positively associated with increased bone loss score were: age; current smoking; use of bisphosphonate drugs; and salivary interleukin-1β levels above the median. Increased levels of salivary osteonectin were associated with less bone loss.

Therefore, the goal of the present investigation was to perform a cross-sectional study to compare the levels of granulocyte–macrophage colony-stimulating factor, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interferon-γ and tumor necrosis factor-α in samples of whole saliva from subjects with chronic periodontitis and periodontally healthy subjects. In addition, we examined correlations between the concentrations of these 10 cytokines and clinical parameters of periodontal disease.

**Material and methods**

**Subject population and study design**

Seventy-four subjects with chronic periodontitis and 44 periodontally healthy subjects were recruited for this cross-sectional study at The Forsyth Institute. The periodontally healthy subjects were 18–65 years of age, with at least 20 natural teeth and no pocket...
depth or attachment level measurements of > 3 mm. The periodontal disease subjects were 18–65 years of age with at least 20 natural teeth and eight or more sites with pocket depths > 4 mm and attachment level > 3 mm. Exclusion criteria included: the presence of orthodontic appliances; abnormal salivary function; use of prescription drugs; use of antibiotics in the month prior to the study; use of any over-the-counter medications other than analgesics; diseases of the soft or hard oral tissues; and systemic conditions that might place subjects in a high-risk category or could influence the course of periodontal diseases and periodontal therapy within 6 mo prior to the baseline examination. Only subjects who were ambulatory and volunteered to come to The Forsyth Institute were included. The Institutional Review Board at The Forsyth Institute approved the study protocols, including the recording of clinical measurements and collection of saliva samples. All subjects signed informed consent prior to entry into the study.

Clinical examination
Clinical measurements were taken at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars (a maximum of 168 sites per subject), as previously described (19). The clinical parameters measured and the order of measurement were as follows: (i) presence of plaque (0 or 1); (ii) pocket depth (mm); (iii) attachment level (mm); (iv) bleeding on probing (0 or 1); and (v) suppuration (0 or 1). Probing pocket depth and attachment level measurements were made to the nearest mm using a North Carolina periodontal probe. Pocket depth and attachment levels were measured twice by the same examiner and the average of the pair of measurements was used for analysis. Saliva samples for cytokine assessment were taken prior to the clinical measurements. All clinical data were recorded on data sheets and then entered into a computer using a prompted data-entry program.

Saliva samples
Subjects refrained from brushing for 12 h and from drinking, eating or chewing gum for 1½ h prior to sample collection. Subjects expectorated 1–2 mL of accumulated whole saliva into a container. Saliva samples were cleared by centrifugation at 9300 g for 10 min to pellet bacteria and the supernatant was kept at −80°C until required for assay.

Quantification of cytokines using Luminex
Cytokine levels were determined using a multiplexed bead immunoassay. Prior to assay, further processing of the saliva samples involved adsorption onto cellulose (Whatman filter paper; VWR, Bridgeport, NJ, USA) followed by elution by centrifugation at 9300 g for 10 min, through a 0.22 μm filter using Spin-X Centrifuge Tube Filters (Corning Incorporated, Billerica, MA, USA). This was required to reduce mucin content and avoid clogging of the Luminex 100™ machine. The levels of 10 cytokines (granulocyte–macrophage colony-stimulating factor, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interferon-γ and tumor necrosis factor-α) were measured using the human ultrasensitive cytokine ten-plex antibody bead kit (BioSource International, Camarillo, CA, USA). The assays were performed in 96-well filter plates, as previously described (20). Briefly, the filter plate was prewetted with washing buffer and the solution was aspirated from the wells using a vacuum manifold (Millipore Corporation, Billerica, MA, USA). Microsphere beads coated with monoclonal antibodies against the 10 different target analytes were added to the wells. Samples and standards were pipetted into the wells and incubated for 2 h with the beads. The wells were washed using a vacuum manifold (Millipore Corporation) and biotinylated secondary antibodies were added. After incubation for 1 h, beads were washed then incubated for 30 min with streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin/R-phycoerythrin). After washing to remove the unbound streptavidin/R-phycoerythrin, the beads (a minimum of 100 per analyte) were analyzed in the Luminex 100™ instrument (MiraiBio, Alameda, CA, USA). The Luminex 100™ monitors the spectral properties of the beads to distinguish the different analytes, while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity. The concentrations of the unknown samples (antigens in gingival crevicular fluid samples) were estimated from the standard curve using a third-order polynomial equation and expressed as pg/mL after adjusting for the dilution factor. Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve.

Data analysis
The outcome variables evaluated in this study were the mean clinical parameters and mean levels of 10 salivary cytokines for each subject. Values for each clinical parameter were averaged within a subject and then averaged across individuals in the periodontitis and periodontally healthy groups separately. Values for the cytokines were averaged across subjects in each clinical group. The differences in mean clinical parameters between periodontally healthy and periodontitis subjects were determined using the unpaired Student’s t-test, while differences in the distribution of male subjects and smokers were examined using the chi-square test. Significance of statistical differences in the levels of salivary cytokines between groups was initially examined using the Mann–Whitney test but ultimately determined using nonparametric analysis of covariance, adjusting for age and smoking status. The Spearman rank correlation coefficient was used to explore associations between levels of salivary cytokines and clinical parameters of disease, such as mean pocket depth, mean attachment level and mean prevalence of bleeding sites.
Correlation coefficients were calculated using the entire data set for periodontally healthy and periodontitis subjects.

Results

Table 1 summarizes the mean demographic and clinical parameters for the two clinical groups. All clinical parameters of periodontal disease were statistically significantly higher in the subjects with chronic periodontitis. In addition, there were statistically significant differences in age, percentage of male subjects and percentage of smokers, which were all higher in the periodontitis group. Further characterization of the level of disease in the periodontitis population revealed that 25% of the subjects presented with a minimum of 18% of sites with pocket depth > 4 mm and CAL > 3 mm. As age is a risk indicator and smoking is a risk factor for periodontal disease, analysis of covariance adjusting for these variables was performed. The results (data not shown) remained statistically significant for mean pocket depth, percentage of sites with bleeding on probing and percentage of sites with plaque, confirming the difference in level of periodontal disease between the groups.

The results from the multiplexed bead immunoassays revealed that the levels of most cytokines were within the range of quantification of the assay. For granulocyte–macrophage colony-stimulating factor, interleukin-1β, interleukin-4, interleukin-6 and tumor necrosis factor-α, over 90% of the samples were within the dynamic range of the standard curves. In the case of interferon-γ, 83% of the samples were quantifiable, while for interleukin-2, interleukin-5, interleukin-8 and interleukin-10, approximately 50% of the samples were in the dynamic range of the assay. Interleukin-10 had the highest percentage of negative samples (33%), while interleukin-8 had the highest percentage of values above the upper limit of quantification (34%).

The mean levels of the 10 biomarkers were initially compared between subjects with periodontitis and periodontally healthy subjects using the Mann–Whitney test. The results showed a statistically significant higher level of mean salivary interleukin-10 ($p < 0.01$) and interleukin-5 ($p < 0.05$) in the healthy group. However, the nonparametric analysis of covariance, adjusted for age and smoking, revealed no statistically significant differences between groups for any of the cytokines (Figs 1 and 2). Interleukin-8 was the most abundant cytokine in saliva, with mean levels (± standard error of the mean) of 1945 ± 181 pg/mL and 2268 ± 111 pg/mL, for periodontally healthy individuals and subjects with periodontitis, respectively.

Discussion

The results of our study did not demonstrate a significant difference in the levels of any of the 10 cytokines tested between patients with chronic periodontitis and periodontally healthy subjects. In addition, only weak statistically significant associations were found among mean clinical parameters of periodontal disease and mean salivary levels of interleukin-8 and interleukin-10. These results cast doubt upon the use of salivary cytokines as biomarkers of periodontal disease. The present results are in contrast to reports by other investigators that have described elevated levels of salivary biomarkers such as interleukin-1β and MMP-8 in subjects with periodontitis compared with periodontally healthy subjects (17,18). In one study, the levels of interleukin-1β and MMP-8, measured using enzyme-linked immunosorbent assay, were statistically significantly higher in subjects with moderate-to-severe periodontitis ($n = 28$) than in control subjects ($n = 29$), and the levels of both analytes correlated with clinical parameters of periodontal disease, such as bleeding on probing and percentage of sites with pocket depth ≥ 4 mm (17). The reported mean levels (± standard
deviation) of interleukin-1β were 213 ± 167 pg/mL and 753 ± 1022 pg/mL for periodontally healthy individuals and subjects with periodontitis, respectively. In the present study, the corresponding values were 633 ± 602 pg/mL and 673 ± 590 pg/mL for periodontally healthy individuals and subjects with chronic periodontitis, respectively. A possible explanation for the discrepancy between these results might reside in the level of disease of the two study populations. Miller’s periodontitis subjects presented with more severe periodontal disease, exemplified by their higher mean percentage of sites with pocket depth (± standard deviation) ≥ 4 mm of 45.2 ± 21.9 compared with 27.6 ± 18.2 in our population. In addition, their mean percentage of sites with bleeding on probing (mean ± standard deviation) was also higher than ours: 45.9 ± 15.6 vs. 28.4 ± 15.3.

Another recent report described a positive association between the mean levels of salivary interleukin-1β and radiographic signs of bone loss in a population of 110 ‘untreated dental patients’ (18). As the authors did not record traditional clinical parameters of periodontal disease, it is difficult to comment on potential differences in the level of disease between their study population and the individuals reported here. However, the authors highlighted in their discussion that several subjects with high salivary levels of interleukin-1β showed no signs of bone loss. Eight subjects in our periodontally healthy group also displayed very high levels of interleukin-1β. Given these observations, it is very unlikely that salivary interleukin-1β could be used as a discriminatory biomarker to distinguish periodontally healthy subjects from subjects with periodontal disease. In addition, differences in methods of saliva collection (stimulated or unstimulated), processing (speed and time of centrifugation), storage (time, temperature and addition or not of protease inhibitors) and in the methodology used for the quantification of the biomarkers (enzyme-linked immunosorbent assay vs. Luminex) might also have had an impact on the difference in results.

**Fig. 1.** Mean levels (± standard error of the mean) of interleukin-1β and interleukin-8 in subjects with chronic periodontitis and periodontally healthy individuals. Whiskers indicate standard error of the mean. The levels of each cytokine were determined (in pg/mL) in each subject and then averaged across subjects in the two clinical groups separately. The significance of differences between the two groups was determined using nonparametric analysis of covariance, adjusting for age and prevalence of smokers. None of the differences were statistically significant. IL-1β, interleukin-1β; IL-8, interleukin-8.

**Fig. 2.** Mean levels (± standard error of the mean) of granulocyte-macrophage colony-stimulating factor, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-10, interferon-γ and tumor necrosis factor-α in subjects with chronic periodontitis and periodontally healthy individuals. Whiskers indicate standard error of the mean. The levels of each cytokine were determined (in pg/mL) in each subject and then averaged across subjects in the two clinical groups separately. The significance of differences between the two groups was determined using nonparametric analysis of covariance, adjusting for age and prevalence of smokers. None of the differences were statistically significant. IL-1β, interleukin-1β; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-10, interleukin-10; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.
Part of the rationale for investigating cytokines present in saliva is based on the concept that these mediators find their way into whole saliva through the gingival crevicular fluid. As the gingival crevicular fluid levels of cytokines are elevated in chronic periodontitis sites compared with periodontally healthy sites (6–9), it was hypothesized that they would also be elevated in the whole saliva of individuals with periodontitis. However, cytokines are also known to be produced by human buccal and gingival epithelial cells, which could provide an additional or alternative source of these mediators in whole saliva (21). Furthermore, there is evidence that the secretion of cytokines by oral epithelial cells increases with an increase in periodontal chronic inflammation and infection with periodontal pathogens (21–23), suggesting that secretion of cytokines by oral epithelia might enhance differences in the levels of cytokines in whole saliva between subjects with chronic periodontitis and periodontally healthy individuals.

The lack of association between the levels of salivary biomarkers and periodontal disease status reported here could be explained, in part, by the extensive dilution of the gingival crevicular fluid containing these cytokines in saliva. The amount of gingival crevicular fluid produced per site per hour has been estimated to be 3 μL/h for healthy sites, 20 μL/h for intermediate pockets and 44 μL/h for deep pockets (24). Considering the turnover of the salivary compartment to be 20 mL/h, this would result in dilution factors of 1:6666, 1:1000 and 1:455 per site, depending on the periodontal condition of the site. The final dilution provided by saliva is difficult to estimate because it would depend on the distribution of healthy, intermediate and deep sites in a subject. However, the mean percentage of sites > 4 mm in our population was 14% (mean of 20 sites per subject), and the percentage of pockets > 6 mm was only 2.7% (mean of 3.8 sites per subject), suggesting a limited contribution of gingival crevicular fluid from pathologically deepened sites to the composition of whole saliva for most patients.

Another possible confounder in the interpretation of data based on levels of cytokines in saliva is the presence of putative inhibitors. The inhibitory effects of whole and parotid saliva on the levels of several cytokines were investigated by Wozniak et al. (14), who concluded that most cytokines tested were statistically significantly reduced in concentration in whole saliva compared with parotid saliva. Only the concentration of interleukin-1α was not affected by the addition of saliva. The authors suggested that the sequestration of cytokines by mucin-like proteins or other large molecules and enzymatic degradation could be potential inhibitory mechanisms present in saliva. The higher level of inhibition demonstrated for whole saliva compared with parotid saliva also suggests that whatever mechanisms are operating, they are either present at higher levels or more efficient in whole saliva.

Similar results were reported by Ng et al. (18). In vitro testing of the inhibitory effects of whole saliva using a similar methodology to Wozniak et al. (14), revealed a 75% reduction in salivary cytokine levels detected using Luminescence compared with samples diluted in buffer. They also reported that the addition of protease inhibitors did not reverse the reduction in levels of cytokines resulting from the addition of whole saliva. They suggested that the reduction in cytokine detection was a consequence of sequestration by large salivary proteins, such as mucins. Also in agreement with Wozniak et al. (14), whole saliva had a greater inhibitory effect than parotid saliva on the detectable levels of pro-inflammatory cytokines such as interleukin-1β, interleukin-6 and tumor necrosis factor-α.

In the present study we did not explore the presence of inhibitory mechanisms in saliva; however, it is fair to assume that they were also in place in our samples. Although inhibitory mechanisms in saliva might have resulted in an underestimation of the salivary levels of the cytokines tested, unless these mechanisms are enhanced by the periodontal disease process, they should have affected both periodontally healthy and periodontitis subjects to the same extent. Therefore, the influence of such inhibitory mechanisms in our results might have been limited.

In summary, we could not find an association between the levels of salivary cytokines and clinical parameters of periodontal disease. The dilution of gingival crevicular fluid components in saliva seems to mask existing differences in the levels of these biomarkers at the site level. Other biomarkers associated with the onset and development of periodontal diseases not examined here might still be present in saliva at levels that could be used to discriminate between periodontal health and disease. Furthermore, other proteomic approaches could be used to explore as-yet-unidentified biomarkers of periodontal disease in saliva.

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References

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