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ORIGINAL ARTICLE

Estimation of sCD14 levels in saliva obtained from patients with various periodontal conditions

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AIM: To assess the concentration of soluble CD14 receptor in saliva of people with periodontal disease and healthy patients and its relationship with periodontal status.

SUBJECTS AND METHODS: Unstimulated whole saliva samples from patients with chronic periodontitis (n = 34), aggressive periodontitis (n = 19) and healthy controls (n = 17) were obtained for the study. The periodontal status of each subject was assessed by criteria based on probing depth, clinical attachment loss and the extent of periodontal breakdown. The levels of sCD14 were measured in saliva samples with an enzyme-linked immunosorbent assay (ELISA).

RESULTS: Although no significant difference ($P > 0.05$) was found for salivary sCD14 levels between periodontitis groups, they were significantly greater ($P < 0.05$) than those detected for healthy controls. Furthermore, Spearman's correlation analysis showed statistically significant correlations ($P < 0.01$) between data from salivary sCD14 levels and clinical measurements.

CONCLUSION: The findings of the present study re-emphasize the importance of whole saliva as sampling method in terms of immunological purposes in periodontal disease and suggest that the elevated sCD14 concentration may be one of the host-response components associated with the clinical manifestations of periodontal disease.

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Introduction

Periodontitis is a disease of a multifactorial nature with pathogenesis related to several risk factors, including bacteria, host responses and genetics (Oliver *et al*, 1998). It is well known that the severity of periodontal disease is dependent on a dynamic equilibrium of interactions between the microbial challenge and host immunoinflammatory responses (Page and Kornman, 1997). Moreover, as various immunopathogenic mechanisms are involved in the disease process, the combination of activity indicators is needed to improve the specificity of diagnosis (Ozmeric, 2004).

Although clinical and radiographic evaluation of periodontal disease remains the basis for patient evaluation, analysis of saliva, a fluid that contains local and systemically derived markers of periodontal disease, may offer a basis for a patient-specific diagnostic test for periodontitis (Kaufman and Lamster, 2000). The use of saliva for periodontal diagnosis has been the subject of considerable research, and proposed markers for disease include proteins of host origin, phenotypic markers, host cells, hormones, bacteria and bacterial by-products (Kaufman and Lamster, 2002).

Periodontal disease is initiated and maintained in the first line by not only gram-negative but also gram-positive bacterial infection of the gingival sulcus (Tietze *et al*, 2006). Recognition of gram-negative bacteria involves membrane-associated lipopolysaccharide (LPS) activation of a series of proinflammatory cytokines and inflammatory mediators from various host cells through a key pathway of cell stimulation: LPS/LPS binding protein (LBP)/CD14 (Darveau *et al*, 1997). In contrast, gram-positive species are recognized by the host through contact with membrane peptidoglycans, lipoteichoic acid, or soluble extracellular toxins (Tietze *et al*, 2006). Host recognition pathways for both gram-negative and gram-positive bacteria comprise pattern recognition receptors among which Toll-like receptors (TLRs) play a pivotal role (Azuma, 2006; Tietze *et al*, 2006). In this way, TLR4 recognizes LPS of

gram-negative bacteria, whereas TLR2 recognizes a variety of cell-wall components from gram-positive bacteria (Tietze *et al*, 2006).

CD14, a 55-kDa glycoprotein, is considered to be an important receptor for initial bacterial recognition (Wright *et al*, 1990). It is predominantly expressed on the surface of various cells, including peripheral blood monocytes, tissue macrophages (Haziot *et al*, 1988), neutrophils, and chondrocytes (Lin *et al*, 2000), as well as gingival fibroblasts (Hatakeyama *et al*, 2003). CD14 can be found in two forms, a glycosylphosphatidylinositol membrane-bound (mCD14) protein (Bažil *et al*, 1989; Wright *et al*, 1990) and a circulating soluble (sCD14) form found in serum and other body fluids (Bažil *et al*, 1986, 1989), which endows cells lacking mCD14, such as epithelial and endothelial cells, with responsiveness to bacterial products (Frey *et al*, 1992). Thus, both mCD14 and sCD14 can function as receptors for LPS of gram-negative bacteria and for various cell-wall products of gram-positive bacteria (Wright *et al*, 1990).

The signal transduction of the LPS/LBP/CD14 ternary complex on effectors cells is then transferred via the TLR4/MD-2 complex (Da Silva Correia *et al*, 2001). Upon stimulation, the TLR4/MD-2 complex leads to the activation of innate host defense mechanisms via the nuclear factor κB (NF-κB) pathway and the release of proinflammatory cytokines, i.e. tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, and interferon-γ (Aderem and Ulevitch, 2000). Although MD-2 has been identified as an essential accessory molecule in TLR4-mediated signaling and intracellular trafficking, a substantial part of the TLR2 and TLR4 signaling pathways coincides (Van Amersfoort *et al*, 2003).

Besides its function in LPS/cell-wall products signaling, sCD14 might play a role in inflammatory diseases by controlling the immune system level of response (Bas *et al*, 2004). It has been demonstrated that sCD14 is a regulatory factor capable of modulating cellular and humoral immune responses by interacting directly (without LPS) with T and B cells, decreasing antigen and mitogen-induced proliferation (Rey Nores *et al*, 1999; Arias *et al*, 2000). Although sCD14 has been involved in periodontal inflammation as playing a crucial role in the subsequent periodontal destruction mediated by LPS (Hayashi *et al*, 1999; Masaka *et al*, 1999; Takayama *et al*, 2003), currently, there are no reports on sCD14 levels in saliva and their associations with different periodontal conditions. The aim of this study was to assess the concentration of sCD14 receptor in saliva of periodontally diseased and healthy patients and their relationship with the periodontal status.

Materials and methods

Patient selection and inclusion/exclusion criteria

A total of 70 patients attending the Graduate Periodontics Clinic at the University of Antioquia as well as patients from the private practices of periodontists, were recruited for the study. All participants provided written informed consent prior to their enrolment into the study. The study conformed to the ethical guidelines of the Helsinki Declaration and was evaluated and approved by the Institutional Research Ethics Board (CIFO).

Participants were privately interviewed to obtain medical and demographic information and were given a clinical screening for oral pathology and a periodontal examination. All clinical periodontal measurements were performed by a single calibrated investigator (DA). The clinical parameters recorded, including probing depth (PD) and clinical attachment loss (CAL), were measured at six surfaces of all teeth (midbuccally, midlingually, and proximally both buccally and lingually) to the nearest millimeter, using a straight periodontal probe (PCP UNC 15, Hu-Friedy, Chicago, IL, USA). PD was defined as the distance from the coronal-most margin of the free gingival to the most apical penetration of periodontal probe. As a reference for CAL measurement, the cemento-enamel junction (CEJ), or the apical border of the restoration, if the CEJ was not visible were used. Following previously defined criteria (Armitage, 1999; Michalowicz *et al*, 2000), these measurements were used to calculate the extent of periodontitis based on the percentage of tooth sites having PD $\geq$ 4 mm along with CAL $\geq$ 2 mm. All calculations were based on data derived from the full-mouth examination.

Subjects for this study comprised 34 chronic periodontitis (CP) and 19 aggressive periodontitis (AgP) patients. These patients were classified as follows: CP, subjects having a minimum of 20 remaining teeth, with periodontal disease as evidenced by at least four tooth sites with PD $\geq$ 4 mm, CAL $\geq$ 3 mm, and radiographic evidence of extensive bone loss (Lindhe *et al*, 1999); AgP, subjects with a pattern of severe destruction, with attachment loss of at least 5 mm on eight or more teeth, at least three of which were not first molars or incisors (generalized) or first molar/incisor presentation with interproximal attachment loss on at least two permanent teeth (localized), one of which was a first molar (Lang *et al*, 1999). As healthy controls, 17 subjects with no evidence of probing depth or clinical attachment loss measurements $>$3 mm, and no clinical gingival inflammation (no bleeding on probing and absence of gingival redness/edema), were also included.

Exclusion criteria included pregnancy; any systemic condition that could affect the host's periodontal status (e.g. diabetes mellitus, immunological disorders, AIDS) or that would require antibiotics for monitoring or treatment procedures (e.g. heart conditions, joint replacements); use of antibiotics and/or anti-inflammatory drugs within the last 3 months; and professional cleaning or periodontal treatment within the last 6 months.

Saliva collection

About 10 ml of unstimulated whole saliva was collected from each subject into a 50-ml sterile plastic centrifuge tube (Greiner Bio-one®, Frickenhausen, Germany) prior to breakfast intake and any dental hygiene procedure. No antiseptic mouthrinse was used prior to collection.

Immediately after collection, whole saliva was clarified by centrifugation for 5 min at 800 *g* (International Equipment Co, Needham, MA, USA). The supernatants were collected and sterile-filtered using a syringe-driven 0.45-μm low-protein binding filter (MILLEX®-HV-Millipore Corporation, Bedford, MA, USA). The filtered saliva was then aliquoted into 500 μl volumes and frozen at −80°C until use.

Enzyme-linked immunosorbent assay (ELISA) for human sCD14

Levels of sCD14 in saliva were measured by an ELISA-based capture assay (Quantikine® Kit; R&D Systems, Minneapolis, MN, USA) following manufacturer's instructions. Filtered saliva was diluted 1:25 in calibrator diluent included in the kit, and samples were incubated for 3 h at room temperature. Optical density was determined within 30 min after stop solution (2 N sulfuric acid) was added, using a microplate reader (EL_x800; NB-Bio-Tek Instruments, Winooski, VT, USA) set to 450 nm with wavelength correction to 490 nm. To calculate the concentrations, a nonlinear regression was performed using GraphPad Prism version 4.03® (GraphPad Software, San Diego, CA, USA). The values obtained from the standard curve were multiplied by the dilution factor so as to find the real concentration of protein. *R*-squared value was 0.9993. The assay used has a sensitivity of less than 125 pg ml^{-1} for sCD14, it does not show any significant cross-reactivity to a wide spectrum of different related cytokines (R&D Systems data), and immunoreactive CD14 fragments, derived from cleavage or hydrolysis by proteolytic enzymes present in saliva, are not detected (Duncan *et al*, 2004).

Statistical analysis

Data collected were analyzed using SPSS 14.0® (SPSS Inc., Chicago, IL, USA) statistical package. All parameters were tested for normal distribution using the Shapiro–Wilk test for small sample size. Because the results for each group did not follow a normal distribution, the variables were analyzed using nonparametric methods. The Pearson chi-squared test (χ^2) was used to assess differences in gender between the clinical groups. To determine the differences in age, PD, CAL, and sCD14 levels between groups, the Kruskal–Wallis *H*-test followed by Mann–Whitney's *post hoc* comparisons were performed. The latter was also used to determine differences between chronic and aggressive periodontitis groups regarding the extent of the disease. Correlations among sCD14 levels and clinical data were assessed using Spearman's rank correlation coefficient. Significance for all tests was established at a *P*-value < 0.05.

Results

Demographic and clinical characteristics of the study groups and controls are displayed in Table 1. Although females comprised about 60% of the patients studied, there were no significant differences between men and women in the study groups compared to healthy controls ($P > 0.05$, chi-squared test). The age of the subjects ranged between 17 and 72 years, and a statistically significant difference was observed in the median ages between the three study groups ($P < 0.001$, Kruskal–Wallis test). While the age of the CP group was significantly greater than that of AgP and healthy subjects ($P < 0.001$, Mann–Whitney *U*-test), there was no significant difference between healthy and AgP patients ($P = 0.452$).

As also shown in Table 1, median values of PD and CAL in healthy subjects were significantly lower than those of periodontitis patients ($P < 0.001$, Mann-Whitney *U*-test). However, no significant difference ($P > 0.05$) was evident for these parameters, or for the extent of periodontitis between the two periodontitis groups. It was also noticeable that severity of periodontitis, that is, the value of CAL of the diseased tooth sites (mm) showed a mean value of 5.41 ± 0.84, ranging from 4.00 to 7.74 with a median at 5.35 for CP subjects, statistically similar to AgP subjects ($P = 0.154$), where the severity was distributed from 3.16 to 7.90 with a median at 5.75 and a mean value of 5.79 ± 1.00.

The median salivary levels of sCD14 in periodontitis patients and healthy controls are displayed in Figure 1. Detectable levels of sCD14 were found in all saliva samples. They were significantly different among the three clinical groups ($P = 0.017$, Kruskal–Wallis test). A *post hoc* Mann–Whitney *U*-test demonstrated significantly greater values in the CP (mean = 15.57 ng ml^{-1},

Table 1 Clinical and demographic characteristics of study subjects

Characteristics	*Clinical groups*		
	Healthy controls (n = 17)	*Chronic periodontitis* (n = 34)	*Aggressive periodontitis* (n = 19)
Gender			
Male	7	13	8
Female	10	21	11
Age (years)[c]	31.0 (26.0–72.0)	44.5 (33.0–66.0)	33.0 (17.0–41.0)
Mean PD[a] (mm)[c]	1.82 (1.24–2.34)	3.42 (1.43–6.66)	3.68 (2.52–6.31)
Mean CAL[a] (mm)[c]	0.85 (0.50–3.60)	3.42 (1.33–6.21)	3.78 (0.39–7.41)
Extent of periodontitis[b] (%)[c]	0	38.65 (3.62–84.0)	38.09 (8.0–94.4)

[a]Data based on measurements obtained from the entire dentition.
[b]Percentage of periodontal pockets ≥ 4 mm deep and attachment loss ≥ 2 mm.
[c]The values are given as median (range).

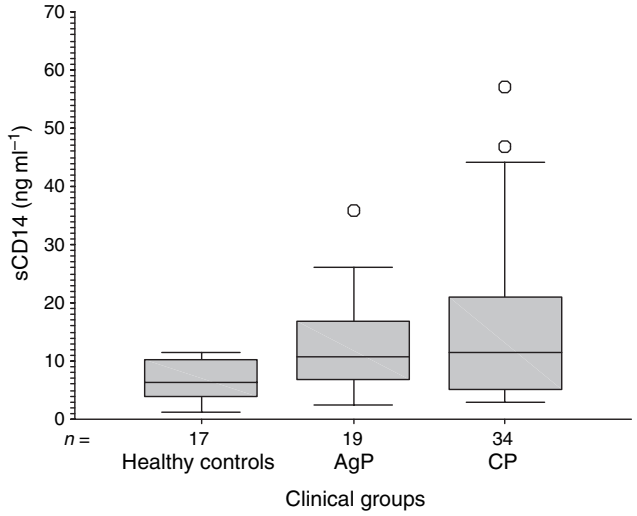

Figure 1 Quantitative comparison of median salivary sCD14 levels of study subjects. The box represents the first and third quartiles (rectangular boxes); the line within the box is the median; vertical bars show the 95% confidence interval. Little circles above these levels represent atypical values that were plotted separately

s.d. = 13.43; $P = 0.011$) and AgP (mean = 12.94 ng ml^{-1}, s.d. = 8.16; $P = 0.008$) groups compared with healthy controls (mean = 6.57 ng ml^{-1}, s.d. = 3.70). Conversely, no significant difference ($P > 0.05$) was observed for salivary sCD14 levels between periodontitis groups. Furthermore, Spearman's correlation analysis showed a highly significant correlation between data from salivary sCD14 levels and clinical measurements (Figure 2).

Discussion

Bacterial infections may cause disease manifestations through multiple mechanisms, including, but not limited to, direct effects of bacterial products, effects of the host immune response to the organism, and the persistent actions of the host immune response after clearance of the organism (Lin *et al*, 2000). Although there is little question that the signs and/or symptoms of periodontal disease result from the presence of the microorganisms, the cause of periodontal breakdown in patients with aggressive and chronic periodontitis is more controversial. Hence, molecular and immunological analyses are essential to verify the effect of other factors associated with variations in clinical characteristics of CP and AgP. Given that inflammatory mediators are continuously washed into saliva by gingival crevicular fluid (GCF), whole saliva sample may constitute an easy alternative to individual pocket samples for determining analytes that are present in periodontal pockets, providing a sensitive and inexpensive detection technique.

The demographic characteristics of the study subjects showed statistically significant differences in age according to the parameters agreed for CP and AgP (Lang *et al*, 1999; Lindhe *et al*, 1999), nevertheless, as a critical concern in periodontal disease is a lack of any reliable criteria to determine the extent of disease activity or rate

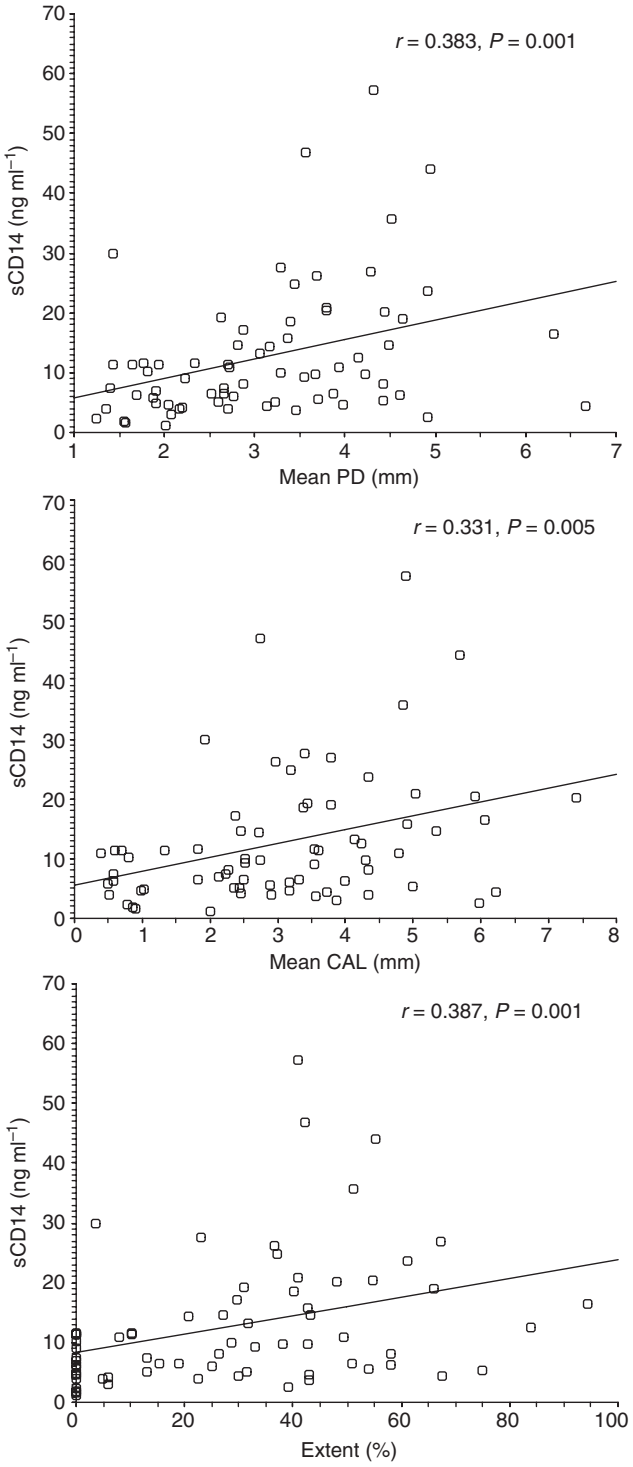

Figure 2 Scatterplot matrix demonstrating the statistical correlation between salivary levels of sCD14 (*y*-axis) and clinical measurements (*x*-axis). Each panel of the matrix is a scatter plot of one variable against another. The regression lines are presented for each plot along the Spearman's rank correlation coefficient (*r*). All correlations were statistically significant at level $P < 0.01$

of disease progression at a given time (Ozmeric, 2004), a full-mouth examination was used to evaluate some clinical parameters associated with the prevalence and

severity of periodontal disease, and to allow direct comparisons among the clinical groups studied, as it could give suggestive information about contrasting patterns of disease among different study groups. Thus, in this study the clinical analysis indicated a severe and generalized form of periodontal disease in both CP and AgP groups (>30% of sites involved/≥5 mm CAL; Armitage, 1999), with amounts of PD and CAL derived from full-mouth examination also statistically similar between them.

It has been demonstrated that sCD14 is produced by proteolytic shedding of CD14 from the cell surface of monocytes and macrophages in a process mediated by host matrix metalloproteinases (MMP)-9 and -12 (Senft *et al*, 2005), and is constantly present in the serum of healthy patients (Nockher *et al*, 1994; Nockher and Scherberich, 1995; Hayashi *et al*, 1999). Moreover, levels of sCD14 have been detected in several corporal fluids such as synovial and cerebrospinal (Lin *et al*, 2000), amniotic (Jones *et al*, 2002), GCF (McNamara *et al*, 1999; Jin and Darveau, 2001; Duncan *et al*, 2004), saliva (Sugawara *et al*, 2002; Takayama *et al*, 2003; Uehara *et al*, 2003), and breast milk (Labéta *et al*, 2000; Jones *et al*, 2002). Although currently no data are available on the salivary levels of sCD14 in periodontitis patients compared to healthy controls, previous data have shown that human salivary glands constitutively express CD14 and that sCD14 is secreted in saliva (Uehara *et al*, 2003) in a functionally active form (Sugawara *et al*, 2002).

Although the function of sCD14 in human disease has not yet been clarified, a potential pathogenic role of sCD14 in several infectious diseases has been proposed (Ayaslioglu *et al*, 2005). In general, increased levels of sCD14 have been found in non-infectious and infectious diseases such as systemic lupus erythematosus (Nockher *et al*, 1994), rheumatoid arthritis (Horneff *et al*, 1993), polytraumatised and severely burned patients (Kruger *et al*, 1991), septic shock (Landmann *et al*, 1995), malaria (Wenisch *et al*, 1996), brucellosis (Ayaslioglu *et al*, 2005), HIV-infection (Lien *et al*, 1998), and tuberculosis with or without HIV infection (Lawn *et al*, 2000). Hayashi *et al* (1999) investigated the concentrations of sCD14 in the sera of patients with adult periodontitis, early-onset periodontitis and compared with periodontally healthy donors. They found that sCD14 concentration in serum was significantly higher in patients with periodontitis than in healthy controls, but did not find significant difference between both clinical forms of periodontitis. In agreement with the former, in this study, although the samples analyzed were different, the levels of sCD14 did not differ significantly between the two periodontitis groups, but were significantly greater than those observed in saliva of healthy controls. Similar results have also been reported by Duncan *et al* (2004), who evaluated the sCD14 levels in GCF by immunoblotting.

It is thought that sCD14 either protects or enhances the host response to microbial LPS. While Maliszewski (1991) hypothesizes that sCD14 can reduce endotoxin-induced activities by competing with mCD14 for LPS and neutralize LPS–LBP complexes thereby prevent responses to LPS, other observations suggest that sCD14 competes inefficiently with cell-bound CD14 and certainly does not block the response of monocytes, which respond briskly to LPS in whole blood (Frey *et al*, 1992). In this study, the correlation between sCD14 concentrations and the clinical parameters was statistically significant. This might be interpreted as more production of sCD14 in cases with moderate-to-severe and generalized periodontal breakdown, which is consistent with the deleterious role of sCD14 because of an LPS potent stimulation of sCD14 release (Hayashi *et al*, 1999) and shedding (Bažil and Strominger, 1991) from monocytes/macrophages and activated neutrophils. As shedding implies the release of the ectodomain of a cell-surface molecule that will keep its biological activity (Duncan *et al*, 2004), once present in the extracellular environment in a soluble and biologically active form, CD14 can interact with cells lacking cell-surface CD14 such as endothelial and epithelial cells (Frey *et al*, 1992; Schumann and Rietschel, 1994). Thus, sCD14 could mediate cell activation induced by endotoxin and whole bacteria, resulting in the production of a potent immune response and proinflammatory mediators (Labéta *et al*, 2000), and amplifying the inflammatory process (Frey *et al*, 1992; Schumann and Rietschel, 1994), which further take part in the tissue destruction and bone resorption observed in periodontitis (Page, 1991). In addition, more recent evidence suggests that when bacteria propagate in the periodontal pocket, salivary sCD14 promotes their invasion and induces production of IL-8 by oral epithelial cells to recruit neutrophils and T cells and activate neutrophils for the initiation and establishment of an innate immune response to the bacteria at the site of infection (Takayama *et al*, 2003).

On the contrary, it was previously shown that the sCD14 expression within the periodontal tissue is negatively correlated with the amount of probing depth, thus suggesting a protective effect for sCD14 (Jin and Darveau, 2001). However, the small sample size in association with a narrow variability of bone defects might have influenced those results, as the size of these defects might not represent critical-size defects to evaluate the levels of sCD14. In other words, not only the probing depth but also the extent of the disease and the amount of attachment loss of the defects might influence the production of sCD14. Furthermore, unlike this study, that investigation had no healthy controls to evaluate the relationship between sCD14 levels and different periodontal conditions. Hence, without control patients conclusions can only be made cautiously.

In conclusion, the findings of the present study reemphasize the importance of whole saliva as sampling method in terms of immunological purposes in periodontal disease and suggest that elevated sCD14 concentration may be one of the host-response components associated with the clinical manifestations of periodontal disease.

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