Immunophenotyping in Saliva as an Alternative Approach for Evaluation of Immunopathogenesis in Chronic Periodontitis

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Background: To date, flow cytometric immunophenotyping has not been used to investigate immune patterns in saliva samples from individuals with inflammatory processes in the oral cavity, such as chronic periodontitis (CP). Saliva analysis could be a non-invasive method for evaluating oral health. The objective of this study is to determine the phenotype of leukocytes and total immunoglobulin A (IgA), IgG, and IgM titers in the saliva of individuals with CP.

Methods: Saliva samples were obtained from patients with CP (n = 12) and from a control group (n = 27) without oral diseases. Flow cytometry was performed to determine the frequency of T cells ($CD4^+$ and $CD8^+$), B cells, and natural killer (NK) cells as well as the total leukocyte population. Immunoglobulin titers were determined by dot enzyme-linked immunosorbent assay.

Results: Cell immunophenotyping revealed that patients with CP had a higher frequency of total leukocytes (47.94% ± 5.1%; $P < 0.001$), B cells (43.93% ± 6.2%; $P = 0.006$), NK cells (0.16% ± 0.04%; $P = 0.03$), and $CD4^+$ T cells (38.99% ± 4.4%; $P = 0.002$) than individuals without oral pathologies (24.75% ± 2.2%, 20.60% ± 2.7%, 0.09% ± 0.03%, and 16.82% ± 3.5%, respectively). No significant differences in salivary total IgA, IgG, and IgM titers were found between the two cohorts studied. Nevertheless, higher total IgG levels were observed in patients with CP, which could indicate a possible correlation between clinical attachment level and salivary IgG ($P = 0.07$; $r^2 = 0.08$).

Conclusion: These results show that cell phenotyping by flow cytometry could be an effective tool for determining leukocyte profiles in saliva samples from patients with CP and healthy individuals. *J Periodontol 2014;85:e111-e120.*

KEY WORDS

Chronic periodontitis; flow cytometry; immunoglobulins; leukocytes; saliva; T-lymphocytes.

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Chronic periodontitis (CP) is an inflammatory oral disease triggered by the host immune/inflammatory response to microorganisms in dental biofilm and calculus. In periodontal tissues, this response may cause the leukocyte population in saliva of individuals with CP to differ from that of periodontally healthy individuals.[1]

The primary immune response in CP occurs after colonization of the gingival sulcus by periodontal pathogens (*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia*, and others) that stimulate the production of, among other inflammatory mediators, cytokines and chemokines by gingival epithelial cells. This results in expression of adhesion molecules, an increase in gingival capillary permeability, and neutrophil chemotaxis and migration from the junctional epithelium to the gingival sulcus. This initial response promotes the migration of T cells and macrophages to the perivascular tissue. If the cellular immune response does not control the infection, B cells are recruited and differentiate into plasma cells, which are responsible for antibody (immunoglobulin) production.[2]

The secretory immunoglobulin A (SIgA) and immunoglobulins M and G (IgM and IgG) are important defense factors present in saliva. They affect the oral microbiota by interfering with its adhesion or inhibiting its cellular metabolism.[3] Patients with periodontal diseases have higher concentrations of these immunoglobulins in their saliva than individuals with healthy periodontium,[3] and the levels of SIgA and IgG decrease significantly after periodontal therapy.[4,5]

Traditional methods for the diagnosis of periodontal diseases, such as CP, are based on clinical and radiographic parameters. Although important, these parameters show only the changes, such as bone loss, that these diseases cause over time. The use of oral fluids for laboratory diagnosis of the disease not only allows a more reliable prognosis to be made but also allows the treatment to be monitored and provides an indicator of the risk of the patient developing CP. By analyzing saliva and gingival fluid, the presence of microorganisms, leukocytes, and inflammatory mediators produced during the host response and specific markers for bone resorption can be identified locally and systemically.[6]

Over the last 40 years, flow cytometry (FCM) has become increasingly important and has helped to advance knowledge in the field of immunology. It has been used as the main tool for the phenotypic and functional characterization of different individual cells and cell populations and has also recently been used in dentistry as a tool to assess the leukocyte population distribution in saliva samples from healthy individuals[7] and individuals submitted to psychologic stress.[8] The objective of the present study is to use FCM to evaluate the frequency of T lymphocytes ($CD4^+$ and $CD8^+$), B lymphocytes, and natural killer (NK) cells, as well as the total leukocyte population, in the saliva of individuals with or without CP to determine whether this approach could be used in the differential diagnosis of this condition. In parallel, the saliva titers of total IgA, IgG, and IgM in these individuals is determined.

MATERIALS AND METHODS

Patients and Controls

This study was approved by the Human Research Ethics Committee, Federal University of Amazonas (UFAM) (reference number CAAE – 0330.0.115.000-10), in accordance with Brazilian law, which complies with the Declaration of Helsinki, as revised in 2000. All individuals in both groups (patients and controls) were individually informed about the proposed study and agreed to participate by signing a written informed consent form.

The 39 participants (eight males and 31 females [CP group: aged 44.33 ± 8.89 years; control group: 40.33 ± 8.71 years]) underwent periodontal examination by a single examiner (PN). They were initially interviewed to obtain clinical and demographic data, including age, sex, and tobacco use. Individuals who had never smoked or had stopped smoking >5 years before the interview were considered non-smokers. In the clinical examination, six sites in each tooth (buccal, mesio-buccal, disto-buccal, lingual, mesio-lingual, and disto-lingual) were analyzed with the aid of a periodontal probe.¶ The parameters analyzed were probing depth (PD), clinical attachment level (CAL), the O'Leary et al. plaque index (PI), [9] and gingival bleeding on probing (BOP) index (Table 1).[10]

Saliva samples were obtained from patients with CP (n = 12) and controls (n = 27) seen at the Periodontal Clinic, State University of Amazonas (UEA), Manaus, Amazonas, Brazil, from April to September 2011. The control group consisted of individuals without evidence of CP. For the patient group, the authors selected individuals aged ≥30 years with a clinical and radiographic diagnosis of CP (at least one site in six different teeth with radiographic evidence of bone loss, PD ≥4 mm, and CAL ≥3 mm). For the control group, the authors selected individuals aged ≥30 years without CP (BOP <10%, CAL <3 mm, and PD ≤3 mm). Exclusion criteria were as follows: 1) other oral pathologies; 2) periodontal treatment in the last 6 months; 3) continuous use of medications such as antibiotics, immunomodulators, or anti-inflammatory drugs in the previous 3 months; 4) systemic diseases that interfere with the condition of the periodontium

¶ Michigan-O with Williams markings, Hu-Friedy, Chicago, IL.

(diabetes, human immunodeficiency virus infection, and immune disorders); 5) pregnancy; and 6) indigenous ethnicity.

Sample Collection

Saliva samples were collected from each individual between 9:00 am and 10:00 am (before breakfast) without any stimulus or prior dental hygiene. Participants remained with their mouths closed and the tips of their tongues on the hard palate for 5 minutes. They were then asked to spit saliva into previously sterilized 50-mL collecting tubes.[#] For cell phenotyping, 500 μL of each sample of saliva was transferred to a 1.5-mL microtube[**] containing 1% paraformaldehyde (PFA) (1:1) and stored at 4°C for up to 7 months. To titrate immunoglobulins, 100 μL saliva was aliquoted into a sterile microtube containing 5 μL phenylmethylsulfonyl fluoride and stored at –80°C for later use.

Dot Enzyme-Linked Immunosorbent Assay

Titration of total IgA, IgG, and IgM in saliva samples was performed by dot enzyme-linked immunosorbent assay (dot-ELISA)[11] with some modifications. Two microliters of each dilution of saliva (serially diluted two-fold from 1:100 to 1:204,800 for IgA and from 1:10 to 1:20,489 for IgG and IgM) was applied to nitrocellulose membranes.[††] The membranes were blocked for 2 hours with 5% powdered skim milk[‡‡] dissolved in Tris-buffered saline (TBS, pH 7.5). They were then incubated for 45 minutes at room temperature with the following peroxidase conjugates: goat anti-human IgA (α chain), goat anti-human IgM (Fc5μ), and goat anti-human IgG (H + L)[§§] (all diluted 1:1,000 in TBS, pH 7.5). After four washes with phosphate-buffered saline (PBS, pH 7.2), the reactions were revealed with 3,3′diaminobenzidine (DAB)[‖‖] diluted in 30 mL TBS, pH 7.5, in the presence of 0.015% hydrogen peroxide. The reactions were stopped with distilled water, and the membranes were dried between sheets of filter paper. Readings were then taken to obtain the final titer of each reaction. To facilitate statistical analysis, Ig titers were obtained with the help of a scale.

FCM

Staining of cells in saliva. FCM staining was carried out following the procedure described elsewhere.[8] Five hundred microliters of PBS, pH 7.0, was added to the saliva samples obtained as described above and then centrifuged at 2,000 rpm for 5 minutes at 4°C to remove mucus. The supernatant was discarded, and the pellet was resuspended in 1 mL of a PBS solution containing 0.1% sodium azide and 2% fetal bovine serum (PBSAz). Duplicate samples were incubated with either 5 μL four-color monoclonal antibody (mAb) reagent[¶¶] containing anti-CD45/fluorescein isothiocyanate (FITC) (for total leukocytes), anti-CD56/phycoerythrin (PE) (for NK cells), anti-CD19/energy coupled dye (for B cells), and anti-CD3/PE-cyanine 5 (for T cells) or three-color mAb reagent[##] containing anti-CD3/peridinin chlorophyll protein complex, anti-CD4/PE, and anti-CD8/FITC. After incubation for 20 minutes at 4°C in the dark, the samples were washed with 500 μL PBSAz (2,000 rpm for 5 minutes at 4°C). After the supernatant was removed, the pellet was resuspended in 500 μL PBS, and samples were promptly submitted to FCM. Fluorochrome-conjugated antibodies against the specific IgG isotype were used as negative-staining controls. PFA fixation of cells after sample collection did not interfere with antibody recognition during immunostaining.

Acquisition and analysis. The samples were acquired at a minimum of 200,000 events in a flow cytometer[***] and analyzed with specific software.[†††] Initially, to normalize the analysis protocol for the saliva samples, a sample of whole peripheral blood from a healthy individual was acquired so that gates could be defined according to standard blood-staining profiles (data not shown). Because not enough cells were obtained after sample preparation, the Ficoll-Hypaque gradient method could not be used to isolate mononuclear cells. The authors therefore used a control cell kit,[‡‡‡] a lyophilized preparation of peripheral blood mononuclear cells, to identify the lymphocyte region in the forward scatter (FSC) × sidescatter (SSC) dot plot.

Phenotyping of cells in saliva was performed using a FCM protocol as shown in Figure 1, where dot plot A represents the scattering profile of cells based on their size (FSC) and granularity (SSC) and was used to analyze the two different staining procedures. An electronic gate (referred to as "Cells") was created to include cells of interest and exclude debris. Figures 1B through 1E represent samples four-fold stained with anti-CD45, anti-CD3, anti-CD56, and anti-CD19 mAbs. Figure 1B shows the dot plot gated on Cells and represents a dual-parametric analysis of SSC vs. CD45, in which two cell populations can be defined: mononuclear cells (low SSC/CD45$^+$) and granulocytes (high SSC/CD45$^+$). Figures 1C through 1E, gated on low SSC/CD45$^+$, represent the fluorescence intensity for anti-CD3, anti-CD56, and anti-CD19,

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*** Epics XL-MCL, Beckman Coulter.
††† Expo 32, Beckman Coulter.
‡‡‡ Coulter Cyto-Trol, Beckman Coulter.

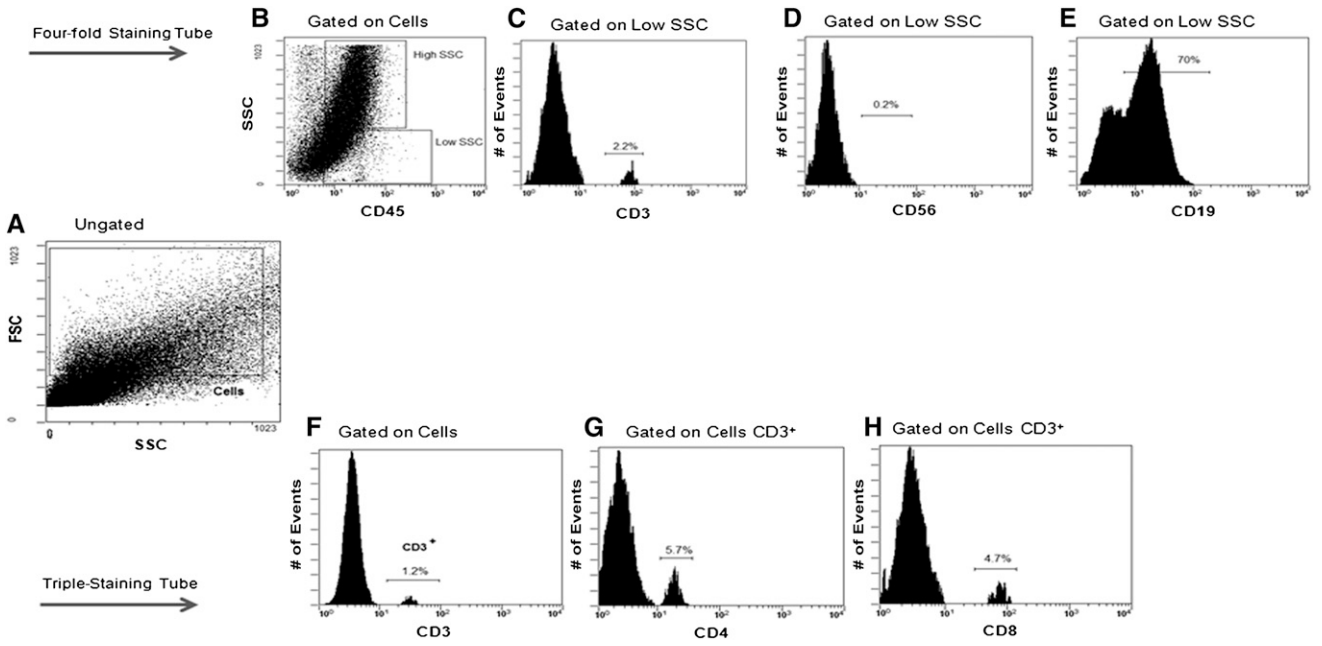

Figure 1.

Flow cytometric protocol for phenotypic analysis of two different saliva samples. ***A)*** *Dot plot represents the scattering profile based on cell size (FSC) and granularity (SSC) common to two different samples. The gate "Cells" includes cells of interest and excludes debris.* ***B through E)*** *Samples four-fold stained with anti-CD45, anti-CD3, anti-CD56, and anti-CD19 mAbs. B) Dot plot gated on Cells represents a dual-parametric analysis of SSC versus CD45, in which two cell populations can be defined: mononuclear cells (low SSC/CD45$^+$) and granulocytes (high SSC/CD45$^+$). C through E) Histograms gated on low SSC/CD45$^+$ represent the fluorescence intensity of anti-CD3, anti-CD56, and anti-CD19 mAbs, respectively.* ***F through H)*** *Histograms of samples triple-stained with anti-CD3, anti-CD4, and anti-CD8 mAbs. F) Histogram gated on Cells represents T lymphocytes (CD3$^+$). G and H) Histograms based on the CD3$^+$ gate represent CD4$^+$ T lymphocytes and CD8$^+$ T lymphocytes, respectively.*

respectively. Figures 1F through 1H represent samples triple-stained with anti-CD3, anti-CD4, and anti-CD8 mAbs. Figure 1F, gated on Cells, represents T lymphocytes (CD3$^+$). Figures 1G and 1H are based on the CD3$^+$ gate and represent CD4$^+$ and CD8$^+$ T lymphocytes, respectively.

Compensation adjustments for interfering signals were performed by acquiring peripheral blood mononuclear cells labeled with a single fluorochrome-conjugated antibody and were based on criteria for mean fluorescence intensity values in quad-stat regions of dual-color dot plots.

Statistical Analyses

Descriptive statistics were used to describe the main clinical and demographic features of patients and controls. Two-tailed *P* values were adopted. The *t* test was used to compare means or medians when the Kolmogorov–Smirnov test revealed a normal distribution. The non-parametric Mann-Whitney test was used to compare means or medians when the data did not have a normal distribution. Linear regression analysis was applied to evaluate the correlation between continuous variables. The D'Agostino and Pearson test was used to check for normality, and the correlation among the percentages of T cell subsets was estimated by Spearman rank correlation coefficient. A significance level of $P < 0.05$ was adopted. Receiver-operating characteristic (ROC) curves allowed the sensitivity, specificity, and area under the ROC curve (AUC) to be determined for CD45, CD4, CD8, CD56, and CD19 analyses.

RESULTS

Clinical Parameters

Table 1 shows the characteristics of the populations studied. No significant difference ($P > 0.05$) in mean salivary flow rate was found between patients and controls. However, as expected, significant differences were observed for PI, BOP, PD, and CAL ($P < 0.05$). Individuals with CP had a higher percentage and number of sites with gingival bleeding, biofilm, and CAL than individuals without the disease.

Dot-ELISA

Patients with CP (n = 12) and healthy individuals (n = 27) were evaluated. Figures 2A through 2C show the titration scale for total salivary IgA, IgG, and IgM, respectively. The data represent the median with the interquartile range. Figure 2A shows the titration scale for IgA (1 to 1:100; 2 to 1:200; 3 to 1:400; 4 to 1:800; 5 to 1:1,600; 6 to 1:3,200; 7 to 1: 6,400; 8 to 1:12,800; 9 to 1:25,600; 10 to 1:51,200; 11 to 1:102,400; and 12

Table 1.

Baseline Characteristics and Parameters (mean ± SD) of Study Participants

Characteristic	CP	Healthy Periodontium
Number of participants	12	27
Males (% [n])	8.30 (1)	26.0 (7)
Females (% [n])	91.70 (11)	74.0 (20)
Smokers (%)	0	0
Age (years)	44.33 ± 8.89	40.33 ± 8.71
Salivary flow rate (mL/minute)	0.85 ± 0.39	0.68 ± 0.30
PI (%)	84.65 ± 25.14*	17.01 ± 23.36
Sites with BOP (%)	53.46 ± 34.41*	6.35 ± 6.64
PD (mm)	5.34 ± 1.018*†	2.66 ± 0.62
CAL (mm)	6.77 ± 1.774*†	2.89 ± 0.32

* $P < 0.05$, significant difference between groups.
† Mean of six sites with highest PD among patients and controls.

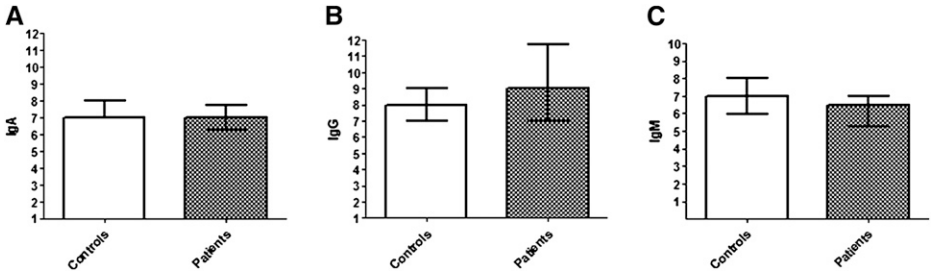

Figure 2.
Evaluation of saliva Ig titers. ***A)*** *Titration scale for IgA.* ***B*** *and* ***C)*** *Titration scales for IgG and IgM, respectively, as described in the text. Patients with CP (patients) (n = 12) and healthy individuals (controls) (n = 27). Data represented by median with interquartile range. Significance level: P <0.05. P values were obtained using a Mann–Whitney U test.*

to 1:204,800); Figures 2B and 2C show the scales for IgG and IgM, respectively (1 to 1:10; 2 to 1:20; 3 to 1:40; 4 to 1:80; 5 to 1:160; 6 to 1:320; 7 to 1:640; 8 to 1:1,280; 9 to 1:2,560; 10 to 1:5,120; 11 to 1:10,240; and 12 to 1:20,480). Figure 2A shows that there was no difference in median between the groups (1:6,400). In Figure 2B there is a small difference in median between controls and patients (1:1,280 and 1:3,200, respectively). The antibody titer mean (± SD) was slightly higher for patients (1:5,947 ± 1:6,363) than for the control group (1:2,262 ± 1:3,871). In Figure 2C there was also a small difference in the median titer between controls and patients (1:640 and 1:480, respectively). The antibody titer mean (± SD) was slightly higher for the control group (1:666 ± 1:560) than for patients (1:536 ± 1:406).

There were no statistically significant differences in IgA, IgG, or IgM titers between patient and control groups (P = 0.46; P = 0.11; P = 0.61, respectively). Total immunoglobulin titers were related to CAL, and although a trend toward a positive correlation with IgG (P = 0.07; r^2 = 0.08) was found (Fig. 3F), there was no correlation among IgA and IgM titers and CAL (P = 0.38; r^2 = 0.02 and P = 0.83; r^2 = 0.001).

Flow Cytometry Analysis

FCM data revealed that patients had a higher total $CD45^+$ leukocyte frequency than the control group (47.94% ± 5.1%, 24.75% ± 2.2%, respectively; P <0.001) (Fig. 4A). T lymphocyte frequencies in both groups were low, and the percentages for the patients (1.02% ± 0.3%) were similar to those for the control group (1.90% ± 0.3%; P = 0.07) (Fig. 4D). NK cell, $CD4^+$ T cell, and B lymphocyte frequencies were higher in the patient group (0.16% ± 0.04%, 38.99% ± 4.4%, 16.82% ± 3.5%, respectively) than in the control group (0.09% ± 0.03, 43.93% ± 6.8%, 20.60% ± 2.7%; P = 0.03, P = 0.002, P = 0.006, respectively) (Figs. 4B, 4C, and 4E). $CD8^+$ T cell frequency (Fig. 4F) was higher in the control group than in the patient group (12.0% ± 1.9% and 4.48% ± 1.4%, respectively; P = 0.003).

The results also showed a positive correlation between CAL and total leukocyte frequency (P = 0.004;

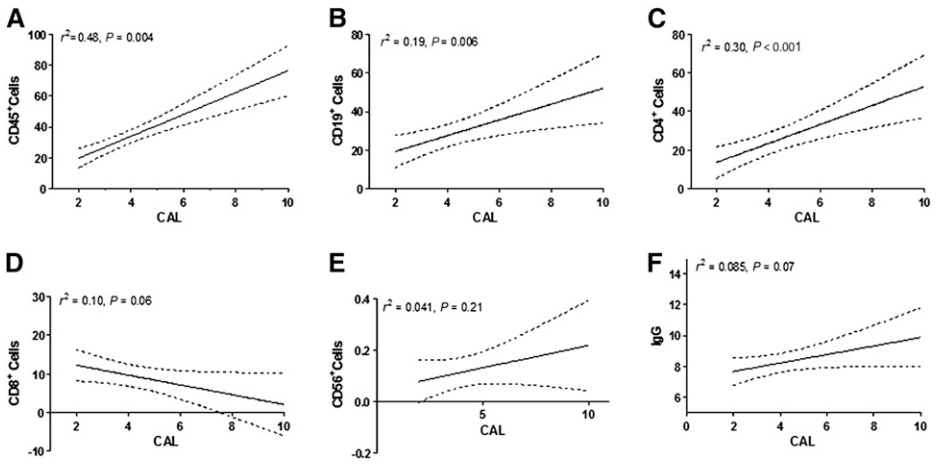

Figure 3.
Correlation analyses between cell frequency and CAL and between IgG and CAL. ***A)*** *CD45+.* ***B)*** *CD19+.* ***C)*** *CD4+.* ***D)*** *CD8+.* ***E)*** *CD56+.* ***F)*** *IgG.*

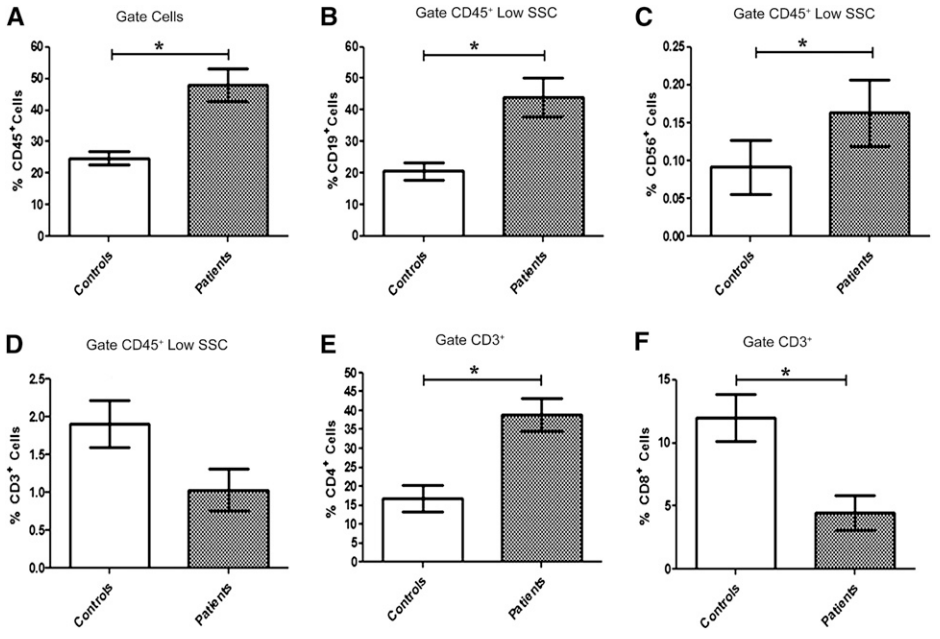

Figure 4.
Results of phenotypic analyses in cells obtained from saliva samples. The bars represent the cell population frequencies determined by FCM. ***A)*** *CD45+.* ***B)*** *CD19+.* ***C)*** *CD56+.* ***D)*** *CD3+.* ***E)*** *CD4+.* ***F)*** *CD8+. Data shown as mean ± SD.* **P <0.05. *P values were obtained using a Mann–Whitney U test.*

$r^2 = 0.2$), CD4+ T cell frequency ($P<0.001$; $r^2 = 0.3$), and B lymphocyte frequency ($P = 0.006$; $r^2 = 0.18$) (Figs. 3A through 3C). No positive correlation between CAL and T lymphocyte (CD3+) ($P = 0.39$; $r^2 = 0.019$), CD8+ T cell ($P = 0.06$; $r^2 = 0.010$), or NK cell ($P = 0.21$; $r^2 = 0.004$) frequencies was observed (Figs. 3D and 3E).

There was no positive correlation between B lymphocyte and CD4+ T cell ($P = 0.13$; $r^2 = 0.06$) or CD8+ T cell ($P = 0.44$; $r^2 = 0.01$) frequencies or between B lymphocyte frequency and total IgA, IgG, or IgM titers ($P = 0.28$, $r^2 = 0.03$; $P = 0.87$, $r^2 = 0.0006$; and $P = 0.69$, $r^2 = 0.004$, respectively).

Figure 5 shows the ROC curve and AUC, which demonstrated that there is a correlation among CP and the increase in the number of CD45+, CD19+, CD4+, and CD56+ cells and decrease in the number of CD8+ cells in the saliva samples.

DISCUSSION

The role of immunoglobulins produced in advanced or progressive CP is not well established. However, there is evidence that these proteins can neutralize pathogens (i.e., act as neutralizing antibodies) or induce persistent infection (i.e., act as non-protective antibodies). In the latter case, regulation of B cell responses to polyclonal B cell activators leads to the production of autoantibodies such as anti-type I and III collagens, which in turn induce pathologic processes in the periodontal lesion.[12-15]

In this study, total salivary IgA, IgM, and IgG are quantified, but their specificity and neutralizing capacity are not assessed. Titration of these immunoglobulins was carried out by dot-ELISA. This method is widely used to express immunoglobulin levels.[11]

Specific antibodies against microorganisms associated with periodontal disease can be used to gain a better understanding of this pathology.[16] Statistically significant positive correlations between the concentrations of IgA and IgG specific for *A. actinomycetemcomitans* in saliva of patients with advanced CP and serum levels of these antibodies have been observed.[16,17] Those authors concluded that saliva samples could be used to evaluate the humoral response to this bacterial species with the same efficacy as serum assessment in adult patients with severe CP.[17]

Most IgG present in saliva is derived from serum and reaches the oral cavity via crevicular fluid.[18] The

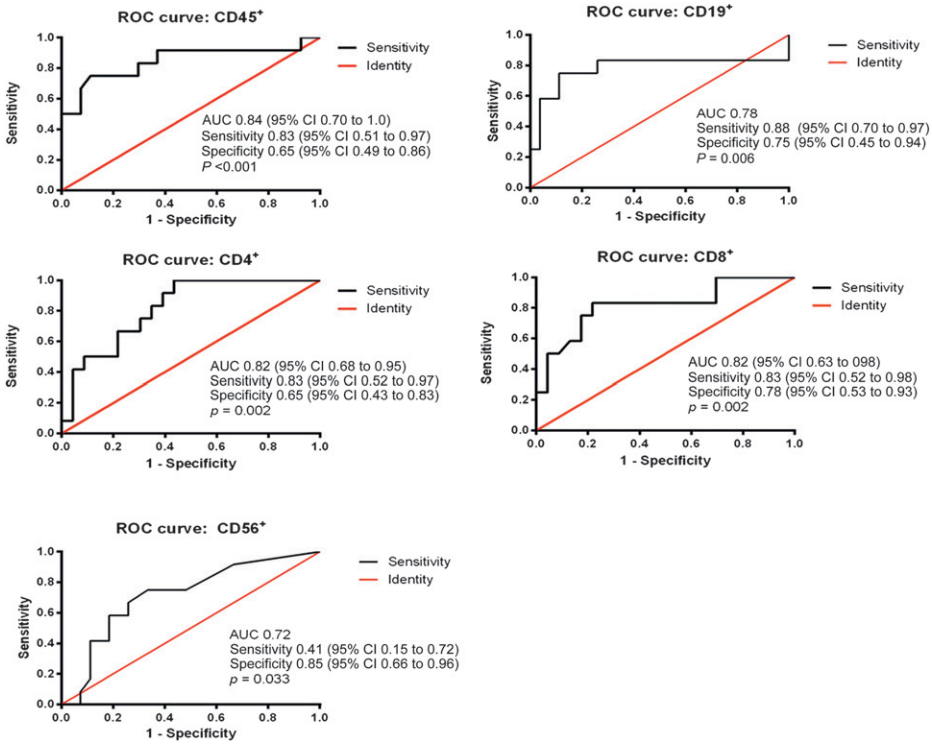

Figure 5.
ROC curve analyses of patients and controls. Calculation of sensitivity, specificity, and AUC for CD45; CD4; CD8; CD56; and CD19 biomarkers. CI = confidence interval.

results of the present study show that there was no significant difference between total Ig titers in saliva from the patient and control groups, although there was a trend toward a positive correlation between IgG titers and CAL. Such a correlation may exist, but because of the small size of the patient cohort, further studies are required to confirm this. The authors therefore decided to consider the median rather than mean titers because large individual variations between the participants could interfere in the analysis. These data confirm the findings from a previous study[19] that found similar total plasma IgA, IgG, and IgM titers in patients with and without periodontal disease. However, in the same study, the authors found that IgG1 and IgG2 serum titers were higher in the control group and that total IgG and IgG2 titers were reduced in smokers with CP.

Because it is a non-invasive method of immune evaluation, FCM has been shown to be a useful tool for studying cell populations in saliva samples.[7,8,20] The technique can therefore be used to evaluate various pathologic conditions of the oral cavity that could interfere with the distribution of leukocytes. In this study, the authors observed a greater percentage of total leukocytes in patients with CP, a finding consistent with data from previous studies demonstrating an increase in the frequency of leukocytes in saliva in clinical gingivitis[20] and CP[21,22] when analyzed by microscopy[21,22] or FCM.[20] The authors also found a higher percentage of NK cells and B lymphocytes in patients with CP than in controls. This finding corroborates those of several studies reporting that lesions in advanced or progressive CP are dependent on NK or plasma cells.[12,23-28]

The roles of T helper 1 (Th1), Th2, Th17, and regulatory T cell cytokine profiles in CP have not been fully clarified.[2,29-34] Positive correlations were found among interleukin (IL)-17, IL-23, IL-1β, IL-6, tumor necrosis factor-α, and CAL in individuals with CP, and the levels of these cytokines were higher in the gingiva of individuals with severe CAL.[35]

Studies to assess cytokine profiles or mRNA expression show that the Th17 lineage plays a role in CP. Differentiation of naive T cells into Th17 cells is activated in the presence of extracellular bacteria and is responsible for the recruitment of neutrophils, which help in the phagocytosis and consequent elimination of these pathogens.[36-42]

The results for the frequency of T cells in saliva in healthy individuals were different from those reported by other authors[7,8] who found a significantly higher percentage of this cell population. Despite the low T lymphocyte frequency in this study, the significant difference in $CD4^+$ T cell frequency between patients and controls is noteworthy because it indicates that there is a greater frequency of this cell subtype in patients with CP. The greater number of $CD4^+$ T cells in individuals with CP could point to involvement of the Th17 lineage, as some reports have shown that this lineage leads to an increase in the number of granulocytes,[37,43] agreeing with the present finding of a larger number of granulocytes in the saliva of patients with CP. Although salivary cytokines were not quantified in the present study, it has been shown that CXCL-8 (a neutrophil chemoattractant), prostaglandin E_2, and IL-6 are released by endothelial and epithelial cells as well as fibroblasts and that this release is induced by IL-17A, which in turn is produced by the Th17 lineage.[43]

Furthermore, unlike an earlier study,[8] these results showed the presence of $CD8^+$ T cells in saliva for the first time, albeit at a low frequency. This is probably

mainly due to the different methods of collecting and processing saliva samples and differences in the ages of participants, as the stimulation of saliva secretion, the collection technique used, and the presence or otherwise of epithelial cells and mucus in the sample could influence the assessment of cell population distribution. Despite the low $CD8^+$ T cell frequency found in this study, a possible correlation between CAL and $CD8^+$ T cells ($P = 0.06$; $r^2 = 0.01$) may exist, indicating that they could be involved in the pathogenesis of periodontitis. For this reason, future studies are required to validate these findings.

The frequency of $CD8^+$ cells in saliva was lower in individuals with CP than in healthy individuals, corroborating the findings of another study[44] in which $CD8^+$ cells were present in smaller numbers in peripheral blood of individuals with this condition. This is probably because the $CD8^+$ T cell response is normally associated with infections of viral origin whereas periodontal diseases are normally associated with bacterial infections. Although patients have a small number of $CD8^+$ T cells, they have a greater number of NK cells, which perform similar functions, although differing in their mechanisms of action. These results for $CD4^+/CD8^+$ cell ratios (data not shown) in patients and controls disagree with those of a study[45] that found a lower ratio in individuals with the disease as well as with the data of another study[46] in which there was no significant difference but a slight increase in the ratio of these cells in the blood of controls in relation to patients. The differences in the results of these studies might be explained by interindividual heterogeneity in $CD4^+/CD8^+$ cell ratios or by the type of periodontopathogen as well as the periodontopathogen load in lesions in CP. This was shown in a study in which higher $CD3^+/CD8^+$ and $CD3^+/CD16^+$ cell ratios were associated with higher *Treponema denticola* and *A. actinomycetemcomitans* loads, respectively, in patients with CP than in controls.[47]

In this study the frequency of $CD3^+$ T cells was approximately 1% and 1.3% in patients and controls, respectively. Most of these cells were made up of a population that did not express CD4 or CD8 coreceptor molecules, i.e., double-negative (DN) T cells. DN T cells have been studied recently in several immunodeficiencies, such as systemic lupus erythematosus[48] and cutaneous leishmaniasis.[49] Studies show that DN T cells in individuals with leishmaniasis[49] and Chagas disease[50] have a proinflammatory cytokine profile (tumor necrosis factor-α,[48-50] interferon-γ,[48-50] and IL-17[48]) when they have αβ receptors, and a regulatory profile (IL-10[49,50]) when they have γδ receptors. The presence of these DN T cell subtypes probably has a modulatory effect. Whereas the characteristics of αβ DN T cells include activation of the inflammatory response to control infection, thereby causing tissue damage, γδ DN T cells regulate the inflammatory process in an attempt to keep it from worsening.[49,50] In healthy patients, these cells have been identified in lower frequencies in peripheral blood and normally express molecules that activate memory cells or have cytotoxic functions.[51] In the present study, the authors found a higher frequency of DN T cells in the saliva of patients than in the saliva of healthy individuals. As the aim of this study is not to investigate DN T cells, a detailed analysis of these cells was not carried out. Further studies are required to investigate T cell receptor chains (α:β or γ:δ) of this DN lineage in the saliva of healthy individuals and individuals with CP.

Hence, in addition to microbial research, knowledge of the cells and different classes of immunoglobulins in saliva of patients with active periodontal infection may help to establish an accurate diagnosis, predict the prognosis of patients with this condition, and identify individuals with a higher propensity to develop these pathologies. It would also allow the effectiveness of periodontal therapy to be more accurately assessed with laboratory rather than clinical parameters.

In recent years, techniques have been developed that use saliva to aid in the diagnosis of certain diseases, such as caries and periodontal disease. This has been accompanied by an increase in the importance of saliva-based diagnostics because of advances made in both dentistry and other areas of healthcare, such as medicine. The techniques used for the collection of saliva and its processing, stabilization, and storage are fundamental for saliva-based diagnostics.[52] Differences in the handling and processing of saliva can mean that the results of different studies cannot be compared.[52] Thus, standardization of these techniques is essential if they are to be successfully developed and used. As a large proportion of the adult population is affected by some kind of periodontal disease, the use of rapid, accurate diagnostic technologies in the treatment of these conditions is essential.[52] This study shows the importance of FCM in the validation of biomarkers in saliva for the diagnosis of different types of periodontal disease. Further studies with a larger study population are required for more accurate validation of these biomarkers.

CONCLUSIONS

Salivary leukocyte count and phenotyping by FCM can be useful in the diagnosis of CP and can also be used to discriminate between CP and other conditions. Standardization of procedures for collecting saliva and subsequent acquisition and analysis by FCM is crucial to achieve consistency between

results in salivary cell phenotyping studies. Once procedures are standardized, immunophenotyping of saliva by FCM will be a promising tool for the diagnosis of CP, as it will allow a more accurate prediction of the prognosis of this disease based on the analysis of salivary cellular profiles and the detection of other parameters such as cytokine production in affected individuals.

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