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Salivary inflammatory mediators and metalloproteinase 3 in patients with chronic severe periodontitis before and after periodontal phase I therapy

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Abstract

Background: The role of IL-1β, PGE_2 and MMP-3 in the pathogenesis of periodontal disease is well researched. This study aimed to asses and compared the salivary IL-1β, PGE_2 and MMP-3 levels in patients with untreated chronic severe periodontitis and those treated with periodontal phase I therapy and periodontally healthy individuals as controls, in relationship to the presence of salivary anti-$β_1$ IgA.

Methods: A total of 30 subjects participate in the study: 15 subjects had chronic severe periodontitis and 15 were healthy individuals used as a control. After saliva collection and its purification, we quantify by enzyme-linked immunosorbent assay (ELISA) procedure using as coating antigen a synthetic $β_1$ peptide with an amino acid sequence identical to the second extracellular loop of the human $β_1$ adrenoceptor ($β_1$ AR), the presence of anti $β_1$ AR antibody (IgA) in the saliva of patients and healthy individuals. Also, IL-1β, PGE_2, nitrites and metalloproteinase 3 (MMP-3) were assessed using ELISA) assay.

Results: Our data indicated that IL-1β, PGE_2, nitrites and MMP-3 levels are elevated in the saliva of patients with untreated chronic severe periodontitis and were significantly higher than in healthy subjects. Also, the amounts of anti- $β_1$ IgA in the saliva was significantly higher compared with that of healthy individuals. After periodontal phase I therapy these levels of inflammatory biomarkers are significantly reduced but the titres of the antibody did not change, suggesting a close association between salivary IL-1β, PGE_2, nitrites and MMP-3 and periodontitis without any changes in the levels of anti $β_1$ IgA.

Conclusions: These results suggest that the abnormal amount of these cytokines and enzymes in saliva has potential monitoring applications as a risk marker of the disease progression but the raised levels of anti $β_1$ IgA present in the saliva of chronic severe periodontitis patient, are not directly associated with the course of the disease. Additional studies are needed to validate this assumption.

Keywords: Periodontitis, IL-1β, PGE_2, antibodies anti-$β_1$ IgA

Introduction

Periondontal disease is a chronic microbial and inflammatory process characterized by the presence of sulcular pathogenic bacteria, impaired host immune response, destruction of the connective tissue involved in tooth attachment, and resorption of alveolar bone. Bacterial pathogens are required to initiate the disease process [1-3].

Circulating substances have been detected at elevated levels in gingival crevicular fluid and whole saliva of patients who have periodontal disease, making them putative biomarkers of the disease [4-6]. Periodontal pathogens activate host cells to produce pro-inflammatory mediators [7,8] and cytoplasmatic enzymes [9], which, in turn, promote the destruction of periodontal tissues. The release of the inflammatory cytokine, IL-1β, PGE_2 and lysosomal and cytoplasmic enzymes, such as metalloproteinases (MMPs), to periodontal tissues is higher in the areas with inflammation [10,11]. In addition, various enzymes, cytokines and biomarkers of bone turnover have been found to be elevated in the saliva of periodontitis patients in comparison with periodontally healthy controls [12-14].

Recently, we reported that in the sera of periodontitis patients we found autoantibodies against atria cardiac $β_1$-adrenoreceptor (anti-$β_1$-AR IgG) that were able to mimic the effect of an authentic $β_1$-AR agonist acting on atria $β_1$-AR [15,16]. However, the release of host-derived inflammatory mediators, such as cytokines from chronically inflamed periodontal tissues, into the circulation together with the sera anti-$β_1$-AR IgG, may provide a link between periodontal disease and cardiovascular disease [17,18]. Moreover, the effect of anti-$β_1$-AR IgG acting on $β_1$-AR in rat atria and its capacity to activate caspase pathway, molecular signals involved in anti-$β_1$-AR IgG - $β_1$-AR-stimulated myocardium apoptosis and increased cAMP production and JNK phosphorylation, and the role of anti-$β_1$-AR IgG in the release of inflammatory mediators (PGE_2, NO, cGMP) that participate in atria $β_1$-AR-stimulated cardiomyocytes apoptosis were also determined [19].

Table 1. Comparison of the Clinical Parameters of the CP patients with or without Conventional Periodontal Treatment.

Characteristics	Healthy subjects (control) (n=15)	Chronic Periodontitis Patients (n=15)	
		Before Treatment	After Treatment
Age (years, SD)	49.6±5.2	47.9±5.6	47.5±5.4
Male (%)	80	75	75
Smoking (non-smoker/smoker)	0/12	0/15	0/15
CAL ≥ 2 mm (%, SD)	0	80.54±12.31	60.33±11.16*
PD ≥ 4 (%, SD)	0	69.40±15.91	47.96±17.71**
BOP (%, SD)	5.37±1.65	88.44±10.76	75.42±10.26
PI (SD)	1.10±0.51	6.14±0.48	2.28±0.38**
GI (SD)	0.55±0.25	1.46±0.18	1.40±0.21

All patients and healthy individuals had at least 20 teeth and no systemic diseases. Values expressed the means±SD of fifteen patients and fifteen healthy normal subjects. CP: chronic Periodontitis; CAL: clinical attachment level; PD: probing depth; BOP: bleeding on probing; PI: periodontal index; GI: gingival index; SD: standard desviation. Mann-Whitney U-test was applied and differences were considered statistically significant when $P<0.05$. *$P<0.001$ comparing CP after versus CP before treatment; **$P<0.001$ comparing CP after versus CP before treatment.

Based on these observations, we considered it of special relevance to investigate whether salivary secretory IgA (anti-β_1 IgA) from patients with chronic severe periodontitis could be a new marker of the pathosiological event that occurs in this disease in relationship with host-derived enzymes (MMPs), cytokines (IL-1β) and PGE$_2$ present in the saliva of the untreated periodontitis patients and those treated with periodontal phase I therapy in its.

Materials and methods
Patients
The patients in this study were 40 to 60 year-old, non-smoker males being treated in the Department of Periodontics, School of Dentistry, University of Buenos Aires.

Thirty patients with pre-existing chronic severe periodontitis were included in the test group before and after conventional periodontal I therapy. The assessment of clinical parameters was carried out by a trained periodontist following criteria based on clinical parameters and the severity of periodontal tissue destruction [20]. The characteristic clinical signs of periodontitis included loss of clinical attachment, horizontal and/or angular alveolar bone loss, periodontal pocket formation, and gingival inflammation. To be included in the study, at least six sites with ongoing periodontal disease were required. Clinical measurements in patients with periodontitis included sites with alveolar bone loss>2mm and a pocket depth>5 mm with bleeding and attachment loss>3 mm. No subject (periodontal patient or healthy individual) had any systemic illness and they were all never-smokers. Patients with periodontitis had

not received periodontal treatment or antibiotics within the preceding 5 months or any anti-inflammatory drug within 3 weeks prior to the study. The clinical characteristics of the study population and the healthy subjects (controls) are shown in (**Table 1**).

The control group consisted of thirty healthy non-smoker volunter males of the same age range, and with a clinically healthy periodontium from the same department. In the healthy subjects (control group), the probing depth was<3 mm and the attachment loss was<2 mm. Moreover, probing pocket depth and clinical attachment level were assessed at six sites per tooth and bleeding on probing at four sites per tooth. This group had<10% of sites with bleeding on probing (BOP), no sites with probing depth (PD)≥4 mm, no clinical attachment loss (AL), <2 mm, and no radiographic evidence of bone loss.

Exclusion criteria were individuals who had undergone periodontal treatment in the last 6 months; history of medications including antibiotics and anti-inflammatory drugs in the last 6 months; history of any systemic disease; and detection of any obvious oral mucosal lesion.

This study was approved by the Ethical Commission of the School of Dentistry, University of Buenos Aires. Written informed consent was obtained from each participant in this study, which was conducted under the rules of the Helsinki Declaration.

Saliva collection
Participants were instructed to refrain from eating, drinking and practicing oral hygiene procedures 12 hours before saliva collection. Whole unstimulated saliva was collected early morning after fasting (8 hours) from all patients by expectoration into sterile bulbs. The total amount of saliva collected was 1 ml. Collected samples were placed immediately on ice and transported to the laboratory, where they were centrifuged at 5,000 g for 10 minutes, and the clear supernatants were stored in aliquots at –80ºC in presence of protease inhibitors. The samples were thawed and the assays were performed with in 2 months of collection.

Clinical parameters
Clinical data included: CAL: clinical attachment level; PD: probing depth; BOP: bleeding on probing; PI: periodontal index; GI: gingival index (**Table 1**). The index measurements were done at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, disto-lingual, mid-lingual and mesio-lingual) and CAL was obtained by measuring only interproximal sites. These data were recorded and the treatment was performed by a trained periodontist. The data were recorded after saliva sample collection.

Periodontal therapy
Periodontal phase I therapy (PPIT) consisted of full-mouth scaling, root planning and oral hygiene instructions. Only the patients with periodontitis received PPIT after saliva

**Table 2. Salivary flow, total IgA and subclasses of IgA
(IgA_1 and IgA_2).**

Group	Basal salivary flow (ml/10 min)	Total IgA (µg/ml)	IgA_1 (µg/ml)	IgA_2 (µg/ml)
Periodontitis patients	1.2±0.6*	164±19	66±5*	98±8*
Healthy subjects	9.7±3.5	180±22	72±6	108±9

Values represent the means±SEM of 15 patients and 15 healthy
normal subjects. *P<0.0001 comparing with healthy subjects.

sample collection and clinical measurements of baseline.
The therapy was completed in two visits, each one week
apart. No antibiotics were prescribed after the treatment. The
patients were re-evaluated for clinical parameters one month
afterwards and saliva samples were taken again.

Purification of saliva IgA

The IgA fractions of 15 patients with chronic periodontitis
untreated and treated with PPIT and 15 healthy individuals
were independently purified by standard diethylaminoethyl
cellulose (DEAE) chromatography. Briefly, saliva samples were
dialyzed against 0.01 M phosphate buffer, pH 8.0, for 18 hours
and then applied to DEAE cellulose columns equilibrated in
the same buffer. The pass-through IgG-rich fractions were
discarded and IgA-rich fractions were eluted with 0.05 M NaCl
in 0.01 M phosphate buffer, pH 8. The IgA concentration in the
enriched fractions was quantified by radial immunodiffusion
method after concentration by ultrafiltration with PM-30
filtering membranes (Amicon, Beverly, MA, USA) (*cutoff
molecular weight, 30,000 Da*). The concentration of IgA was
35.8±15.2 mg/dl in the IgA-enriched fractions. IgA was also
purified by affinity chromatography of different saliva on
Jacalin agarose beads following the recommendations of the
supplier (ICN Pharmaceuticals, Irvine, CA, USA) and previously
described methods [21].

ELISA procedure

Fifty microliters of synthetic β_1 peptide solution (20 µg/ml)
in 0.1 M Na_2CO_3 buffer, pH 9.6, was used to coat microtiter
plates (NUNC, Kastrup, Denmark) at 4°C overnight.

After blocking the wells, diluted sera from patients with
chronic severe periodontitis and healthy individuals (control)
were added in triplicate and allowed to react with the peptide
for 2 hours at 37°C. After the wells were thoroughly washed
with 0.05% Tween 20 in phosphate buffered solution (PBS)
100 µl of 1:6000 biotinylated goat anti-human IgA antibodies
(Sigma Chemical Co., St. Louis, MO, USA) was added and
incubated for 1 hour at 37°C. Then, a 1:6000 dilution of
extravidin-alkaline phosphatase (Sigma) was allowed to
react an extra 30 minutes at 37°C. After extensive washings,
p-nitrophenylphosphate (1 mg/ml) was added as the substrate,
and the reaction was stopped at 30 minutes. Finally, the plates
were read at 405 nm and the results for each sample were

expressed as the means and SD of triplicate values.

Biomarker analysis

Concentration of salivary IL-1β (pg/ml), PGE_2 (ng/ml), nitrites
(µM) and MMP-3 (ng/ml) were determined in duplicate using
ELISA assay for each patient and the healthy individuals of
the human colorimetric immunoreactive kits from Cayman
Chemical (Ann Arbor, MI, USA), respectively.

Drugs

The synthetic β_1 peptide corresponded to the sequence of
the second extracellular loop of the human β_1-AR (H-W-W-R-
A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C) and a 27-mer
non-related peptide S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-S-G-
S-G-S-G-S-G-S-G-S, as a negative control, were synthesized
by Peptide Genetic Research Company (Livermore, CA, USA)
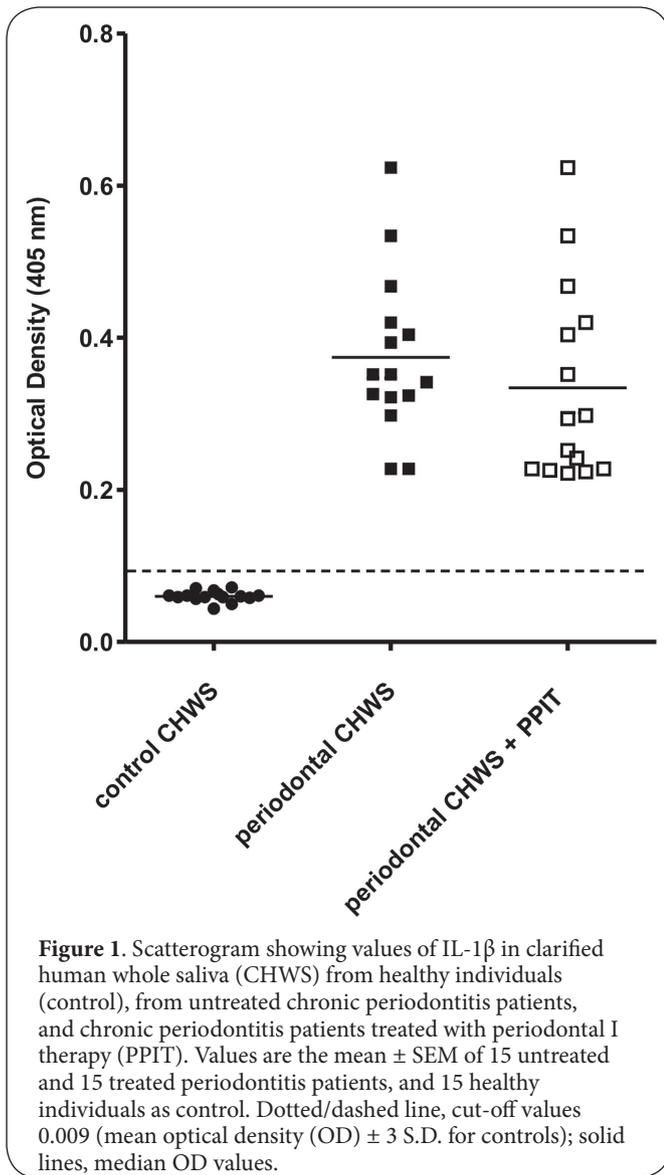as described previously [22].

Statistical analysis

Student's *t*-test for unpaired values was used to determine
the levels of significance. Analysis of variance (ANOVA) and a
post hoc test (Dunnett's method and Student-Newman-Keuls
test) were employed when pair-wise multiple comparison
procedures were necessary. Differences between means
were considered significant at P<0.05.

Results

It can be seen in (**Table 2**) the total salivary IgA (Total IgA
(µg/ml) in periodontitis patients: 164±19; healthy subjects:
180±22; n=15 in each group) and its subtypes: IgA_1 (µg/
ml): periodontitis patients: 66±5*; healthy subjects: 72±6;
*P<0.0001 comparing with healthy subjects; n=15 in each
group and IgA_2 (µg/ml): periodontitis patients: 98±8*; healthy
subjects: 108±9; *P<0.0001 comparing with healthy subjects;
n=15 in each group. Also, the values of the salivary flow
(Basal salivary flow (ml/10 min) in periodontitis patients:
1.2±0.6*; healthy subjects: 9.7±3.5; *P<0.0001 comparing
with healthy subjects; n=15 in each group) were performed
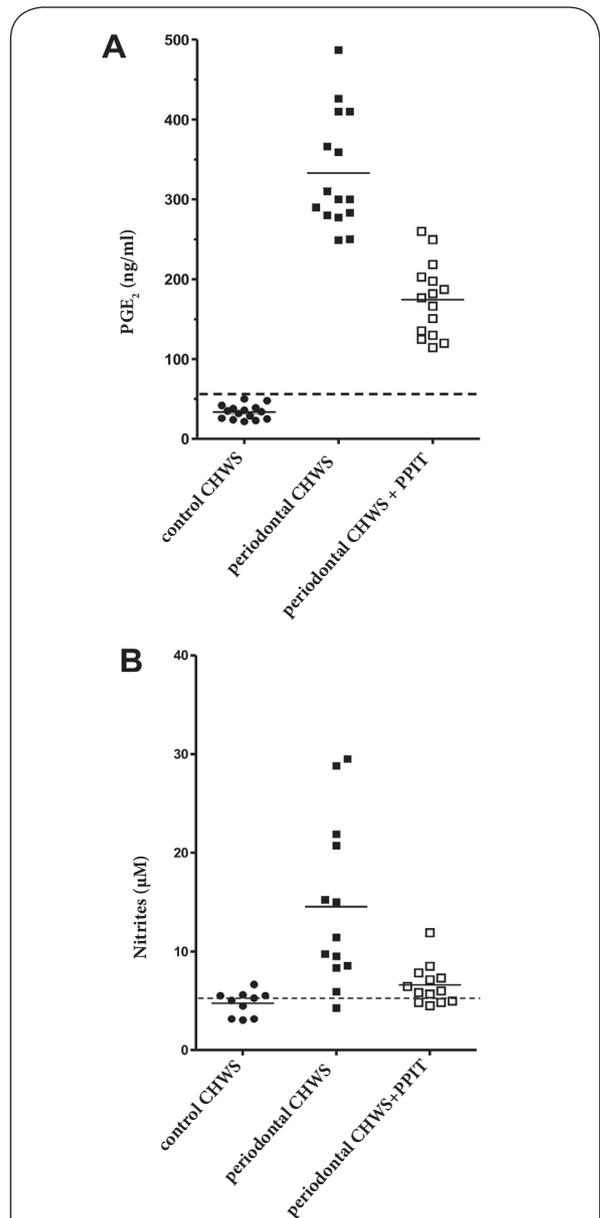with commercial plates for radial immunodiffusion containing
anti-IgA.

The distribution of anti-β_1-AR IgA was studied in clarified
human whole saliva (CHWS) from untreated chronic peri-
odontitis patients, treated withPPIT chronic severe perio-
dontitis patients and in healthy individuals (control). The
scattergram of (**Figure 1**) shows that the optical density
values (at 405 nm) of salivary anti-β_1-AR IgA from CHWS
of the untreated chronic severe periodontitis patients
(periodontal CHWS) and those from chronic severe
periodontitis patients treated with PPIT. Also, the optical
density values in healthy individuals (control CHWS) was
shown. It can be seen that the data from periodontal CHWS and
periodontal CHWS+PPIT were significantly higher (P<0.0001)
than those of control CHWS. It is important to note that there
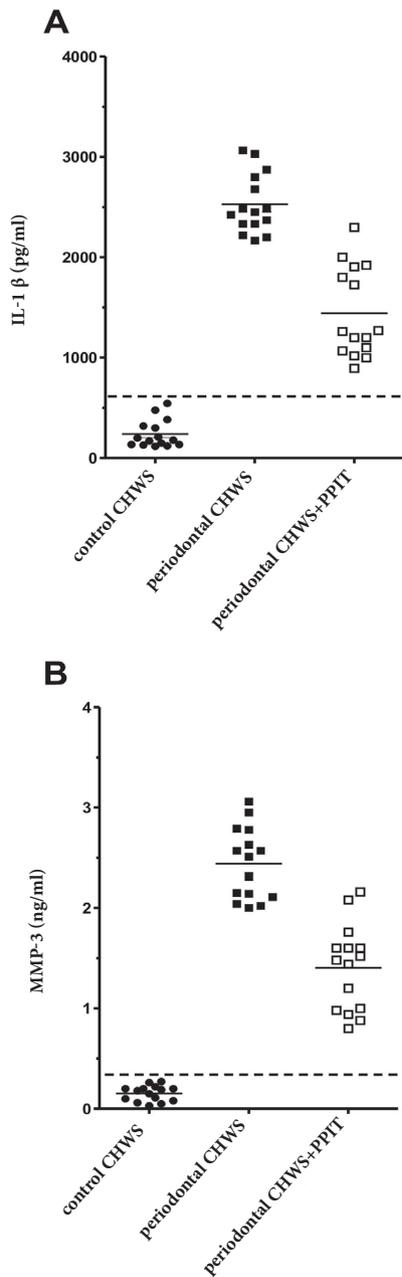is no significant difference between patients with chronic

Figure 1. Scatterogram showing values of IL-1β in clarified human whole saliva (CHWS) from healthy individuals (control), from untreated chronic periodontitis patients, and chronic periodontitis patients treated with periodontal I therapy (PPIT). Values are the mean ± SEM of 15 untreated and 15 treated periodontitis patients, and 15 healthy individuals as control. Dotted/dashed line, cut-off values 0.009 (mean optical density (OD) ± 3 S.D. for controls); solid lines, median OD values.

Figure 2. Scatterogram showing values of PGE_2 and nitrites in clarified human whole saliva (CHWS) from healthy individuals (control), from untreated chronic periodontitis patients, and chronic treated periodontitis patients. (**A**) Distribution of the values of PGE_2 (ng/ml) in CHWS from healthy individuals (control), from untreated chronic periodontitis patients, and treated chronic periodontitis patients. (**B**) Distribution of the values of nitrites (μM) in CHWS from healthy individuals (control) and from untreated chronic periodontitis patients, and treated chronic periodontitis patients. Values are mean ± SEM of 15 untreated and 15 treated periodontitis patients and 15 healthy individuals as control. Dotted/dashed line, cutoff values of 53.8 ng/ml for PGE_2; solid lines, median PGE_2 values; dotted/dashed line, cut off values of 6.01 μM for nitrites; solid lines, median nitrite values.

periodontitis before and after PPIT in the amount of this autoantibody. On the other hand, the immunoreactivity of saliva fron untrated and treated patients were negative in the presence of non-related peptide (data not shown), asserting the immunological recognition of the anti-β$_1$ AR IgA salivary only when the coating antigen is the synthetic β$_1$ peptide.

(**Figure 2**) shows the distributions of PGE_2 (A) and nitrite (B) concentrations in CHWS from untreated patients, treated patients and healthy individuals. As shown in the scatterogram of (**Figure 2A**), PGE_2 concentrations were significantly higher in chronic periodontitis patients than that in control CHWS (P<0.0001). Moreover, the concentration of PGE_2 in CHWS from untreated chronic periodontitis patients was statistically significantly higher than that from treated patients (P<0.001). It can be seen in (**Figure 2B**) that the concentration of nitrites

Figure 3. Scattergram showing values of IL-1β and MMP-3 in clarified human whole saliva (CHWS) from healthy individuals (control) and from untreated chronic periodontitis patients treated with periodontal I therapy (PPIT) chronic periodontitis patients. (**A**) Distribution of the values of IL-1β (pg/ml) in CHWS from healthy individuals (control) and from untreated chronic periodontitis patients, and chronic periodontitis patients treated with PPIT. (**B**) Distribution of the values of MMP-3 (ng/ml) in CHWS from healthy individuals (control) and from untreated chronic periodontitis patients treated with PPIT chronic periodontitis patients. Values are the mean ± SEM of 15 untreated and 15 treated periodontitis patients and 15 healthy individuals as control. Dotted/dashed line, cutoff values of 610.9 pg/ml for IL-1β and solid lines; median IL-1β values; dotted/dashed line, cutoff values of 0.25 ng/ml for MMP-3; solid lines, median, MMP-3 values.

Table 3. Mean values of the proinflammatory substances in whole human saliva.

Groups	anti-β₁ IgA (OD 405 nm)	PGE₂ (ng/ml)	IL-1β (pg/ml)	MMP-3 (ng/ml)
Healthy subjects	0.060±0,001	33.53±2,28	237.90±35,34	0.153±0,019
Untreated periodontitis patients	0.374±0,027*	333.12±18,64*	2528.00±76,01*	2.442±0,09*
Treated periodontitis patients	0.353±0.026	174.4±12.04*	1444.00±80.02 *	1.402±0.12*

Values expressed the means±SEM of fifteen patients and fifteen healthy normal subjects. *P<0.0001 comparing with healthy subjects.

in untreated patients were significantly increased when compared with control CHWS (P<0.0001) and the treated chronic periodontitis CHWS (P<0.001). The nitrite concentration from treated chronic periodontitis CHWS was not significantly different from that in the control group (P>0.05).

It can be seen in (**Figure 3A**) that the concentration of IL-1β in CHWS from untreated patients and patients treated with HBPT, the concentration was significantly higher that in normal individuals. Again, the levels of this cytokine in treated CHWS were significantly different from that in untreated patients.

We next investigated the level of MMP-3 in CHWS from the three different groups. Significant values were obtained in untreated and treated chronic periodontitis patients compared to healthy subjects (P<0.0001). Again, as shown above, MMP-3 levels in untreated and treated chronic periodontitis patients were significantly different (P<0.001; (**Figure 3B**).
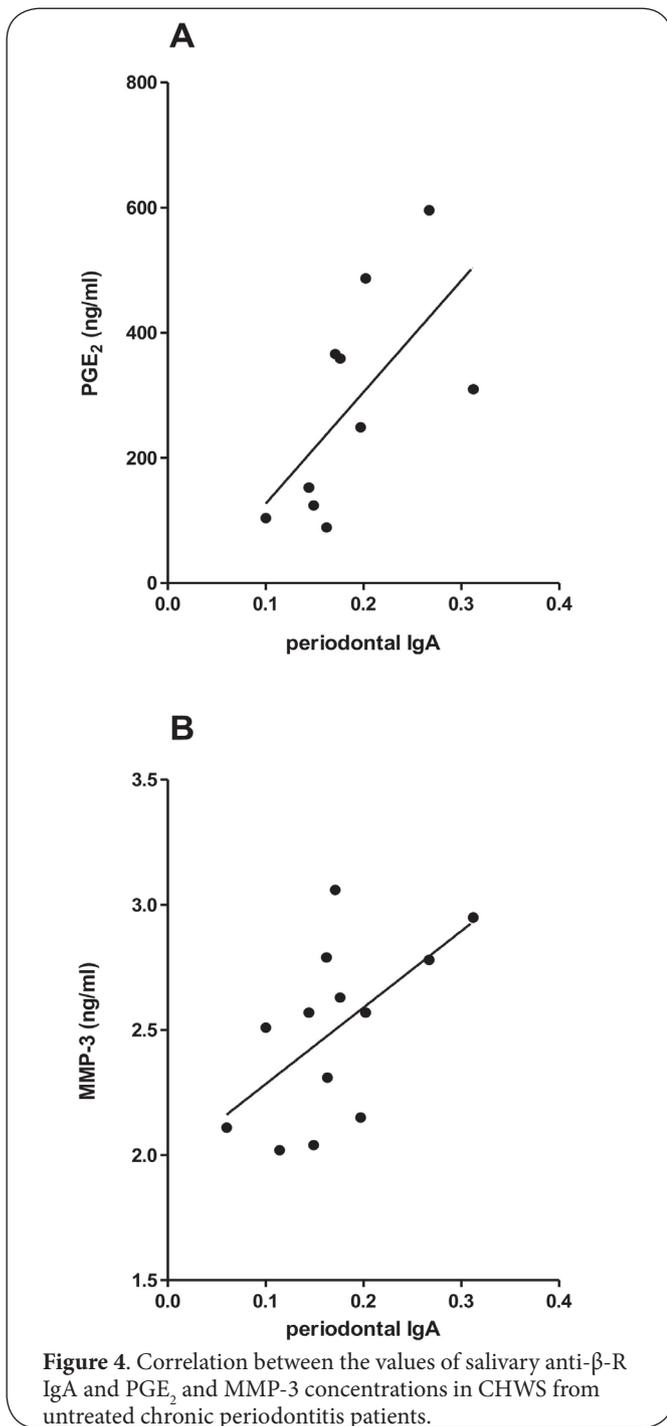
All of these findings are summarized in (**Table 3**), consistently indicating that the mean values of the different proinflammatory substances were significantly reduced after periodontal phase I therapy. But, the amount of anti-β₁-AR IgA was not significantly different between untreated and treated chronic severe periodontitis patients.

(**Figure 4**) shows a positive correlation (r=0.05) between the increment of PGE₂ production and MMP-3 concentrations in the function of the presence in the saliva of the anti-β₁-AR.

Discussion

The fair amount is known about the immunological mechanisms responsible for the pathology observed in the disease. The morphology of chronic periodontitis lesions, and the clinical signs and symptoms of the disease suggest that cytokines (IL-1β, PGE₂) [23,24], nitric oxide levels [25] and cytoplasmic enzymes (MMP-3) [26] are important in the pathogenesis of the disorders. But, we considerer important to determine if the anti β₁ adrenoceptor IgA present in the saliva of chronic severe patients, participates in the pathophysiology of the disease.

In this study we demonstrated high concentrations of IL-

Figure 4. Correlation between the values of salivary anti-β-R IgA and PGE_2 and MMP-3 concentrations in CHWS from untreated chronic periodontitis patients.

mononuclear cells and fibroblasts from gingival tissues and endothelial cells are responsible for the increase in IL-1β production [28]; thus, there is a close association between IL-1β levels and periodontal disease status. After periodontal based therapy in patients with chronic periodontitis, IL-1β levels are reduced in all patients tested, which is correlated with clinical improvement. PGE_2 is thought to be involved in the pathogenesis of the oral lesions observed in untreated chronic periodontitis, because of its role as a potent stimulator of bone resorption and association with attachment loss was published [29]. Therefore, there is a reciprocal interaction between PGE_2 and IL-1β; IL-1β is a potent stimulator of PGE_2 production in human gingival fibroblasts. However, PGE_2 differential regulates IL-1β-induced matrix metalloproteinase (MMP-3) production. In human gingival fibroblasts from healthy gingiva, PGE_2 down-regulates IL-1β-induced MMP-3 production, whereas in human gingival fibroblasts from periodontitis patients, PGE_2 enhances it [29]. These data may reflect the heterogeneity of immuno-inflammatory responses in healthy and disease conditions, in which the concentrations of IL-1β, PGE_2 MMP [30-31] may play a critical role as a marker of chronic severe periodontitis disease progression and oral manifestations.

The correlation between the amount of PGE_2 and MMP-3 in the saliva of each patient studied in this study has demonstrated an important relationship with the amount of anti-$β_1$ IgA. Analysis of this result, PGE_2 and MMP-3 performer on follow up studies underlines the correlation on the levels of the cytokine and the enzyme in the saliva of untreated patients with chronic periodontitis and the presence of salivary anti-$β_1$ IgA. It is important to note that, as previously reported, the pathogenesis of periodontal disease involves essential immunologic factors associated with infections caused by bacteria in sub-gingival plaques. The level of nitrite in saliva and its increment in patients with untreated chronic periodontitis was observed, and also an increased expression of iNOS in periodontal disease biopsy samples as well as in gingival fibroblast cell culture was described [22,32]. NO levels are associated with the severity of periodontitis, allowing differentiation between moderate and advanced generalized chronic periodontitis and NO levels were correlated with probing depth [25].

The biological plausibility of the differences observed in this study indicated that nitric oxide levels may be important in the pathogenesis of the disorders, and may be only in partly explained, by periodontal bacterial components triggering the host-immune response and causing inflammation and activation of pro-inflammatory mediators (IL-1β, PGE_2 and MMP-3). All of these molecules travelling in blood, together with those produced locally by the inflammatory process in the soft and hard oral tissues, might influence the pathophysiology of chronic periodontitis, but the real importance of the presence in the saliva of an anti $β_1$ adrenoceptor IgA remain to be determined.

1β, PGE_2, nitrites and MMP-3 in saliva from untreated chronic severe periodontitis patients. Moreover, the highest levels of IL-1β and PGE_2 were found in the saliva of these patients compared with those detected in healthy individuals (control). Previously was demonstrated that in human gingival fibroblasts are able to produce large amounts of PGE_2 in response to inflammatory cytokines, and the increased PGE_2 would be a potent stimulator of bone resorption [27]. Macrophages,

Conclusions

The findings of the markedly elevated salivary IL-1β, PGE$_2$ and MMP-3 levels and their significant reduction post therapy in patients with chronic periodontitis compared to healthy individuals (control), suggest a close association between salivary cytokines and enzymes and the periodontal status. But, the real participation of an anti β$_1$ adrenoceptor IgA present in the saliva of patients with severe chronic periodontitis, incorporated another pathophysiological factor in this multifactorial disease was not clarified yet in this present work. Future longitudinal studies with larger sample sizes are needed to validate in saliva not only if IL-1β, PGE$_2$ and MMP-3 are "real biomarkers" for periodontal disease but to know what role would play the autoantibody (β$_1$ adrenoceptor IgA) present in the saliva of these patients in the course of the periodontal disease or in its pathophysiology.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	SR	FH	NC	EB
Research concept and design	--	--	--	✓
Collection and/or assembly of data	--	✓	✓	--
Data analysis and interpretation	✓	--	--	--
Writing the article	--	--	--	✓
Critical revision of the article	--	--	--	✓
Final approval of article	--	--	--	✓
Statistical analysis	✓	--	--	--

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