

Research Article

Serum Adipokines, IL-1 Beta and Osteocalcin are not Associated with Optical Alveolar Density in Periodontal Disease Patients - a Pilot Study

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Abstract

Background: Adipokine can influence insulin resistance, inflammation and affect the osteoclast activity, which might increase the individual susceptibility to the development of chronic periodontitis.

Aim: We aimed to analyze the serum levels of Adiponectin, leptin, resistin, Interleukin (IL)-1 β and Osteocalcin (OC) in non-treated Generalized Chronic Periodontitis (GCP) patients. As a secondary aim, we correlate such biomarkers with the Optical Alveolar Density (OAD).

Material and Methods: Twenty-one patients with untreated GCP (mean age 46.1 \pm 8.8) composed the test group. Sixteen gingivitis patients (mean age 32.3 \pm 7.6) were used as controls. The periodontal examination included Pocket Depth (PD), Clinical Attachment Level (CAL), Bleeding on Probing (BOP) and Plaque Index (PI). The serum levels of leptin, Adiponectin, resistin and IL-1 β were analyzed via multiplexed bead immunoassay, whereas OC was analyzed by ELISA. The OAD was calculated using digital intra-oral radiographs and a specific software.

Results: The median values for Adiponectin was twice lower and leptin was twice higher in GCP when compared to the gingivitis group, but, not-statistically significant ($p= 0.07$ and $p= 0.28$, respectively). Resistin, IL-1 β , OC levels and OAD were very similar in GCP when compared to gingivitis patients. No significant correlation was observed between Adiponectin, leptin, resistin, IL-1 β and OC with OAD.

Conclusion: We were unable to demonstrate any association between the serum levels of adipokines with the optical alveolar density. In the other hand, the tendency of lower values of Adiponectin observed in GCP patients deserves a further investigation using a larger group.

Keywords: Adipokines; Alveolar bone loss; Cytokines; Digital; Periodontal disease; Radiography

Abbreviations

ANCOVA: Analysis Of Covariance; ADIPOQ: Adiponectin; % β : β -Cell Function; BOP: Bled On Probing; CAL: Clinical Attachment Level; CRP: C - Reactive Protein; ELISA: Enzyme Linked Immunosorbent Assay; GCP: Generalized Chronic Periodontitis; HDL-c: High Density Levels Cholesterol; HOMA: Homeostasis Model Assessment; IL: Interleukin; IQR: Interquartile Range; IR: Insulin Resistance; %S: Insulin Sensitivity; LDL-c: Low Density Level Cholesterol; OC: Osteocalcin; OAD: Optical Alveolar Density; PI: Plaque Index; PD: Probing Depth; ROI: Regions Of Interest ; UERJ: Rio De Janeiro State University; SD: Standard Deviation; VLDL-c: Very Low Density Level Cholesterol.

Introduction

Adipose tissue produces and releases a variety of pro- and anti-inflammatory factors. Adipokines, or adipose-derived hormones, might influence the insulin resistance and play an important role in inflammation/immune responses [1] with an effect on the osteoclast

activity [2]. Hypoadiponectinemia, a decreased level of Adiponectin, has been observed in obesity, dyslipidemia, essential hypertension, coronary heart disease, and insulin-resistant states, such as type 2 diabetes, lipodystrophy and metabolic syndrome [3-6]. Moreover, the levels of Adiponectin have also a trend to decrease in periodontitis patients [7-10].

Current data suggest that the adipokines effects and interactions with bone metabolism are not fully understood [11]. Available data *in vitro* suggests that Adiponectin has an anabolic effect on osteoblasts and inhibits osteoclastogenesis [12,13]. These actions would be expected to result in a positive effect of Adiponectin on bone mass *in vivo*. A previous animal study has shown that over expression of Adiponectin enhanced bone formation and inhibited bone resorption, resulting in an increase in bone mineral density [13]. On the other hand, several clinical studies suggested that circulating adipokines, especially Adiponectin, might have a negative impact on bone mineral density in healthy subjects and chronic disease patients [14-16]. Based on these studies, we hypothesized that the circulating adipokines

levels might influence the optical alveolar density in patients with generalized chronic periodontitis.

Osteoclastogenesis is activated by several pathways, including Interleukin (IL)-1 β , which has been shown to act synergistically with Adiponectin to affect the prostaglandin expression [17,18]. Osteocalcin, an osteoblast-derived hormone, has a role in the regulation of bone mineralization and promotes Adiponectin and leptin secretion [19]. Previous studies reported its positive association with Adiponectin levels [20-24].

Therefore, we aimed to analyze the serum levels of Adiponectin, leptin, resistin, IL-1 β and Osteocalcin (OC) in non-treated Generalized Chronic Periodontitis (GCP) patients. As a secondary aim, we correlate such biomarkers with the Optical Alveolar Density (OAD).

Material and Methods

Patient selection

Thirty-seven untreated patients previously diagnosed for periodontal disease participated in the study. Twenty-one patients presented marked traits of bone loss and were diagnosed as GCP (mean age 46.1 \pm 8.8). Sixteen subjects with gingivitis alone (mean age 32.3 \pm 7.6) were used as controls. All individuals were seeking treatment at the dental school of the Rio De Janeiro State University (UERJ), Rio de Janeiro, Brazil. Patients were diagnosed according to criteria described by the American Academy of Periodontology [25]. The participants reported no ongoing systemic disease, no measurement \geq 30 kg/m² and were not under any medication that could affect the periodontal conditions. They were at least 6 months without taking any antibiotics and 3 months without taking any non-steroidal anti-inflammatory drugs. All individuals signed an informed consent prior to enrollment. The Ethics Committee of the Pedro Ernesto University Hospital (UERJ, Rio de Janeiro, Brazil) approved the study protocol (2714/2010).

Clinical measurements and radiographic alveolar bone density

The clinical periodontal parameters measured were percentage of sites that bled on Probing (BOP), O'Leary's Plaque Index based on the visible continuous plaque along the gingival margin after staining (PI), Probing Depth (PD) and Clinical Attachment Level (CAL). The PD and CAL were taken at 6 sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual) except for third molars. A periodontal computerized probe (Florida Probe[®], Gainesville, Florida, USA) was used together with a stent of silicone (1.0 mm plates) (Bio Art, São Paulo, Brazil).

The Optical Alveolar Density (OAD) was analyzed in the interproximal standard Regions of Interest (ROI). Digital intra-oral radiographs were taken using the Kodak Digital Radiography System RVG 6100[®] (Rochester, NY, USA). The X-ray apparatus was the KODAK Intra-Oral 2200[®] (Rochester, NY, USA). Radiographs were captured at 70kV, 7 mA and adjusted to factor 3. The exposure time was set for each region from the dental arch according to the manufacturer.

The values of OAD (pixels/mm²) from each patient were assessed by the mean of the four interproximal sites inflamed shallow site (PD

\leq 3 mm, and CAL \leq 1 mm) selected in different teeth. The site clinical inflammation was defined by the presence of clinical signs of redness, swelling, or BOP.

The ROIs were drawn using a metal grid with several 1.0-mm² squares attached to the sensor (KODAK[®], Rochester). The ROIs were defined and marked in the most coronal portion of the alveolar bone crest in the digital image. It must not overlap any portion of the tooth surface, periodontal ligament or *lamina dura*. The first ROI (ROI 1) was placed in the most coronal portion of alveolar bone crest. The second ROI (ROI 2) was placed 5 mm apical to ROI 1, using the upper edges of each ROI as reference. The OAD value is the change (\otimes) between ROI2-ROI1 to represent the demineralization in the ROIs. The OAD evaluation was performed using two calibrated examiners (GLM and FB). There was 99% concordance within \pm 5 pixels between the examiners.

Blood collection

The individuals were instructed not to eat for 12h before sample collection. Blood samples (20 ml) were obtained in the morning (8:00 AM) by venous puncture into tubes containing or not anticoagulant. Eight mL were transferred to glass tubes containing 7.2 mg K2-EDTA (BD Vacutainer, Franklin Lakes NJ, USA) for glycated hemoglobin, blood count and OC. Another 4 mL were transferred to glass tubes with 6 mg of NaF and 12mg Na₂EDTA (BD Vacutainer) for glucose analysis. Eight mL were transferred to Clot activator glass tubes (BD Vacutainer) for lipid profile, High-Sensitive C-Reactive Protein (hs-CRP), insulin, leptin, adiponectin, resistin and IL-1 β . All samples were immediately centrifuged for 5 min, except the K2-EDTA, and used for immediate analysis or stored at -70°C pending analyses.

Laboratory assays

Plasma glucose, triacylglycerols, total cholesterol, High Density Levels Cholesterol (HDL-c), Low Density Level Cholesterol (LDL-c), Very Low Density Level Cholesterol (VLDL-c), glycated hemoglobin, insulin and leukocytes count were measured using an automatic analyzer from the laboratory routine (DLE - Diagnostics Laboratories

Especializados-Medicina laboratorial, Rio de Janeiro, RJ, Brazil). C - Reactive Protein (CRP) levels were determined using a highly sensitive immunoturbidimetric assay (DiaSys Diagnostic Systems, Holzheim, Germany).

A total of 50 μ L of serum samples was used for each analysis. Leptin, Adiponectin, resistin and IL-1 β were detected using commercially available kits (Milliplex[®], Billerica, MA, USA) in a Luminex 200 analyzer[®] (Alameda, CA, USA) according to the manufacturer's instructions. Briefly, two 96-well magnetic filter plates were used. The plates were pre-wetted with washing buffer. The microsphere beads were coated with monoclonal antibodies against the Adiponectin and resistin and added to the wells of one magnetic plate. Beads coated with antibodies against leptin and IL-1 β were added to separate plates. Samples and standards were added into the wells and incubated overnight at 4°C. The wells were washed using a vacuum manifold and secondary antibodies were added. After incubation for 1 hour, streptavidin conjugated to the fluorescent protein R-phycoerythrin was added to the beads and incubated for 30 minutes. After washing to remove unbound reagents, a sheath fluid was added to the wells, and the microspheres were analyzed in a bead analyzer (Luminex[®],

Alameda, CA, USA). The concentrations of the unknown samples (antigens in serum samples) were estimated from the standard curve using a software program Milliplex Analyst[®] (Milliplex, Billerica, MA, USA), and the cytokine levels were expressed as the amount pg.

Osteocalcin was measured using an Enzyme Linked Immunosorbent Assay (ELISA) development it (Biosource Europe S.A., Nivelles, Belgium). Manufacturers' guidelines were followed for each assay. Concentrations of osteocalcin in the serum samples were then determined by comparing the average sample optical density readings with the concentrations from the assay standard curve. The lower detection thresholds for the osteocalcin assays were 0.08 ng ml⁻¹.

Homeostasis model assessment calculation

Homeostasis Model Assessment (HOMA) describes the glucose-insulin homeostasis by a set of empirically derived nonlinear equations. The model predicts fasting steady-state levels of plasma glucose and insulin for any given combination of pancreatic β-cell function (%β), Insulin Sensitivity (%S) and the index of Insulin Resistance (IR). Computer simulations have been used to generate a norm gram from which mathematical transformations using the HOMA Calculator software (Heading ton, Oxford, UK).

Statistical analysis

Data is presented as mean or median and Inter Quartile Range (IQR) or Standard Deviation (SD). The significance of differences between groups for gender, race and smoking were tested using Fisher Exact Test. The significance of differences between groups was calculated using Mann-Whitney U Test, the significance was determined at 5% (p < 0.05). The Analysis of Covariance (ANCOVA) was used to adjust for age. The Spearman's rho correlation coefficient was use and the significance of the correlations were arbitrary stipulated in r=0.6, p≤0.01. Statistical analyses were performed using the software SPSS v.19.0, (IBM, Chicago, IL, US).

Results

All the clinical data were presented in Table 1. Besides the expected differences for PD and CAL, the percentage of positive sites with plaque was significantly higher in GCP (p<0.001) where as there was no significant difference in the percentage of BOP between the groups (Table 1).

The median values for Adiponectin was twice lower and leptin was twice higher in GCP when compared to the gingivitis group, but, not-statistically significant (p= 0.07 and p= 0.28, respectively). Resist in, IL-1β, OC levels and OAD were very similar in GCP compared to gingivitis patients (Table 2).

The glycated hemoglobin was higher and leucocytes was lower in GCP when compared to gingivitis patients after the adjustment for age (p= 0.011, and p= 0.021) (Table 3). No significant correlation was observed between the biomarkers and OAD (Table 4 and 5).

Discussion

Our results showed no significant difference in Adiponectin, leptin and resist in levels when patients with GCP were compared to subjects with gingivitis. Moreover, we were unable to demonstrate any association between such biomarkers with the optical alveolar density. Leptin, Adiponectin and resistin modulates bone metabolism

Table 1: Mean values (±SD) for PD, CAL, PI and BOP for Generalized Chronic Periodontitis (GCP) and gingivitis patients.

| | | GCP (n=21) | GINGIVITIS (n=16) | P value |
|----------|-------------|------------|-------------------|---------|
| Age | | 46.1±8.8 | 32.3±7.6 | ≤0.001 |
| *Gender | Woman | 14 | 13 | |
| | Man | 7 | 3 | |
| *Race | Non-white | 9 | 11 | |
| | White | 12 | 5 | |
| *Smoking | Non-smoking | 14 | 16 | |
| | Smoking | 2 | 0 | |
| | Ex-smoking | 5 | 0 | |
| PD (mm) | | 3.2 ±0.5 | 1.4±0.6 | ≤0.001 |
| CAL (mm) | | 2.0±0.7 | 0.2±0.2 | ≤0.001 |
| BOP(%) | | 19.0 ±16.6 | 15.0±11.6 | 0.639 |
| PI(%) | | 59.0±24.8 | 27.2±18.7 | ≤0.001 |

PD: Probing Depth total; CAL: Clinical Attachment Level total BOP: Bleeding on Probing; VPI: Visible Plaque Index
Compared GCP and gingivitis groups (Mann-Whitney U Test and *Fisher Exact Test).

Table 2: Median values (Interquartile Range - IQR) for biochemical index in Generalized Chronic Periodontitis (GCP) and gingivitis patients.

| | GCP (n= 21) | | Gingivitis (n= 16) | | P value | ANCOVA |
|-------------------------------|-------------|----------|--------------------|-----------|---------|--------|
| | Median | (IQR) | Median | (IQR) | | |
| ADIPONECTIN (ng/ml) | 9.212.2 | (6963.4) | 18.560.3 | (12653.9) | 0.073 | 0.078 |
| RESISTIN (ng/ml) | 6.3 | (2.6) | 6.3 | (4.8) | 0.575 | 0.849 |
| LEPTIN (ng/ml) | 21.0 | (23.6) | 10.4 | (25.3) | 0.280 | 0.609 |
| IL1β (pg/ml) | 0.8 | (2.0) | 1.2 | (1.4) | 0.657 | 0.786 |
| OC (mg/ml) | 0.4 | (3.0) | 0.6 | (1.2) | 0.838 | 0.975 |
| OAD (pixels/mm ²) | 89.3 | (19.6) | 99.7 | (14.8) | 0.567 | 0.075 |

Compared GCP and gingivitis groups (Mann-Whitney U Test). ANCOVA: Adjusting for differences in age.

[13]. Several clinical studies have shown a negative correlation between Adiponectin and bone mineral density [14-16]. Regarding Leptin, the results are very controversial. Some human studies have failed to show any association with bone mineral density [26,27], whereas other shave reported a positive [28,29] and even negative [30,31] association with bone mineral density.

Our results showed no significant difference in Adiponectin, leptin and resistin levels when patients with GCP were compared to gingivitis. However, Adiponectin levels were twice lower in GCP patients. Such results are in agreement with the literature, which shows decreased levels of Adiponectin in subjects with periodontitis without reaching statistical significance [7-19]. Furthermore, lower Adiponectin serum levels in overweight periodontitis patients when compared to normal weight suggesting the influence of periodontal inflammation [32]. Hypoadiponectinemia play a significant role in the development of the metabolic syndrome [6] and might be affecting the course of the periodontal disease.

Leptin levels were twice higher in GCP when compared to gingivitis group. Elevated serum levels of leptin have been correlated with the amount of periodontal destruction previously [33-35].

Table 3: Median values (Inter Quartile Range - IQR) for biochemical index in Generalized Chronic Periodontitis (GCP) and gingivitis patients.

| Variables | GCP (n= 21) | | Gingivitis (n= 16) | | P value | ANCOVA |
|--------------------------------|----------------|--------------|-----------------------|--------------|--------------|--------------|
| | Median (IQR) | Median (IQR) | Median (IQR) | Median (IQR) | | |
| Triglyceride (mg/dl) | 95.0 | (70.0) | 70.5 | (27.0) | 0.108 | 0.616 |
| Cholesterol (mg/dl) | 201.0 | (50.5) | 188.0 | (62.3) | 0.639 | 0.888 |
| HDL Cholesterol (mg/dl) | 44.0 | (14.0) | 49.5 | (21.5) | 0.078 | 0.763 |
| LDL Cholesterol (mg/dl) | 131.0 | (44.5) | 113.5 | (48.5) | 0.217 | 0.631 |
| VLDL Cholesterol (mg/dl) | 19.0 | (14.0) | 14.5 | (12.0) | 0.280 | 0.633 |
| hsCRP (mg/dl) | 0.1 | (0.3) | 0.2 | (0.7) | 0.639 | 0.311 |
| Leucocytes (/mm ³) | 6.660.0 | (3290.0) | 8.135.0 | (3850.0) | 0.095 | 0.021 |
| Glycatedhemoglobin (%HbA1c) | 5.5 | (0.4) | 5.3 | (0.3) | 0.012 | 0.011 |
| Insulin (mcUI/ml) | 5.4 | (7.8) | 8.0 | (10.8) | 0.138 | 0.737 |
| Glucose (mg/dl) | 100.0 | (9.5) | 91.5 | (12.5) | 0.011 | 0.412 |
| %β HOMA | 63.1 | (38.8) | 105.5 | (55.6) | 0.003 | 0.538 |
| %S HOMA | 136.0 | (157.9) | 98.3 | (161.3) | 0.165 | 0.589 |
| IR HOMA | 0.7 | (0.1) | 1.1 | (1.1) | 0.108 | 0.963 |

Compared GCP and gingivitis groups (Mann-Whitney U Test). ANCOVA, adjusting for differences in age.

Table 4: Spearman's rho correlation Amongprobing Depth (PD), Clinical Attachment Level (CAL), the mean Optical Alveolar Density (OAD), Adipokines (ADIPOQ; Adiponectin), Interleukin(IL)-1β and Osteocalcin (OC)in GCP and gingivitis patients.

| | | ADIPOQ. | RESISTIN | LEPTIN | IL-1β | OC |
|----------------|---|---------|----------|--------|-------|-------|
| PD total mean | r | -0.32 | -0.22 | 0.27 | -0.05 | 0.07 |
| | p | 0.05 | 0.20 | 0.11 | 0.79 | 0.69 |
| CAL total mean | r | -0.29 | -0.12 | 0.17 | -0.11 | -0.04 |
| | p | 0.08 | 0.49 | 0.32 | 0.52 | 0.84 |
| OAD | r | 0.33 | 0.03 | 0.28 | -0.11 | -0.38 |
| | p | 0.85 | 0.88 | 0.09 | 0.57 | 0.02 |
| OC | r | -0.01 | -0.17 | -0.30 | 0.08 | |
| | p | 0.94 | 0.34 | 0.08 | 0.67 | |
| IL-1β | r | 0.41 | 0.27 | -0.19 | | |
| | p | 0.01 | 0.11 | 0.27 | | |
| LEPTIN | r | -0.15 | 0.15 | | | |
| | p | 0.37 | 0.39 | | | |
| RESISTIN | r | -0.03 | | | | |
| | p | 0.88 | | | | |

Moreover, authors previously reported that increased plasma leptin concentrations are associated with chronic inflammation [36,37]. Leptin stimulate the immune system by enhancing pro-inflammatory cytokine production, as TNF-α and IL-6 [38,39] and growth factors, which may contribute to endothelial dysfunction, atherosclerosis and insulin resistance [40]. The rise in serum leptin concentration above 10,000 pg/mL is considered as a risk factor for cardiovascular disease. An increased serum leptin levels due periodontitis could have an important role in the risk for cardiovascular disease [41].

The complementary circulating measurements of metabolic disturbances was performed and could be observed that only the level of glycated hemoglobin higher and leucocytes lower in GCP when compared to gingivitis patients after the adjustment for age (p= 0.011, and p= 0.021). However, major of complementary

circulating measurements of metabolic disturbances still inside the reference values for human health and no significant differences were observed between the groups. The reciprocal endocrine regulation of bone and adipose tissue function is proposed, the mainly mediators suggested are insulin, osteocalcin and leptin. Insulin may stimulate leptin production which stimuli osteoclast-mediated desorption to releases osteocalcin into the circulation, where it ultimately enhances pancreatic insulin production. Along these lines, an endocrine feedback system is formed whereby bone regulates adipose tissue function indirectly via the actions of osteocalcin on the pancreas, and adipose tissue reciprocally regulates bone homeostasis through leptin and other secreted factors [19].

In conclusion, we were unable to demonstrate any association between the serum levels of adipokines with de optical alveolar density.

Table 5: Spearman's rho correlation Among adipokines (ADIPOQ: Adiponectin; RES: Resistin; LEP: Leptin), Interleukin (IL)-1 β , Osteocalcin (OC) and biochemical index in GCP and gingivitis patients.

| | | ADIPOQ. | RES. | LEP. | IL-1 β | OC |
|---------------------|---|---------|-------|-------|--------------|-------|
| Triglyceride | r | -0.50 | -0.02 | 0.09 | -0.30 | -0.08 |
| | p | <0.001 | 0.90 | 0.59 | 0.08 | 0.66 |
| Cholesterol | r | -0.04 | 0.06 | 0.11 | 0.04 | -0.22 |
| | p | 0.82 | 0.73 | 0.52 | 0.81 | 0.21 |
| HDL | r | 0.40 | -0.02 | -0.08 | 0.04 | 0.06 |
| | p | 0.01 | 0.91 | 0.66 | 0.83 | 0.73 |
| LDL | r | -0.16 | 0.23 | 0.24 | -0.13 | -0.30 |
| | p | 0.34 | 0.18 | 0.15 | 0.45 | 0.08 |
| VLDL | r | -0.44 | -0.12 | 0.00 | -0.39 | -0.08 |
| | p | 0.01 | 0.49 | 1.00 | 0.02 | 0.67 |
| hsCRP | r | -0.47 | 0.12 | 0.30 | -0.24 | -0.02 |
| | p | <0.001 | 0.48 | 0.07 | 0.17 | 0.93 |
| Leucocytes | r | 0.08 | 0.20 | 0.19 | 0.09 | -0.10 |
| | p | 0.64 | 0.25 | 0.25 | 0.58 | 0.57 |
| Glycated hemoglobin | r | -0.35 | -0.23 | 0.13 | -0.31 | 0.20 |
| | p | 0.03 | 0.17 | 0.43 | 0.07 | 0.24 |
| Insulin | r | -0.15 | 0.09 | 0.08 | -0.03 | -0.19 |
| | p | 0.39 | 0.59 | 0.65 | 0.86 | 0.27 |
| Glucose | r | -0.29 | -0.31 | -0.15 | -0.14 | 0.04 |
| | p | 0.08 | 0.06 | 0.38 | 0.43 | 0.80 |
| % β HOMA | r | 0.02 | 0.25 | 0.10 | 0.06 | -0.17 |
| | p | 0.93 | 0.13 | 0.55 | 0.72 | 0.32 |
| % s HOMA | r | 0.12 | -0.05 | -0.02 | -0.01 | 0.20 |
| | p | 0.48 | 0.78 | 0.90 | 0.94 | 0.23 |
| IR HOMA | r | -0.13 | 0.10 | 0.07 | -0.03 | -0.20 |
| | p | 0.45 | 0.55 | 0.70 | 0.85 | 0.25 |

On the other hand, the tendency of lower values of Adiponectin observed in GCP patients deserves a further investigation using a larger group.

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