Serum, Saliva, and Gingival Crevicular Fluid Osteocalcin: Their Relation to Periodontal Status and Bone Mineral Density in Postmenopausal Women

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Background: Periodontitis and osteoporosis are characterized by the loss of bone mass. Osteocalcin levels have been postulated as a marker of inhibition of bone formation. The aim of the present study was to assess plasma, saliva, and gingival crevicular fluid (GCF) levels of osteocalcin and correlate them with periodontitis and osteoporosis.

Methods: Seventy-three postmenopausal women, over 35 years old, were recruited for the study. Serum, saliva, and GCF osteocalcin were measured. Vertebral bone mineral density was measured by dual-energy x-ray absorptiometry. Differences between groups were assessed by analysis of variance (ANOVA), chi-square test, and non-parametric Kruskal-Wallis test.

Results: Thirty-four (46.6%) were classified in the normal healthy bone group, 11 women (15.1%) in the osteopenic group, and 28 women (38.4%) in the osteoporotic group. No statistically significant differences between these densitometric groups were observed in probing depth ($P = 0.24$); clinical attachment level ($P = 0.11$); or mean osteocalcin concentrations in serum, saliva, and GCF. Twenty-seven (37.0%) of the women were classified without periodontitis (NPG) and 63.0% (N = 46) with periodontal disease (PG). There were no statistical differences in serum and saliva osteocalcin concentrations between these two groups. GCF osteocalcin concentrations were significantly higher in the PG women than in the NPG group ($P = 0.008$). Mean probing depth correlated significantly with GCF osteocalcin concentrations ($r = 0.35$; $P = 0.002$).

Conclusion: The results further support the concept that osteocalcin levels in GCF correlates with periodontal but not with osteoporosis status. J Periodontol 2005;76:513-519.

KEY WORDS
Bone resorption/prevention and control; gingival crevicular fluid/analysis; osteocalcin; osteopenia; osteoporosis; periodontitis; plasma/analysis; saliva/analysis.

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing it to increase risk of fracture, with bone strength determined by both bone density and bone quality.¹ Periodontitis is an inflammatory disease usually leading to bone loss. In periodontitis, there is an increased turnover of alveolar bone although there may be a dominance of bone resorption over bone formation, leading to alveolar bone loss and loss of attachment.² Based upon knowledge of the etiology and the common risk factors involved, some authors³-⁵ have studied the association between both processes. The majority of studies³-⁵ have shown low bone mass to be independently associated with loss of alveolar crestal height and tooth loss. However, studies that focus on the relation of clinical attachment loss and osteoporosis are less consistent.³ Many factors have been identified which contribute to bone mass loss, including natural or surgically created menopause at an early age.⁴ Increase in bone resorption due to gonadal reasons and accelerated bone loss in the first decade after menopause appear to be the main pathogenic factors in women.⁵ Therefore, postmenopausal women are one of the risk groups for periodontitis and osteoporosis.

Biochemical methods of measuring specific markers are used routinely to provide information on bone resorption and formation. While many substances...
are being investigated for their potential to aid in the assessment and monitoring of tissue loss in periodontitis, few are so directly related to bone metabolism. Osteocalcin, a 49 amino acid non-collagenous matrix protein of calcified tissue, is synthesized by osteoblasts. It has been postulated to have a role in both bone resorption and mineralization,\(^5\) is currently described as the most specific marker of osteoblast function,\(^7\) and has been identified as the current best marker in plasma of spontaneous bone loss in untreated postmenopausal women.\(^8\) The serum level of this protein is considered to be a marker of bone formation\(^9,10\) and is routinely assayed in investigation and monitoring of metabolic bone disorders.\(^7\) Serum osteocalcin is presently considered a valid marker of bone turnover when resorption and formation are coupled, and a specific marker of bone formation when formation and resorption are uncoupled.\(^11\)

Gingival crevicular fluid, which is an exudate that can be harvested from the sulcus or periodontal pocket, has been regarded as a promising medium for the detection of periodontal disease activity.\(^12,13\) The majority of new diagnostic tests for periodontal disease use GCF.\(^14,15\) However, of the more than 50 different components in GCF evaluated to date, the majority lack specificity to alveolar bone destruction and essentially constitute soft-tissue inflammatory events.\(^16,17\) Several investigations on osteocalcin levels in GCF from periodontal patients\(^2,16,18-21\) have been reported, suggesting that osteocalcin levels in GCF may reflect inflammation at diseased sites, and there has been interest in osteocalcin as a potential marker of bone turnover in periodontal disease.\(^2,10,11\) However, the role of osteocalcin in periodontal disease progression is still unclear.

The aim of the present study was to assess plasma, saliva, and GCF levels of osteocalcin and correlate them with periodontitis and osteoporosis.

**MATERIALS AND METHODS**

**Study Population and Clinical Examination**

For 3 years, all the postmenopausal women >35 years old who were patients at our Dental School were invited to participate in the study, and 73 patients were recruited. Our protocol and consent forms were approved by the Committee of Ethics and Research of the University of Seville Hospital Virgen Macarena. All the women met the following inclusion criteria: at least 12 months since the last menstrual period; at least seven teeth; no hormone therapy; no medical treatment influencing bone metabolism; and no metabolic diseases.

General data were recorded: age, age at menopause, years since menopause, and history of cigarette smoking. One clinician examined all the patients, recording the following measurements for all teeth: 1) O’Leary plaque index;\(^22\) 2) van der Velden bleeding on probing (BOP) index;\(^23\) 3) probing depth (PD), measured from the gingival margin to the most apical penetration of the probe; and 4) recession measured from the cemento-enamel junction to the gingival margin. Clinical attachment level (CAL) was calculated by adding recession to PD. PD and CAL were assessed at six sites per tooth: mesial edge, midtooth, and distal edge on both facial and lingual sides. According to the criteria established by Machtei et al.,\(^24\) the clinical entity of periodontitis is based on the presence of CAL ≥6 mm in two or more teeth and one or more sites with PD ≥5 mm.

**Sample Collection and Analytical Determinations**

Blood was centrifuged to obtain plasma. GCF sampling was performed based on the method previously described by Nomura et al.\(^25\) Each patient was monitored at four sites in the upper incisor teeth. In the periodontitis group the sites were always PD ≥5 mm. After isolation from saliva, the area around each site sampled was dried and supragingival plaque removed. GCF was collected for 2 minutes using four strips (20 × 4 mm) of paper.\(^8\) Each strip was placed gently into the gingival crevice until slight resistance was felt, and GCF volume was collected. The GCF absorbed (µl)/wet area (mm²) in the strip paper was calculated by comparing it to the amount of a standard plasma containing a low concentration of \(^{125}\)I used as a tracer that the strip can absorb, similar to what has been described for blood specimens.\(^26,27\) A saliva sample was also collected from the sublingual zone with the same paper strips. The plasma, saliva, and GCF samples were placed in a microcentrifuge tube and vortexed vigorously in 200 µl of extraction buffer consisting of 0.5 NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM CaCl\(_2\), and 0.05% Brij 35 (polyoxyethylene\(^23\) lauryl ether\(^6\)) by a modification of the method described by Lee et al.\(^28\)

**Osteocalcin Assay**

Osteocalcin measurements in serum, saliva, and GCF were made using an electrochemiluminescence technique.\(^\dagger\) Results were obtained from a calibration curve performed in the system by means of a two-point calibration and a master curve included into the reactive bar code. The linearity defined by the detection limit and the calibration curve maximum concentration is established in 0.500 to 300 ng/ml. The detection limit, the lowest measurable osteocalcin concentration, is calculated as the concentration situated two standard deviations above the minor concentration calibrator value of the master curve is 0.5 ng/ml. The minimum volume used by the analyst to carry out a determination was 20 µl sample.

\(^5\) Whatman Lab Sales Ltd., Maidstone, Kent, U.K.
\(^6\) Nacalai Tesque Inc., Kyoto, Japan.
\(^\dagger\) Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA.
Bone Mineral Density Measurements

Vertebral bone mineral density (BMD) was measured in each woman by dual-energy x-ray absorptiometry.\(^8\) We used antero-posterior projections of L2-L4. BMD was calculated as the average of density between L2 to L4, and expressed as g/cm\(^2\). The World Health Organization\(^9\) defines osteoporosis by a BMD measured as two-and-one-half standard deviations \((-2.5\ SD)\) below average peak bone density achieved in young adults, matched by gender and race. Based on the values obtained, three groups were established: normal healthy group (BMD \(\geq -1\ SD\)), osteopenic group (BMD between \(< -1\ SD\) and \(> -2.5\ SD\)), and osteoporotic group (BMD \(\leq -2.5\ SD\)).

Statistical Analysis

Data are expressed as mean \(\pm\) standard deviation. Differences between groups in normally continuous variables were assessed by analysis of variance (ANOVA), using the Bonferroni test to establish differences between groups. Discrete variables were analyzed by means of frequency distribution. The comparative analysis of discrete variables was carried out using the chi-square test. Non-parametric Kruskal-Wallis test was used to analyze non-normally distributed variables. The Pearson correlation coefficient was used to analyze correlation between variables. \(P<0.05\) was considered significant.

RESULTS

The mean age of the 73 women was 57.3 \(\pm\) 7.7 years (range 38 to 76 years). Only four were smokers, one smoked \(>20\) cigarettes/day and three \(<20\) cigarettes/day. Mean menopausal age was 48.1 \(\pm\) 7.5 years (range 1 to 29 years).

According to the criteria established by Machtei et al.\(^{24}\) 37.0\% (\(N = 27\)) of the women were classified without periodontitis (NPG) and 63.0\% (\(N = 46\)) with periodontal disease (PG) (Table 1). Mean PD, mean CAL, and BOP were statistically higher in PG than in the NPG group (\(P<0.05\)). There were no significant differences in the percentage of sites with plaque between both groups (\(P = 0.48\)).

According to bone mineral density assessed by densitometry, 34 women (46.6\%) were classified in the normal (healthy) bone group (NBG), 11 (15.1\%) in the osteopenic group (OEG), and 28 (38.4\%) in the osteoporotic group (OPG) (Table 2). There was no significant difference between NPG and PG groups in the distribution of women according to their densitometric results (\(P = 0.36\)). No statistically significant differences between groups were observed in probing depth (\(P = 0.24\)) or in CAL (\(P = 0.11\)). Women in osteopenic and osteoporotic groups showed more BOP than those in the healthy bone group, but differences were not statistically significant (\(P = 0.04\); non-significant Bonferroni test). On the contrary, the mean percentage of sites with plaque in osteoporotic women (81.2\% \(\pm\) 12.5\%) was significantly higher than that in the osteopenic and normal healthy bone groups (63.0\% \(\pm\) 32.6\% and 59.0\% \(\pm\) 35.8\%, respectively) (\(P = 0.01\); with significant differences between the normal healthy bone group and osteoporotic group in Bonferroni test).

Mean osteocalcin concentrations in serum, saliva, and GCF in correlation with periodontal status of the women are shown in Table 3. Serum and saliva osteocalcin concentrations did not show statistical differences between the two groups (\(P>0.05\)). On the contrary, GCF osteocalcin concentrations were significantly higher in the periodontally diseased group (35.0 \(\pm\) 30.3 ng/ml) than in the healthy group (18.1 \(\pm\) 15.2 ng/ml) (\(P = 0.008\)).

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<th>Table 1. Clinical Characteristics by Periodontal Disease Status (mean (\pm) SD)</th>
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\# DQR 1000, Hologic Inc., Waltham, MA.
shown in Table 4. No statistically significant differences between densitometric groups were observed ($P>0.05$).

Women were classified according to their serum osteocalcin concentration into two groups: normal osteocalcin concentration group (osteocalcin concentration between 3.5 to 10 ng/ml) and increased osteocalcin concentration group (osteocalcin concentration $\geq$10 ng/ml). The correlation between serum osteocalcin concentrations and periodontal clinical parameters was examined (Table 5). No statistically significant differences were observed ($P>0.05$). Using the Pearson lineal correlation test, serum osteocalcin concentration did not correlate with any periodontal clinical parameter.

To study the correlation between saliva osteocalcin concentrations and periodontal clinical parameters, women were classified according to their serum osteocalcin concentration in three groups: 1) osteocalcin concentration $<3$ ng/ml; 2) osteocalcin concentration ranging from 3 to 7 ng/ml; and 3) osteocalcin concentration $>7$ ng/ml (Table 6). No statistically significant differences in probing depth, bleeding on probing, or percentage of dental plaque between the three groups were observed ($P>0.05$). On the contrary, there were significant differences between the three groups in clinical attachment loss ($P = 0.01$; with significant differences between the group $<3$ OC and $>7$ OC in Bonferroni test). Using the Pearson lineal correlation test, saliva osteocalcin concentrations did not correlate with any periodontal clinical parameter.

To study the correlation between GCF osteocalcin concentrations and periodontal clinical parameters, women were classified in three groups according to their GCF osteocalcin concentration: 1) osteocalcin concentration $<12$ ng/ml; 2) osteocalcin concentration ranging from 12 to 30 ng/ml; and 3) osteocalcin concentration $>30$ ng/ml (Table 7). Mean probing depth
correlated significantly with GCF osteocalcin concentrations \((r = 0.35; \ P = 0.002)\). Women with low GCF osteocalcin concentrations showed a significantly lesser probing depth \((2.2 \pm 0.3 \ \text{ng/ml})\) than those with higher GCF osteocalcin concentrations \((P = 0.03)\). Bonferroni test also showed significant differences between the group with 12 to 30 ng/ml and the group with >30 ng/ml. Mean CAL did not correlate with GCF osteocalcin concentrations \((r = 0.18; \ P = 0.119)\) but CAL was significantly higher in the group with >30 ng/ml osteocalcin concentration \((P = 0.08)\). Bleeding on probing percentage increased in correlation with GCF osteocalcin concentration \((r = 0.25; \ P = 0.02\) Bonferroni test showed statistical differences between the group with <12 ng/ml GCF osteocalcin and the group with >30 ng/ml \((P = 0.01)\). No statistically significant differences in percentage of dental plaque between the three groups were observed \((P >0.05)\).

**DISCUSSION**

Osteocalcin levels in serum, saliva, and gingival crevicular fluid were determined in the present cross-sectional study. To our knowledge, this is the first study to measure osteocalcin concentrations in these three fluids in both healthy and periodontally diseased subjects. We showed that OC in GCF correlated significantly \((P = 0.008)\) with the periodontal status of the patients. Moreover, in the present study, OC in GCF correlated significantly with probing depth, clinical attachment level, and bleeding on probing, three main clinical parameters of periodontal disease. Additionally, saliva OC has been shown to correlate significantly with clinical attachment level.

It is unclear where osteocalcin in GCF originates. Some of this protein will be derived from circulating levels. However, it is also possible that osteocalcin will be produced locally, either alveolar bone is resorbed or from active local osteoblast synthesis. The reservoir of osteocalcin locked into the alveolar bone and released into the GCF by osteoblast activity will, most likely, be correlated with the local osteoblast activity. The localization of osteocalcin in the entire human periodontium including gingiva, periodontal ligament, cementum, and alveolar bone has been studied. The authors demonstrated its presence within the alveolar bone, cementum, and periodontal ligament and a high expression of osteocalcin in periodontal ligament fibroblasts. Since osteocalcin may play important roles in cementogenesis, osteoblast differentiation, and bone mineralization, it may express hard tissue metabolism. Furthermore, the finding of increased bone density in osteocalcin null mutant mice implicates osteocalcin as a potential inhibitor of bone formation.

GCF was thought to be an inflammatory exude capable of detecting episodes of periodontitis, but it has not been determined whether GCF markers in humans directly correlate with those at the underlying bone. There are clear diagnostic advantages in identifying an unambiguous marker of periodontal disease. We therefore investigated GCF osteocalcin levels as a marker of periodontitis. Osteocalcin is a specific osteoblast product and raised levels in adult periodontitis may be related to the severity of breakdown and/or repair of alveolar bone. During active bone resorption, osteocalcin and osteocalcin fragments are likely to be released from the extracellular matrix into the GCF. The fact that bone remodeling occurs simultaneously in multiple skeletal location is generally viewed as evidence that it is locally controlled, through autocrine and/or paracrine mechanisms. So, osteocalcin levels in GCF are more informative than serum or saliva levels regarding bone turnover in the periodontium.

In the present study, patients with the highest OC in GCF had significantly more probing depth, clinical attachment level, and bleeding on probing, all clinical parameters that involve periodontal disease. Moreover, periodontally diseased women showed 2-fold higher OC in GCF than healthy women \((P = 0.008)\). These results agree with previous data reported by other investigators who studied the relationship between GCF osteocalcin levels and periodontal disease. Kunimatsu et al. reported a positive correlation between GCF osteocalcin N-terminal peptide levels and clinical parameters in a cross-sectional study of 14 patients with periodontitis and five with gingivitis. Nakashima et al. reported that OC in GCF correlated significantly with the gingival index, but not with probing depth. A longitudinal study using an experimental periodontitis model in beagle dogs reported a strong correlation between GCF osteocalcin levels and active bone turnover as assessed by bone-seeking radiopharmaceutical uptake. The results of the present study, together with those of previous studies, support the concept that OC in GCF can be used as a marker of periodontal disease.

On the other hand, several studies have found that OC in GCF did not correlate with periodontal status. Treatment of adult periodontitis patients with subantimicrobial doxycycline failed to reduce GCF osteocalcin levels. A cross-sectional study of 20 periodontitis patients reported no differences in GCF osteocalcin levels between deep and shallow sites in the same patients. Moreover, in a longitudinal study of untreated periodontitis patients with ≥1.5 mm attachment loss during the monitoring period, GCF osteocalcin levels alone were unable to distinguish between active and inactive sites. In another study, osteocalcin levels in the GCF during orthodontic tooth movement were highly variable between subjects and lacked a consistent pattern related to the stages of tooth movement. Recently, Wilson et al. did not detect...
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The levels of serum total osteocalcin were similar among and this would facilitate the periodontal bone resorption that an inhibition of bone formation could take place, with the patient periodontal status. It could be deduced from previous reports,

contribution from bone remodeling would be masked. Any data that reflect periodontal disease, as circulating levels of osteocalcin are related to previous reports,

10,18,19 and this protein are considered to be a marker of bone remodeling. The serum levels of osteocalcin levels have been shown in periods of rapid bone turnover such as osteoporosis, multiple myeloma, and fracture repair.34 However, in the present study no correlation between serum, saliva, and gingival crevicular fluid osteocalcin concentrations and bone mineral density assessed by densitometry was found. The finding that serum OC did not correlate with bone mineral density could be explained because of the age of the study population (57.3 ± 7.7 years) and the relatively high number of years that passed since menopause (9.15 ± 7.5 years). In agreement with these results, osteocalcin is predominantly synthesized by osteoblasts and it plays an important role in both bone resorption and mineralization.30 The serum levels of this protein are considered to be a marker of bone remodeling and are routinely assayed in the investigation and monitoring of metabolic bone disorders, especially in osteoporosis.7 Elevated serum osteocalcin levels have been shown in periods of rapid bone turnover but not as a predictive indicator of periodontal disease.31 It could be deduced that an inhibition of bone formation could take place, and this would facilitate the periodontal bone resorption. Additional longitudinal studies may be warranted to elucidate more fully the usefulness of osteocalcin as a diagnostic aid of periodontal disease activity.

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