ÖZET
Amaç: Yaşlı bireylerde yaşla birlikte kemik metabolizmasında değişiklikler görülebilir. Araştırmamızın amacı farklı periodontal hastalığa sahip yaşlı bireylerde dişeti oluğu sıvısı (DOS) kemik yapım ve yıkım seviyelerinin karşılaştırılmasıdır.

Yöntem: 65 yaş üstü, sistemik sağlıklı toplam 39 birey (periodontal sağlıklı (n=16), gingivitis (n=13) ve kronik periodontitisli (n=10) dahil edildi. Klinik ölçümlerde sondalanan cep derinliği (SCD), klinik ataşman seviyesi (KAS), sondalamada kanama (SK) ve plak indeksi (PI) kaydedildi. DOS örneklerinde kemik markırları alkalen fosfataz (ALP), osteokalsin (OC), osteopontin (OPN), osteoprotegerin (OPG) ve paratiroid hormon (PTH) seviyeleri enzyme-linked immunosorbent assay (ELISA) yöntemi ile analiz edildi. Elde edilen veriler non-parametrik testler ile değerlendirildi.

Bulgular: Çalışma grupları arasında DOS ALP, OC, OPN, OPG ve PTH seviyelerinde fark bulunmadı (p>0.05). DOS ALP seviyesi, SCD ve KAS klinik parametreleriyle pozitif korelasyon gösterdi (p<0.05).

Sonuç: Araştırmamız sınırları dahilinde, yaşlı bireylerde DOS'ta bulunan kemik yapım ve yıkım markırlarının lokal seviyelerinin periodontal hastalık patogenezinde rol oynamadığını düşündürmektedir.

Anahtar Kelimeler: periodontitis, gingivitis, kemik yapım-yıkım, yaşlanma

ABSTRACT
Objectives: Elderly individuals may exhibit altered bone metabolism due to aging. The purpose of this study was to investigate bone formation and resorption levels in gingival crevicular fluid (GCF) of elderly subjects with different periodontal diseases.

Methods: This study included 39 systemically healthy elderly participants aged > 65 years, including 10 with chronic periodontitis, 13 with gingivitis and 16 with healthy periodontium. Clinical periodontal parameters including probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP) and plaque index (PI) were recorded. GCF alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OPN), osteoprotegerin (OPG), and parathyroid hormone (PTH) were analyzed using enzyme-linked immunosorbent assay (ELISA). Statistical tests were performed using non-parametric methods.

Results: There were no significant differences in GCF ALP, OC, OPN, OPG and PTH levels among the study groups (p>0.05). Moreover, ALP levels in GCF were positively correlated with site-specific clinical parameters including PD and CAL.

Conclusion: Within the limitations of the present study, it may be concluded that investigated markers were able to detected in GCF however they may not play a role as bone turnover biomarkers in elderly individuals with different periodontal diseases.

Keywords: periodontitis, gingivitis, bone turnover markers, aging.

INTRODUCTION
Current knowledge about the pathogenesis of periodontal disease suggests that it results from the loss of a healthy balance between microbial virulence factors and host inflammatory response. Inflammation and tissue destruction are a part of host-mediated process in response to bacterial infection. Periodontal diseases differ in their etiology and pattern of progression, and this variability can be attributed to differences in the presence of factors that may modify the host response to microbial pathogens. Periodontitis is associated with destruction of soft and hard tissues caused by a large number of cytokines as well as the presence of other effector molecules released by resident and migrating cells.
Bone homeostasis is maintained by a coupled process of resorption and formation, reflecting a change in bone turnover.\(^1\)\(^2\) Markers of bone formation and resorption include proteins revealing osteoblast/osteoclast activity, matrix proteins or osteoblastic enzymes.\(^1\)\(^2\) The activity of osteoblast and osteoclast cells could better understand within biochemical assays. For bone remodeling cycle activity ALP, OC, OPN, OPG and PTH were investigated in systemic bone changes.

Aging is associated with a decrease in mental and physical health and an increase in the risk of chronic diseases, such as atherosclerosis, cancer, diabetes and periodontitis.\(^3\)\(^4\) It also affect the human immune response, particularly the T-lymphocyte system regarding to diminished count of T-cells in blood cells.\(^3\)\(^4\) It has been suggested that abnormal higher interleukin-6 levels may be related to the pathogenesis of periodontal diseases in the elderly.\(^3\) Among individuals undergoing dialysis serum OPG levels were observed to be higher in elderly patient than in adult patients.\(^5\) It has also been shown that serum OC levels generally increased with age in post-menopausal women.\(^3\)\(^6\)

Periodontal disease is common chronic inflammatory disease in the elderly.\(^4\) However, little is known about the role and levels of biochemical markers in the elderly patients with periodontal diseases.\(^5\)\(^7\)\(^8\) Yoshihara et al. reported that there was a significant relationship between serum OC levels and systemic bone mineral density in elderly patients.\(^9\) Yoshihara et al. showed a weak significant relationship between the clinical attachment loss and serum OC levels.\(^5\) Since ALP, OC, OPN, OPG and PTH are associated with bone metabolism we hypothesized that bone turnover markers may be associated with periodontal disease in the elderly. The aim of the study was to investigate GCF ALP, OC, OPN, OPG and PTH levels in elderly patients with different periodontal diseases and to best of our knowlegde, there are no other studies that have focus on this.

**MATERIALS AND METHODS**

**Study population**

A total of 39 elderly participants (age, >65 years) who were non-smokers and systemical healthy, having at least 16 teeth in their mouth were included in this study. All subjects were recruited from the Department of Periodontology, School of Dentistry, Ege University, İzmir, Turkey, between April 2013 and September 2013. The purpose of the study and procedures used explained to all subjects prior to participation, and written informed consent in accordance with the Helsinki declaration was collected from all of them. The study protocol was approved by the Ethics Committee of the Ege University School of Medicine (ethics approval number: 11–13.1/11). Complete medical and dental histories were collected from all participants and none of them had a history of any systemic diseases such as diabetes mellitus or immunological disorders and had not been prescribed any antibiotics or other medications and nonsurgical periodontal treatment within the past 4 months.

Patient were selected according to the clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions.\(^9\) Chronic periodontitis group (CP). The patients in the CP group had moderate-to-severe alveolar bone loss, CAL of ≥ 5 mm and PD of ≥ 6 mm in multiple sites in all four quadrants of the mouth, but exhibited no evidence of rapid progression. This group consisted of 6 females and 4 males (mean age = 67.72 ± 2.7 years, range 65-78). Gingivitis group (G). The patients in the G group had varying degrees of gingival inflammatory, but no CAL > 2 mm and no evidence of alveolar bone loss in radiographs (i.e., distance between the cemento-enamel junction and bone crest at >95% of the proximal tooth sites ≤ 3 mm). This group consisted of 6 females and 7 males (mean age of 68.62 ± 2.8 years, range 65-74).

Healthy group (H). The H group consisted of healthy volunteers from the Department of Periodontology. There was no radiographic evidence of alveolar bone loss, clinical inflammation or sulcular bleeding in these patients. This group comprised of 7 females and 9 males ( mean age of 67.86 ± 1.9 years, range 65-88 years).

**Determination of periodontal status**

One examiner (V.O.O) carried out a clinical periodontal examination including PD, CAL, BOP (dichotomous measurement) and PI in all subjects.\(^10\) PD measurements were performed manual using Williams probe. All measurements were performed at 6 sites per tooth for the whole mouth.

**Collection of GCF samples**

After selection, subjects were recalled for collection of GCF samples from two single-rooted anterior teeth in each group. GCF samples were collected from teeth with PD ≥ 6 mm in the CP, teeth exhibited bleeding on probing and PD ≥ 2 mm in the G group and teeth with PD ≤ 2 mm and no BOP in the H group. Prior to sampling, the supragingival plaque was removed from the interproximal surfaces with a sterile curette; and the teeth were dried gently using an air syringe and isolated.
with cotton rolls. GCF was sampled with filter paper strips. (Periopaper, ProFlow, Inc., Amityville, NY, USA). That were carefully inserted into the crevice/pocket until mild resistance was felt and left for 30 seconds. Care was taken to avoid mechanical injury and strips contaminated with blood were discarded. The absorbed GCF volume in each strip was determined by electronic impedance (Periotron 8000, ProFlow, Inc., and Amityville, NY, USA). The paper strips were pooled, placed in a sterile Eppendorf vial and kept at -40°C until analysis.

Analysis of bone turnover markers
GCF samples were eluted from the strips by placing them in 600 μl of PBS. GCF ALP (Usclife, Wuhan, Hubei, China), OC (Ebioscience, San Diego, USA), OPG (Raybiotech, Norcross, GA, USA), OPN (Raybiotech, Norcross, GA, USA) and PTH (Diasource, Belgium) levels were assayed using commercially available ELISA kits. Procedures were performed according to the instructions provided in the kit. The minimum detectable limits of ALP, OC, OPG, OPN and PTH were 0.312 ng/ml, 1.2 ng/ml, 1 pg/ml, 50 pg/ml and 15 pg/mL, respectively. The amounts of ALP, OC, OPN, OPG and PTH in each sample was calculated based on the dilutions and the results were expressed as the total amount in the 30 second of the two GCF sample.

Statistical analysis:
Minimum sample size was calculated assuming a difference of 50% in mean levels of the biochemical marker (OC) in GCF, maximum standard deviations of 80% of the mean values and accepting a power of 90%, p-value of 5% in healthy and periodontitis groups and a power of the study 80%. Power calculations revealed that the minimum required sample size was nine subjects for each group. Kruskal-Wallis test was used for comparisons of the study groups. When significant differences (p<0.05) were observed, post-hoc 2-group comparisons carried out using Bonferroni-corrected Mann-Whitney U tests, and p-values <0.02 were considered to be statistically significant. Spearman rank correlation analysis was used to examine the relation between biochemical levels and clinical parameters (p<0.05). GraphPad (Prism6 for Mac, USA) was used for data analysis.

RESULTS
Clinical findings
The age and gender distribution of the subjects were similar among the study groups. The whole-mouth clinical parameters of the study groups are presented in Table 1. The CP group had significantly higher mean PD and CAL scores compared to the G and H groups (PD: p=0.0003, p=0.0001, respectively; CAL p=0.0001, p=0.0001, respectively). The total number of teeth and single-rooted teeth were similar among the three groups.
Both periodontitis and gingivitis groups had BOP and PI scores significantly higher than those of the H group (BOP: $p<0.0001$, $p<0.0001$, respectively, PI: $p=0.01$, $p=0.02$, respectively).

The mean clinical data for the sampling areas are shown in Table 2. The mean PD scores of the sampling sites of the G and CP groups were significantly higher than those of the H group ($p=0.0003$, $p<0.0001$, respectively) and the CP group had higher PD scores compared to the G group ($p=0.01$). The mean CAL of the sampling sites in the CP group was significantly higher than those of the G and H groups ($p=0.01$, $p=0.001$, respectively). The CP and G groups had significantly higher PI scores compared to the H group ($p=0.001$, $p=0.02$) and the percentage of BOP-positive sites was similar in CP and G groups ($p>0.05$). PI scores and the percentage of BOP-positive sites were significantly higher in the study group than in the healthy group. The CP and G groups had significantly higher PI scores compared to the H group ($p=0.001$, $p=0.02$, respectively). CP group had significantly elevated GCF scores compared to the G group ($p=0.04$).

The mean PD scores of the sampling sites of the G and CP groups were significantly higher than those of the H group ($p=0.0003$, $p<0.0001$, respectively) and the CP group had higher PD scores compared to the G group ($p=0.01$). The mean CAL of the sampling sites in the CP group was significantly higher than those of the G and H groups ($p=0.01$, $p=0.001$, respectively). The CP and G groups had significantly higher PI scores compared to the H group ($p=0.001$, $p=0.02$) and the percentage of BOP-positive sites was similar in CP and G groups ($p>0.05$). PI scores and the percentage of BOP-positive sites were significantly higher in the study group than in the healthy group. The CP and G groups had significantly higher PI scores compared to the H group ($p=0.001$, $p=0.02$, respectively). CP group had significantly elevated GCF scores compared to the G group ($p=0.04$).

Biochemical findings
The distribution of the total amount of GCF ALP is shown in Figure 1, and no significant differences in the total amount of GCF ALP were observed between the study groups ($p>0.05$). ALP amounts in GCF (mean ± SD) were 4.89 ± 3.90 pg in the healthy sites, 6.10 ± 4.06 pg in the gingivitis sites, and 5.77 ± 3.98 pg in the CP sites.

The distribution of the total amount of GCF OC is shown in Figure 2. There were no significant differences in total amount of GCF OC between the study groups ($p>0.05$). OC amounts in GCF (mean ± SD) were 0.64 ± 0.09 pg in the healthy sites, 0.58 ± 0.07 pg in the gingivitis sites, and 0.56 ± 0.08 pg in the CP sites.

The distribution of the total amount of GCF OPN is shown in Figure 3, and it was similar in the study groups ($p>0.05$). OPN amounts in GCF (mean ± SD) were 3708 ± 1488 pg in the healthy sites, 3828 ± 1177 pg in the gingivitis sites, and 3913 ± 1164 pg in the CP sites.

The distribution of the total amount of GCF OPG is shown in Figure 4, and no significant differences were observed in between the study groups ($p>0.05$). OPG amounts in GCF (mean ± SD) were 257.6 ± 65.06 pg in the healthy sites, 273.1 ± 60.01 pg in the gingivitis sites, and 271.3 ± 55.73 pg in the CP sites.

The distribution of the total amount of GCF PTH is shown in Figure 5, and all study groups exhibited similar level of total amount of GCF PTH ($p>0.05$). PTH amounts in GCF (mean ± SD) were 9.17 ± 3.87 pg in the healthy sites, 7.81 ± 4.96 pg in the gingivitis sites, and 9.28 ± 4.51 pg in the CP sites.

Correlation between bone turnover markers and site-specific clinical parameters
The Spearman rank correlation analysis was used to investigate the association between the total amount of bone turnover markers in GCF and site-specific clinical periodontal measurements (Table 3). The GCF ALP levels were positively correlated with PD and CAL (p<0.01), whereas OC, OPN, OPG and PTH did not exhibit any correlation with any of the clinical parameters (p>0.05) (Table 3).

**DISCUSSION**

Physiological changes during aging include reduction in bone mass, weakening of the immune system and increasing risk of developing cancer. There is a strong relationship between aging, inflammation, response to infection, and progression of chronic inflammatory diseases. Edentulousness is common among the elderly populations globally, making the evaluation of periodontal status in these subjects important. Bone turnover in periodontitis is associated with increased bone resorption markers with lower bone formation markers contrary to expectations our results showed that GCF ALP, OC, OPN, OPG and PTH levels are similar in all study groups. Differences in dietary habits, increased flow of gingival exudate from the inflamed gingiva, and possible age-related changes in salivary gland secretions may alter the level of GCF biomarkers similarly. In the present study only total amount of biochemical datas was shown due to the total amount of GCF is more sensitive than expressing its as a concentration.

The present study included only elderly subjects visiting a dental facility, and none of the subjects were hospitalized or institutionalized. Ogawa et al. reported that institutionalized elderly subjects were less active and more prone to higher risk of periodontal disease than non-institutionalized subjects. However, it is extremely difficult to identify elderly individuals with no systemic diseases. Jung et al. identified the elderly systemically healthy subjects as “healthy survivors”.

Age-related diseases often occur in combination with other chronic conditions resulting in an increase in the prevalence of diabetes mellitus, cardiovascular disease, and osteoporosis in elderly population each year. However, patient selections were made questionable medical and dental history, instead of systematic biochemical data. Blood were not obtained for participants due to ethical reasons, this could be one of major limitation of the present study. Although the inclusion of more number of elderly participants would be ideal, this study comprised 39 systemically healthy elderly subjects in accordance with the minimum sample size calculated. Limited number participants in each group may reflect similar results on biochemical analysis.

ALP is metalloenzymes responsible for catalyzing the hydrolysis of phosphate esters, and is present as multiple isoforms in human tissues. It is present as a glycosyl-phosphatidyl-inositol-anchored ectoenzyme on the membranes of osteoblastic cells. Although its exact function is not fully understood, ALP is required on the cell membrane for bone mineralization. Ishikawa et al. showed that GCF ALP levels were significantly higher in both chronic and aggressive periodontitis patients than healthy subjects. GCF ALP is correlated with increased gingival index. Daltaban et al. reported higher GCF ALP levels in post-menopausal women with periodontitis than in the clinically healthy control group. A recent study, demonstrated low levels of GCF ALP in non-destructive and slightly destructive periodontitis sites, whereas significantly high levels of ALP were observed in moderately and severely destructive sites. In the present study, there was no significant difference between the study groups including between CP and H suggesting that the destruction was moderate in the sampling sites of the CP group or might be even a little destruction or subclinical inflammation in H group. Similar total amounts of ALP in GCF may be a reflection of the multitude of complex age-related changes including reduced inflammatory response occurring in elderly.

![Figure 5](image.png)

**Figure 5.** Total amount of GCF parathyroid hormone levels Total amount of GCF Parathyroid hormone in the study groups. Box plots show medians, 25th and 75th percentiles as boxes, and 10th and 90th percentiles as whiskers.
Serum OC levels increase in osteoporosis, making it a valid marker of this process. It has previously been shown that bone turnover profiles from periodontal bone surfaces and GCF differed from systemic bone turnover profiles. In elderly women, Gerdhem et al. showed higher serum OC levels, whereas Garnero et al. showed similar levels in postmenopausal women. The present study showed that OC levels in all geriatric groups were similar. Several studies have found that GCF OC levels were not correlated with periodontal status; our results were in agreement with this. These results may be a result of lower osteoblastic activity in geriatric subjects. Moreover, similarities in OC levels in the present study may reflect an inability effect of clinical signs of periodontal disease (CAL, increased PD, BOP) are present in inflamed sites, but with no evidence of activity. This may also have resulted in similar GCF OC levels in all groups.

OPN, is mainly involved in enhancing osteoclastic action and has also been detected in GCF in some aforementioned studies. The effects of initial periodontal therapy also confirmed that OPN levels significantly decreased and were correlated with decreasing clinical periodontal parameters. Kido et al. reported that OPN in GCF may be considered as a marker of alveolar bone loss in periodontitis, while Sharma and Pradeep reported that GCF OPN levels were higher in periodontitis patients compared to those with gingivitis and healthy subjects. Conversely, the present study showed similar total amounts of OPN GCF levels in all geriatric groups. Neighboring tissues including alveolar bone and cementum, macrophages in periodontal tissues, blood and salivary glands function as the source of OPN in GCF. Moreover, the reduced inflammatory response may also play a role of similar pattern of OPN in GCF. It appears that GCF OPN levels are not related to inflammatory changes in elderly individuals with periodontal disease, so this could partly explain the similar results observed in all groups.

OPG is continually produced by resident periodontal connective tissue fibroblasts and potentially by endothelial cells. The levels of serum calcium, and phosphorus are controlled by PTH, which also plays an important role in bone formation because of the receptor present in the membrane of osteoblastic cells. Meanwhile, recent literature has shown that the adjunctive action of OPG-PTH can shift bone turnover toward an osteogenic environment. This could be related to the fact that OPG eliminates the PTH receptor active in osteoclasts, potentially being responsible for the balance between these markers observed in the present study.

It is well known that the homeostasis in bone turnover is disturbed during the progression of periodontal disease and leads to irreversible bone resorption where the immune cells and cytokines released play an important role. The presence of measurable amounts of GCF ALP, OC, OPN, OPG and PTH in the CP, G and H groups might be due to the bone homeostasis process independently ageing.

Our study results, for the first time, demonstrated the presence of bone turnover markers in the GCF of elderly individuals with different periodontal disease. The findings should be confirmed by longitudinal studies investigating the levels of these biomarkers in saliva and serum before and after periodontal treatment.

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