Journal of
PERIODONTAL RESEARCH

J Periodont Res 2014

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/jre.12182

Monitoring bone morphogenetic protein-2 and -7, soluble receptor activator of nuclear factor-κB ligand and osteoprotegerin levels in the peri-implant sulcular fluid during the osseointegration of hydrophilic-modified sandblasted acid-etched and sandblasted acid-etched surface dental implants

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Dolanmaz D, Saglam M, Inan O, Dundar N, Alniacık G, Gursoy Trak B, Kocak E, Hakki SS. Monitoring bone morphogenetic protein-2 and -7, soluble receptor activator of nuclear factor-κB ligand and osteoprotegerin levels in the peri-implant sulcular fluid during the osseointegration of hydrophilic-modified sandblasted acid-etched and sandblasted acid-etched surface dental implants. J Periodont Res 2014; doi: 10.1111/jre.12182 © 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd.

Background and Objective: The implant surface plays a major role in the biological response to titanium dental implants. The aim of this study was to investigate levels of soluble receptor activator of nuclear factor-κB ligand (sRANKL), osteoprotegerin (OPG), bone morphogenetic protein-2 (BMP-2) and -7 (BMP-7) in the peri-implant crevicular fluid (PICF) of different implants during the osseointegration period.

Material and Methods: Forty-seven patients (22 females and 25 males, mean age 47.34 ± 10.11) were included in this study. Forty-seven implants from two implant systems (group A1 (sandblasted acid-etched [SLA]-16), group A2 (hydrophilic-modified SLA [SLActive]-16), and group B (sandblasted acid-etched [SLA]-15) were placed using standard surgical protocols. PICF samples, plaque index, gingival index and probing depth measurements were obtained at

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Key words: bone morphogenetic proteins; dental implant; osteoprotegerin; receptor activator of nuclear factor-kappa B

Accepted for publication February 06, 2014

1 and 3 mo after surgery. PICF levels of sRANKL, OPG, BMP-2/-7 were analyzed by ELISA.

Results: No complications were observed during the healing period. No significant differences were observed in the PICF levels of sRANKL, OPG, BMP-2 and BMP-7 for all groups at any time point ($p > 0.05$). A significant decrease was observed in BMP-2 levels in group A1 ($p < 0.05$). A significant increase in BMP-7 levels was observed only for group A2 ($p < 0.05$). There was a strong negative correlation between OPG and gingival index and a negative correlation between BMP-7 and plaque index ($p < 0.05$).

Conclusion: Considering the correlations between clinical and biochemical parameters, the levels of these cytokines in PICF during early healing of implants reflects the degree of peri-implant inflammation, rather than differences in the implant surfaces.

Introduction

The term "osseointegration" defines a direct structural and functional connection between bone tissue and the implant surface (1). The properties of the implant surface, including topography, degree of roughness, chemistry, surface free energy and wettability (hydrophilicity), have been described as important factors in osseointegration (2,3). Surface topography plays a prominent role in the entire osseointegration process and influences cell behavior such as proliferation, differentiation, initial attachment and the production of growth factors and cytokines (4,5). Irregularities and roughness of the dental implant surface have an effect on cell differentiation and secretion (6,7). These effects are mediated by "improved adsorption, configuration and bioactivity of proteins that mediate osteoblast adhesion" (8–10) and upregulation of signaling cytokines, such as interleukin-1β and transforming growth factor (TGF)β1 (11). Rough surfaces have been reported to increase the amount of implant-to-bone contact during the initial bone healing process compared to machined or polished titanium surfaces (12–15), which provide a firmer mechanical link (16), and enhance and accelerate the cell-mediated osseointegration process (4). Different techniques have been developed to produce new microrough Ti implant surfaces, including sandblasting, acid-etching, combinations of these, or surface oxidation, to modify surface topography (17). Among these new surfaces, the sandblasted and acid-etched (SLA) surface results in a superior bone-to-implant contact (BIC) relative to titanium plasma sprayed, Al_2O_3-blasted and polished implants (13,18). The SLA surface has been shown to promote osteoblast differentiation, increased production of osteogenic factors, cytokines and growth factors, and increased BIC compared with a relatively smooth-machined surface implant (19). Considering these effects of the SLA surface, several clinical studies investigating this surface showed that healing periods with this surface were shorter (6–8 wk) than conventional healing periods of 3–6 mo (20–22). Surface free energy, hydrophilicity and wettability of the implant surface are crucial factors for the initial attachment of cellular components and macromolecules to the implant surface (5). A chemically modified version of SLA surface (SLActive surface) demonstrated improved wettability and hydrophilic properties; it was produced through sandblasting and etching procedures similar to those of SLA. The native oxide chemistry is preserved by processing the implant under N_2 protection and immediately storing it in an isotonic N_2 solution (23). Studies have demonstrated that the SLActive surface presents increased hydrophilicity and induces greater BIC, osteoblast differentiation, growth factor production and osteogenic gene expression at 2 and 4 wk of healing than the SLA surface (24–27).

RANKL and OPG are critical factors in the control of osseous healing, which are both produced by osteoblasts. sRANKL is a natural and necessary surface-bound molecule found on osteoblasts and is stimulated to activate osteoclasts, cells involved in bone resorption. OPG (osteoclastogenesis inhibitory factor) counteracts the biological activities of RANKL by preventing its interaction with its receptor (RANK) (28–30).

Other important bone biological factors that play essential roles in osteogenesis are bone morphogenetic proteins (BMPs). BMPs are members of secreted signaling proteins that belong to the TGFβ superfamily (31). It has been demonstrated that BMPs induce bone formation by differentiating mesenchymal stem cells to chondroblastic and osteoblastic cells (32). BMP-2 and BMP-7 were shown to be the most effective types that induce complete bone morphogenesis (33). BMP-2, like other BMPs, plays an important role in the development of bone and cartilage (34). The primary effect of BMP-2 on pluripotent cells that are capable of differentiating into osteoblasts, adipocytes or muscle cells appears to be its ability to commit the cell to an osteoblastic pathway (35). BMP-7 is also known as osteogenic protein-1 (36). It plays a key role in the transformation of mesenchymal stem cells into bone and cartilage (34).

Biological factors play an important role in osseointegration, and little is known about the biological factors involved in bone healing and remodeling around dental implants. Our hypothesis was that levels of RANK, RANKL, OPG, BMP-2 and BMP-7 found in peri-implant crevicular fluid (PICF) might differ between two otherwise identical dental implants that have slightly different surfaces and a third implant system with different macroscopic configuration and different surface. Therefore, the aim of the present study was to evaluate and compare levels of BMP-2/-7, sRANKL and OPG in crevicular fluid collected from implants with SLActive and SLA surfaces during the osseointegration process.

Material and methods

Forty-seven patients (22 women and 25 men; mean age 47.34 ± 10.11 years) who were attending the Oral and Maxillofacial Surgery Department at the Selçuk University Faculty of Dentistry (Konya, Turkey; during the 2011–2013 academic years) and scheduled for implant placement surgery were included in this study. The study protocol was approved by the Ethics Commission of Selçuk University, Selçuklu Faculty of Medicine for human subjects. Patients with systemic health problems that might affect the healing process, smokers or alcohol and drug users (anti-inflammatory agents, biphosphonates), and pregnant patients were excluded from the study. Information about the study that was related to the specific instrumentation was given to each patient before surgery and a verbal and written consent was obtained. Periodontal therapy was performed in all patients before implant surgery. Phase I periodontal therapy was performed for patients with chronic periodontitis. Patients with gingivitis have undergone scaling only. All patients also received oral hygiene instruction. No patient had ≥ 4 mm periodontal pocket depth after periodontal therapy. Forty-seven implants from two implant systems (group A1 [16 implants; Standard plus SLA; Institute Straumann, Basel, Switzerland], group A2 [16; Standard plus SLActive; Institute Straumann] and group B [15; SLA; Nucleoss, Izmir, Turkey]) were placed using standard surgical protocols. Tissue-level implants (grade 4 titanium) were used for group A1 and A2 in this study. These implants have a buttress-shaped thread pattern and a cylindrical shape. Thread pitch is 1.25 mm and thread depth is 0.3 mm. In our study, group B implants were made out of grade 4 titanium. These implants have two-start V-shaped thread pattern and root form body shape. Thread pitch is 0.75 mm and thread depth is 0.3 mm. Bone-level implants were used in group B.

The SLA surface is sandblasted with large grit (250–500 μm), which results in a peak-to-peak macro-roughness of approximately 20–40 μm, followed by micro-roughness of approximately 2–4 μm upon acid etching. Average surface roughness (R_a) is 2.93 ± 0.46 (37).

The SLActive surface has been produced via a similar sandblasting and etching procedure as SLA, except for the fact that the implants were rinsed in water under N_2 protection and directly stored in an isotonic NaCl solution, again protected by N_2 filling (23). R_a of the SLActive surface ranges between 1.2 and 3.99 mm (15).

Group B implants exhibited "Maxicell" surface technology, in which the surface is first sandblasted by large grain Al_2O_3, as in the SLA surface, and then is thermal acid etched for surface cleaning. This produces fine 1–2 μm micropits on the rough-blasted surface. Figure 1 presents scanning electron microscope images of the group B implant system. R_a of group B is 3.2361 ± 0.2315, the root mean square of the values of all points of the surface (R_q) is 4.1316 ± 0.3085, maximum peak-to-valley height of the entire measurement trace (R_t) is 27.1536 ± 3.1756 and arithmetic average of the maximum peak-to-valley height of the five greatest values (R_z) is 21.9079 ± 1.6022.

Implant therapy

Patients who were scheduled for single tooth implant placement surgery in the Department of Oral and Maxillofacial Surgery were randomly allocated to one of the three treatment groups by an experienced investigator (DD). The implants from the distributors of Straumann Company were provided by the Research Project Coordination of Selçuk University support and the implants from the Nucleoss Company were kindly provided; group sizes were determined according to provided implants. Group A1 and group A2 implants were only placed in the mandible. Group B1 implants were placed in both the mandible and maxilla. However, the number of implants placed in the mandible is 15. For standardization, we used these 15 implants for group B.

Dental implants were placed using a one-stage protocol. After administration of local anesthesia (Ultracain DS; Aventis Pharmaceuticals, Istanbul, Turkey), an incision was made on the alveolar ridge. Forty-seven implants were placed (group A1 [16], group A2 [16] and group B [15]) in the mandible. Good primary stability was obtained for each implant. The mucoperiosteal flaps were adapted around the implant neck to allow non-submerged healing and were sutured with silk sutures (Sterisilk; SSM Sterile Health Products Inc, Istanbul, Turkey). A healing cap (gingival former) was placed for all implants. Postoperative medication included amoxicillin 500 mg three times a day for 10 d, paracetamol 500 mg twice a day for 5 d and 0.2% chlorhexidine mouthwash (Corsodyl; GlaxoSmithKline Consumer Healthcare, Brentford, Middlesex UK) twice a day for 10 d. The silk sutures were removed 10 d after surgery. Resonance frequency analysis (implant stability quotient [ISQ]) (Osstell ISQ; Integration Diagnostics AB, Savedalen, Sweden) was used to measure implant stability for each implant at the time of surgery before flap closure and at 1 and 3 mo (loading time) postoperatively. The device was used in both buccolingual and mesiodistal direction. Three measurements were obtained for group A1 and group A2 at each time point and average ISQ readings were ≥ 80 at 1

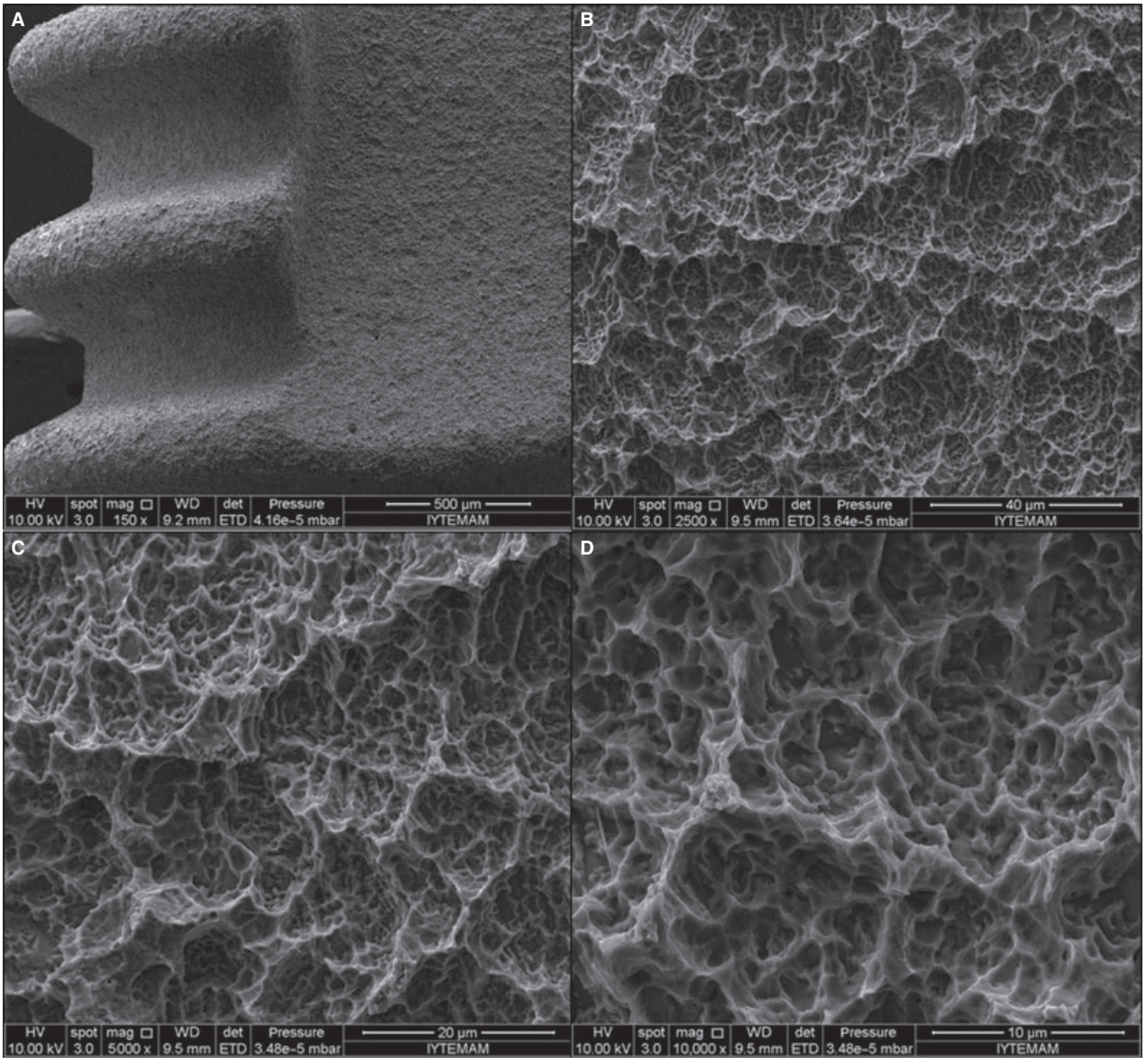

Fig. 1. Scanning electron microscope images of group B implant system. (A) 150×, (B) 2500×, (C) 5000×, (D) 10,000×.

and 3 mo after surgery for all implants. Group B implants had no probe for resonance frequency analysis. The primary stability was evaluated clinically and by using periapical radiography.

Evaluation of the implant status by clinical parameters

The clinical examination included the assessment of pocket depth and the measurement of the modified plaque index (MPI) (38) and the gingival index (GI) (39). MPI and GI measurements were performed at four sites around each implant and the probing depth was performed at six sites around each implant using a periodontal probe with a plastic tip (Hu-Friedy, Chicago, IL, USA). PICF sampling and clinical measurements were recorded at 1 and 3 mo after implant therapy.

Collection of peri-implant crevicular fluid

PICF was collected via the intracrevicular method (40). Implant surfaces were air-dried and isolated by cotton rolls. Paper strips (Periopaper; ProFlow, Inc., Amityville, NY, USA) were inserted into the implant crevices for 30 s. The adsorbed volume was determined by impedance measurements (Periotron 8000; Oraflow, Inc., Plainview, NY, USA). Two strips (mesial and distal sites) were pooled for each implant. Paper strips were placed into 1.5 mL plastic tubes containing 500 µL of phosphate-buffered saline and stored at −80°C before ELISA analysis.

Osteoprotegerin determination

OPG levels were determined by using a commercial ELISA kit (Human

Osteoprotegerin ELISA; BioVendor, Brno, Czech Republic) according to the manufacturer's instructions. In human OPG ELISA, the standards and samples (1 : 3 dilution) were incubated with a mouse monoclonal anti-human OPG antibody coated in wells. After washing, biotin-labeled polyclonal antihuman OPG antibody was added and incubated with captured OPG. After adding streptavidin-horseradish peroxidase conjugate and following the stop solution, the absorbance values (optical densities) were measured spectrophotometrically at a wavelength of 450 nm, and the samples were compared with the standards. Data were then calculated and obtained by methods of interpolation of a predetermined standard curve.

Soluble receptor activator of nuclear factor-κB ligand determination

The sRANKL levels in the samples were determined by commercial ELISA kits in accordance with the manufacturer's instructions (total sRANKL ELISA kit; BioVendor). One hundred μL of standards and samples were added to the wells and the plates were incubated for 18 h. Then the wells were washed five times with the wash solution. Afterwards biotin-labeled antibody was added to each well and incubated for 60 min at room temperature. Then the wells were washed again five times. One hundred μL streptavidin-horseradish peroxidase conjugate was added to each plate followed by incubation at room temperature for 1 h and washing the wells again. Then the wells were developed with 100 μL tetramethylbenzidine for 10 min at room temperature in the dark. The reaction was then stopped by the addition of 100 μL stop solution and the color developed was measured in an automated microplate spectrophotometer set to 450 nm, and the samples were compared with the standards. Data were then calculated and obtained by methods of interpolation of a predetermined standard curve.

Bone morphogenetic protein-2/-7 determination

BMP-2 (BMP-2 Immunoassay, Quantikine; R&D Systems, Minneapolis, MN, USA) and BMP-7 (Human BMP-7 Immunoassay, Quantikine; R&D Systems) levels were determined by using a commercial ELISA kit according to the manufacturer's instructions. One hundred μL of assay diluent was added to each well. Fifty μL of standards and samples were added to the wells and the plates were incubated for 2 h at room temperature. Then the wells were washed four times. Two hundred μL of BMP-2/BMP-7 conjugate was added to each well followed by incubation at room temperature for 2 h and washing the wells again four times. Two hundred μL of substrate solution was added to each well and the plates incubated for 30 min at room temperature. The reaction was then stopped by the addition of 50 μL stop solution and the color developed was measured in an automated microplate spectrophotometer set to 450 nm, and the samples were compared with the standards. Data were then calculated and obtained by methods of interpolation of a predetermined standard curve.

The results of sRANKL, OPG, BMP-2 and BMP-7 were expressed as pg/30 s for the total amounts and as pg/μL for concentrations when adjusted for PICF volume. The data were combined as mean per group. The primary outcome was BMP-2 and BMP-7 and the secondary outcome was clinical parameters. The groups were compared with each other at 1 and 3 mo in terms of clinical and biochemical markers. The results between the first and third month were also evaluated for each group.

Statistical analysis

The statistical analysis was performed using commercially available software (SPSS v.20.0; IBM, Chicago, IL, USA). The Shapiro–Wilk normality test was used to verify the normality of the data. Parametric tests were used for statistical analyses. One-way ANOVA and Tukey's test were used to compare the groups at each time point. The paired *t*-test was used to compare between the two time points for the same group.

Results

Forty-seven implants were placed using a one-stage protocol. The patient demographic data and implant site distribution were presented in Table 1. The implants showed no clinical signs of peri-implant infection or detectable mobility throughout the healing period.

Clinical assessments

The results of the clinical measurements (mean ± SD) at 1 and 3 mo in implant groups are displayed in Fig. 2. The changes in probing depth were not significant for group A1 and A2 ($p > 0.05$). There was a significant increase in probing depth for group B ($p < 0.05$). The probing depth was significantly higher in group B compared to groups A1 and A2 at 1 and 3 mo ($p < 0.05$). The changes in GI were

Table 1. Demographic data and implant site distribution

	Sex			Implant site distribution	
	Male	Female	Mean age ± SD	Premolar	Molar
Group A1	8	7	48.25 ± 10.16	6	10
Group A2	8	7	47.18 ± 10.08	6	10
Group B	7	8	46.53 ± 10.70	5	10
Total	25	22	47.34 ± 10.11	17	30

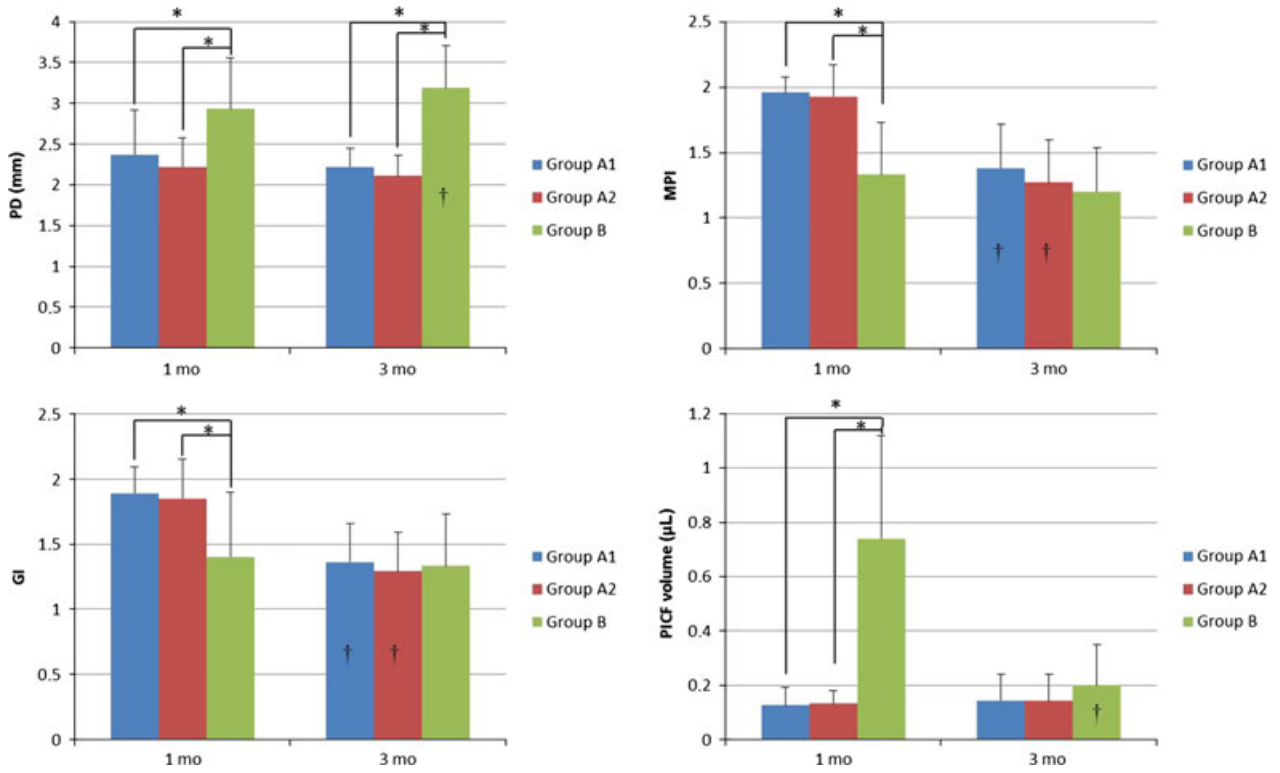

Fig. 2. (A) PD (mm) values for each group at 1 and 3 mo. (B) MPI values for each group at 1 and 3 mo. (C) GI values for each group at 1 and 3 mo. (D) PICF volumes (μL) for each group at 1 and 3 mo. *Significant difference between groups ($p < 0.05$). †Significant difference compared to the first month. GI, gingival index; MPI, modified plaque index; PD, probing depth; PICF, peri-implant crevicular fluid.

significant for group A1 and A2 ($p < 0.05$) but not significant for group B ($p > 0.05$). The GI was significantly lower in group B compared to groups A1 and A2 at 1 mo. No statistically significant differences were observed in GI between the groups at 3 mo ($p > 0.05$). The changes in MPI were significant for groups A1 and A2 ($p < 0.05$) but not significant for group B ($p > 0.05$). The MPI was significantly lower in group B compared to groups A1 and A2 at 1 mo. No statistically significant differences were observed in MPI between the groups at 3 mo ($p > 0.05$).

Biochemical assessments

Total amount (pg/30 s)— The results of the total amounts of biochemical markers (mean ± SD) between 1 and 3 mo in implant groups are displayed in Fig. 3. The OPG levels between 1 and 3 mo were not significant for all groups ($p > 0.05$). No statistically significant differences were observed in OPG levels between groups at 1 and 3 mo ($p > 0.05$). The sRANKL levels between 1 and 3 mo were not significant for all groups ($p > 0.05$). No statistically significant differences were observed in sRANKL levels between groups at 1 and 3 mo ($p > 0.05$). The sRANKL/OPG ratios were similar between groups at all time points ($p > 0.05$) (data not shown). The BMP-2 levels between 1 and 3 mo were not significant for groups A2 and B ($p > 0.05$). There was a significant decrease in the BMP-2 level for group A1 ($p < 0.05$). No statistically significant differences were observed in BMP-2 levels between groups at 1 and 3 mo ($p > 0.05$). The BMP-7 levels between 1 and 3 mo were not significant for groups A1 and B ($p > 0.05$). There was a significant increase in the BMP-7 level for group A2 ($p < 0.05$). No statistically significant differences were observed in BMP-7 levels between groups at 1 and 3 mo ($p > 0.05$).

There was a strong negative correlation between OPG and GI ($p < 0.05$). There was no correlation between sRANKL and clinical parameters ($p > 0.05$). There was no correlation between BMP-2 and clinical parameters ($p > 0.05$). There was a negative correlation between BMP-7 and PI ($p < 0.05$). There was a positive correlation between BMP-2 and sRANKL ($p < 0.05$).

Concentration (pg/μL)— The results of the concentration of biochemical markers (mean ± SD) between 1 and 3 mo in implant groups are displayed in Fig. 4. The OPG levels between 1 and 3 mo were not significant for groups A1 and A2 ($p > 0.05$). There was a significant increase in OPG levels for group B ($p < 0.05$). The OPG level was significantly lower in group B compared to groups A1 and A2 at 1 mo ($p < 0.05$). No statistically significant differences were observed in OPG levels between groups at 3 mo ($p > 0.05$). The sRANKL levels between 1 and 3 mo were not significant for groups A1 and A2 ($p > 0.05$).

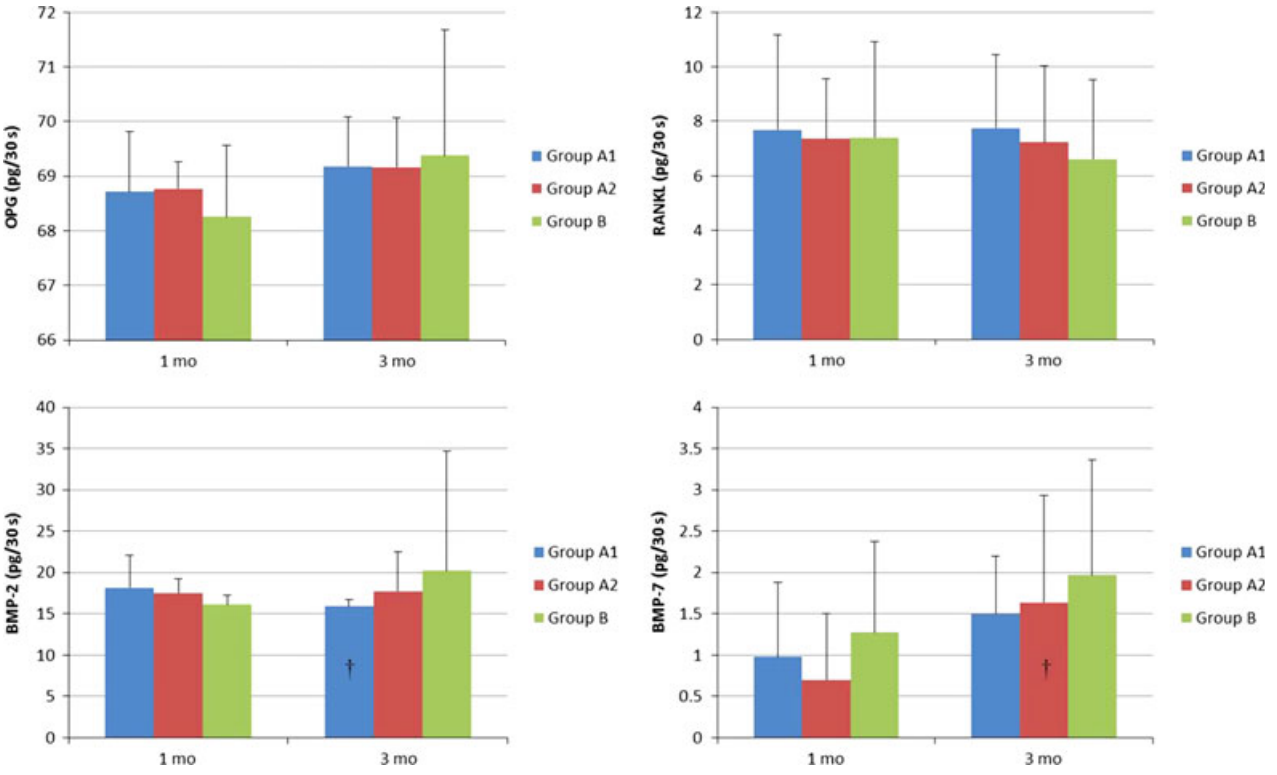

Fig. 3. (A) Total amount of OPG in PICF samples for each group at 1 and 3 mo. (B) Total amount of soluble RANKL in PICF samples for each group at 1 and 3 mo. (C) Total amount of BMP-2 in PICF samples for each group at 1 and 3 mo. (D) Total amount of BMP-7 in PICF samples for each group at 1 and 3 mo. BMP-2/-7, bone morphogenetic protein-2/-7; OPG, osteoprotegerin; PICF, peri-implant crevicular fluid; RANKL, receptor activator of nuclear factor-κB ligand.

There was a significant increase in sRANKL levels for group B ($p < 0.05$). The sRANKL level was significantly lower in group B compared to groups A1 and A2 at 1 mo ($p < 0.05$). No statistically significant differences were observed in sRANKL levels between groups at 3 mo ($p > 0.05$). The BMP-2 levels between 1 and 3 mo were not significant for groups A1 and A2 ($p > 0.05$). There was a significant increase in BMP-2 levels for group B ($p < 0.05$). The BMP-2 level was significantly lower in group B compared to groups A1 and A2 at 1 mo ($p < 0.05$). No statistically significant differences were observed in BMP-2 levels between groups at 3 mo ($p > 0.05$). The BMP-7 levels between 1 and 3 mo were not significant for group A1 ($p > 0.05$). There was a significant increase in BMP-7 levels for groups A2 and B ($p < 0.05$). No statistically significant differences were observed in BMP-7 levels between groups at 1 and 3 mo ($p > 0.05$).

There was a strong negative correlation between OPG and GI and a negative correlation between OPG and PI ($p < 0.05$). There was a strong negative correlation between sRANKL and GI ($p < 0.05$). There was a strong negative correlation between BMP-2 and GI ($p < 0.05$). There was a strong negative correlation between BMP-7 and GI and BMP-7 and PI ($p < 0.05$). There was a strong positive correlation between each biochemical parameter ($p < 0.001$).

Discussion

It has been shown that surface topography is an important factor for the successful osteointegration of titanium implants (2,4). Biochemical markers within the PICF provide information about the microenvironment around dental implants. In the present study, PICF levels of sRANKL, OPG, BMP-2 and BMP-7 were analyzed in 47 titanium implants in 47 systemically healthy patients. To our knowledge, however, there are several reports presenting sRANKL and OPG, and this is the first study to evaluate PICF levels of BMP-2 and BMP-7 in humans. It has been suggested that total amounts of cytokines in the gingival crevicular fluid sample per sampling time rather than concentration is a better indicator of relative gingival crevicular fluid constituent activity because concentrations are directly affected by the sample volume (41,42). Accordingly, we based our discussion mainly on the total amount of data, although both total amounts and concentrations were calculated and presented. RANKL is the key molecule of osteoclastogenesis in bone metabolism and in pathologic conditions associated with chronic inflammation (43). OPG is a soluble decoy receptor for RANKL and prevents the interaction with RANK (43). To our knowledge, some studies (30,44–46) sought to identify sRANKL and OPG in the PICF. In two of them (30,44), no significant correlation was found between

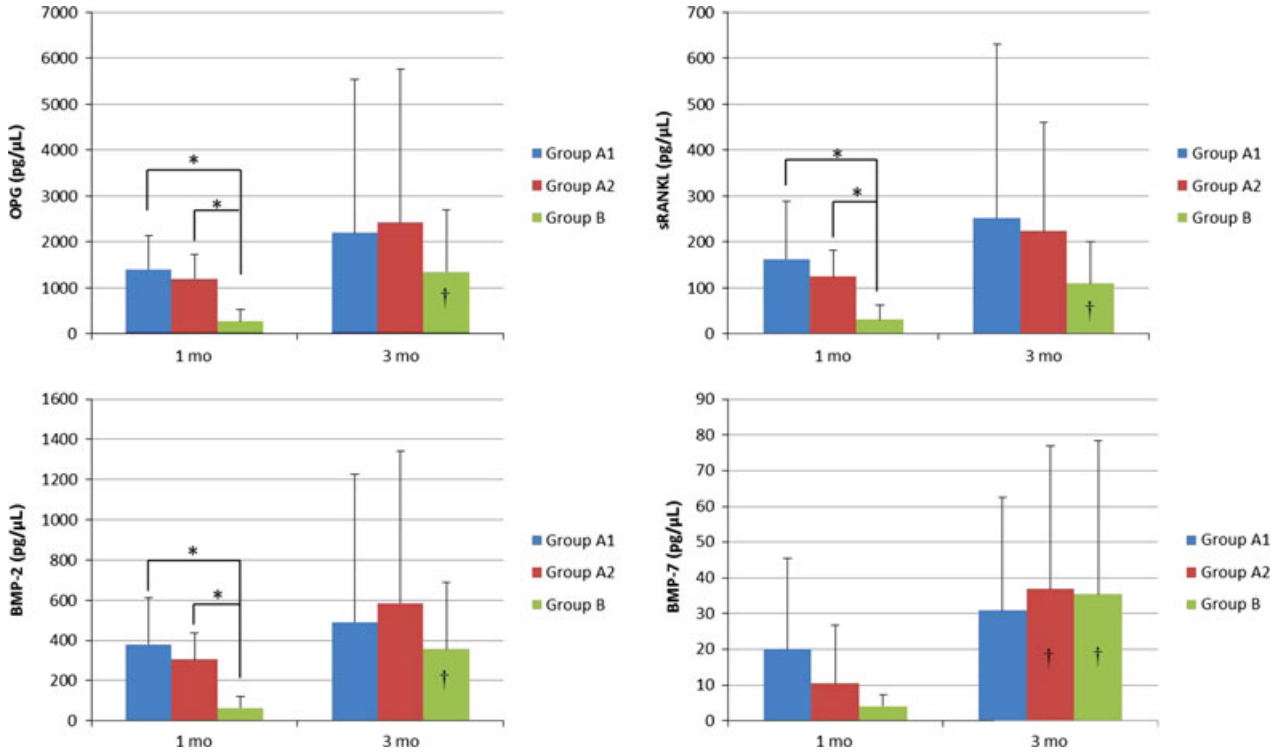

Fig. 4. (A) Concentration of OPG in PICF samples for each group at 1 and 3 mo. (B) Concentration of sRANKL in PICF samples for each group at 1 and 3 mo. (C) Concentration of BMP-2 in PICF samples for each group at 1 and 3 mo. (D) Concentration of BMP-7 in PICF samples for each group at 1 and 3 mo. *Significant difference between groups ($p < 0.05$). †Significant difference compared to the first month. BMP-2/-7, bone morphogenetic protein-2/-7; OPG, osteoprotegerin; PICF, peri-implant crevicular fluid; sRANKL, soluble receptor activator of nuclear factor-κB ligand.

the PICF levels of sRANKL and the clinical parameters measured around the dental implants. Our present findings are in agreement with these studies. Monov *et al*. detected sRANKL in 35% of their samples (30), whereas Arikan *et al*. detected sRANKL in 12% of the samples in their studies (44). We detected sRANKL in all of the samples as in the study of Sarlati *et al*. (47). Our findings can be explained by the periodontal health status of the dental implants. Such a difference may account for different levels of subclinical inflammation among healthy subjects or differences in the sensitivity of various ELISA kits employed in each study (48). The detected sRANKL levels may be associated with subclinical inflammation or bone remodeling in the dental implants in our study. No significant difference was observed between groups in terms of sRANKL level. Güncü *et al*. reported that although the PICF RANKL level in gingivitis/inflamed group was higher than the level of healthy/non-inflamed group, the difference between groups did not reach the statistically significant level (45). Sarlati *et al*. also demonstrated that there were no statistically significant differences in sRANKL concentration between healthy group, peri-implant mucositis and peri-implantitis (47). In our study, there was no clinical inflammation signs (edema, bleeding or change in color) in the placed implants, thus we think that it is difficult to find a difference in RANKL levels of healthy implants considering the findings mentioned above. Nicu *et al*. have shown that no statistically significant differences were observed in PICF RANKL levels when comparing the minimally (turned) and moderately (TiUnite®, from Nobel Biocare, Göteborg, Sweden) rough implant surfaces (46). Lossdörfer *et al*. reported that RANKL mRNA levels expressed by human osteoblast-like cells (MG63) were low and independent of surface microtopography (49). These data might explain why no significant difference was observed between groups in our present study.

In an *in vitro* study, it was demonstrated that OPG production of primary human alveolar osteoblasts and human osteoblast-like MG63 cells were higher in SLActive surfaces compared to SLA surfaces (27). Mamalis *et al*. reported that the gene expression of OPG by human periodontal ligament cells, which have osteoblast-like properties, was significantly upregulated in response to the SLActive surface compared to the SLA surface (3). In our study, the SLActive surface (group A2) and SLA surface (groups A1 and B) implants were similar in terms of OPG levels at all time points. It was demonstrated that a chemically modified SLA surface promoted enhanced bone apposition during the early stages of osseointegration compared with hydrophobic SLA surface after 2

and 4 wk (24). Additionally, it was reported that surface hydrophilicity affects the early phases of osseointegration in an *in vivo* rat model that examined healing during the first 3 wk in bone (25). Perhaps we may have observed significant differences between SLActive and SLA surfaces if we had collected the PICF samples before the first month.

Güncü *et al*. demonstrated that OPG levels in PICF, by using ELISA, were significantly higher in the gingivitis/inflamed group compared to the healthy/non-inflamed group (45). However, Hall *et al*. demonstrated that OPG levels in PICF were similar for the subjects in the healthy and peri-implantitis group by using quantitative polymerase chain reaction (50). The differences in these studies may be due to the method of analysis, PICF sampling procedure and study population. We did not observe any differences in OPG level between groups. All implants were clinically healthy although significant differences were observed in clinical parameters at 1 mo. Arikan *et al*. showed that the total amount of OPG was positively correlated with PICF volume, GI and bleeding on probing (44). They suggested that locally produced OPG correlated with the local signs of inflammation in periodontal and/or peri-implant tissues. However, we observed that there was a strong negative correlation between OPG and GI, and no significant correlation between PICF volume and OPG in this present study. Thus, it can be speculated that the decrease in GI may have a beneficial effect on osteointegration by increasing OPG levels. The population of a study by Arikan *et al*. had an unbalanced distribution of samples into three health categories (44). They investigated 79 healthy implants, four implants with peri-implant mucositis and three implants with peri-implantitis. In our study, all implants were healthy. The differences in correlations between their study and ours may be due to this situation.

Nicu *et al*. reported that no statistically significant differences were observed in PICF OPG levels when comparing the minimally (turned) and moderately (TiUnite®, from Nobel Biocare) rough implant surfaces (46). In our study, OPG levels in PICF were also independent of surface microtopography.

It was reported that the epithelial components around Straumann implants appear to be consistent with epithelial components around teeth and connective tissue fibers are in direct contact with the implant surface (51). The biologic width of Straumann implants is also similar to the biologic width of teeth (51). However, there are no data about the relationship between soft tissue (epithelium and connective tissue) and group B implants in the literature. The differences in probing depth between implant groups may be related to the relationship between soft tissue and implant systems. Gingival thickness and type of healing abutment may also affect this difference in probing depth.

The differences in GI and PI between implant groups at 1 mo were related to individual plaque control ability. Although GI and PI were lower in group B compared to others, the PICF volume was greater in group B. This was an interesting result for us. Mokeem reported that implants with deep probing depth ($\geq$ 3 mm) had a significantly higher mean PICF volume (52). The greater PICF volume in group B may be associated to greater probing depth in group B compared to others or this group included one or more patients with a different inflammatory response possibly because of previous periodontitis. Furthermore, paper strips were inserted into the crevices of the implants until feeling a mild resistance. Thus, paper strips may have absorbed more fluid in deeper implant crevices for 30 s.

The BMPs are a family of growth factors with osteoinductive activity. BMP directly affects osteoblasts by stimulating the differentiation of osteoblast precursor cells into more mature osteoblasts (53). Mamalis *et al*. reported that after 7 d of culture, the gene expression of BMP-7 by human periodontal ligament cells was significantly upregulated in response to the SLActive surface compared to the SLA surface (3). In our study, the SLActive surface (group A2) and SLA surface (groups A1 and B) implants were similar in terms of BMP-7 levels at all time points. Vlacic-Zischke *et al*. reported that a number of genes associated with the TGFβ-BMP signaling cascade (BMP2, BMP6, SP1, CREBBP, RBL2, TBS3, ACVR1 and ZFYVE16) were significantly differentially upregulated with culture on the SLActive surface and BMP2 was shown to have the largest fold change increase in expression, which was subsequently confirmed at the protein level by ELISA (54). Eriksson *et al*. reported that more BMP-2-positive cells were found on hydrophilic discs than on hydrophobic ones after 1 wk (25). We observed that PICF levels of BMP-2 were similar between SLActive surface (group A2) and SLA surface groups (group A1 and group B) at all time points. Takebe *et al*. suggested that macrophage secretion of TGFβ1 and BMPs may affect osteogenesis at endosseous implant surfaces and indicated that endosseous implant surface topography induced changes in macrophage shape that were associated with changes in BMP-2 expression in the J774A.1 mouse macrophage cell line (55). In another study by Takebe *et al*., it was demonstrated that the expression of BMP-2 by macrophages is influenced by hydroxyapatite-coated commercially pure titanium surface topography and physicochemical properties (56). Additionally, it was demonstrated that surface roughness induced BMP2 mRNA expression, especially at the early time point of 24 h. However, the study mentioned above was a cell culture study and may not always mimic clinical conditions; the authors observed differences in BMP2 mRNA expression at 24 h. Under clinical conditions, it was not possible to conduct PICF sampling before complete epithelial attachment healing to avoid interfering with wound healing. In our study, we waited to examine the biochemical markers for at least 1 mo to allow the appropriate shaping of the attachment. Thus, these selected time points

may have prevented the detection of a difference between SLActive and SLA surface groups in terms of BMP-2/-7. In another study that supported our findings, Lang *et al*. reported that healing showed similar characteristics with bone resorptive and appositional events for both SLActive and SLA surfaces between 7 and 42 d (57).

In our study, the chosen PICF sampling times were at 1 and 3 mo after surgery. We waited for complete epithelial healing to prevent the possible effect of inflammatory events on biomarkers in PICF. A fully epithelialized gingival crevice with a well-defined epithelial attachment can be present 1 mo after flap surgery. Furthermore, woven bone is the first bone tissue that is formed in osseointegration and its formation clearly dominates the healing area within the first 4–6 wk after surgery (58). Thus, the first month after surgery was decided to be the first time point for measurement. The conventional protocol of implant loading recommended a healing time of 3–6 mo (59). In our study, implant loading was performed 3 mo after surgery and this time period was decided to be the second PICF sampling time. Prosthetic appointments were arranged for the patients after completing PICF samplings.

Implants were not manufactured by a single company and the population presented a balanced distribution of samples into three implant surface categories in our study. We used two implant systems, which have different macro and micro designs. Implant macro design includes thread pattern (shape), body shape and thread design (thread geometry, face angle, thread pitch, thread depth, thickness and thread helix angle) (60). These properties are important factors in the number of different forces to the bone–implant interface, implant stability and extent of the BIC area (60). Group A1 and A2 implants were tissue-level implants consisting of a smooth neck section of 1.8 mm that allows flexible coronoapical implant placement in combination with trans- or subgingival healing. However, group B implants were bone-level implants with microthreads in the coronal portion. The addition of microthreads to an implant might provide a potential positive contribution on BIC, as well as, on the preservation of marginal bone. Nonetheless, this remains to be determined (61). The differences in macro design between two implant systems may be the reason for different findings in evaluated parameters.

Chemical and physical surface properties such as ionic composition, hydrophilicity and roughness are parameters that play a major role in implant–tissue interaction (62). Although significant differences between SLA and SLActive are well documented in the literature, our study did not demonstrate a significant difference between SLActive and SLA implants in terms of bone metabolism-related biomarkers.

To our knowledge, however, there are several reports presenting sRANKL and OPG, this is the first study to evaluate PICF levels of BMP-2 and BMP-7 during the osseointegration of dental implants in humans. According to the relationships between clinical and biochemical parameters, the levels of these cytokines in PICF during early healing of implants reflects the degree of peri-implant inflammation, rather than differences in the implant surfaces.

This was a pilot study and it was not possible to calculate a power analysis to determine the number of implants in each group. In view of our findings, further well-designed studies with sample size needed for $\geq 80\%$ statistical power could be conducted, and different time points (earlier time points) should be chosen for PICF sampling to evaluate the relationship between microtopographic/chemical characteristics of the implants and bone remodeling parameters. It seems more likely that changes in OPG/sRANKL and BMP-2 and -7 during early osseointegration are short term and transient. Immunohistochemical animal studies could be conducted to detect these biomarkers precisely. Moreover, whether clinical parameters affect the bone remodeling process should be further clarified for other implant systems.

Acknowledgements

This study was supported by the Research Project Coordination of Selçuk University (project no. 08401093). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article. The authors are grateful to Bulent Ozkan (Department of Biostatistics, Izmir Kâtip Çelebi University) for his help in statistical analyses.

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