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Emerging roles of Interleukin-34 together with receptor activator of nuclear factor-kB ligand and osteoprotegerin levels in periodontal disease

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ABSTRACT

Objective: The dependence between gingival crevicular fluid (GCF) of Interleukin-34 (IL-34) level and Receptor activator of nuclear factor -kB ligand/ osteoprotegerin (RANKL/OPG) ratio in the severity of periodontitis might reveal an unknown pathway of diseases with bone destruction. There is no study about the evaluation of IL-34 levels together with GCF RANKL and OPG levels in periodontitis patients before and after non-surgical periodontal treatment (NSPT). The objectives of this research were to investigate changes in the levels and relative ratios of IL-34, OPG, and RANKL in the GCF of patients with periodontitis before and after NSPT.

Materials and Methods: 20 healthy participants (CTRL), 20 patients with stage 3-grade B periodontitis and 20 with stage 3-grade C periodontitis were recruited. GCF and clinical periodontal recordings were investigated at the baseline and 6 weeks after NSPT. Enzyme-linked immunosorbent assay (ELISA) were used for quantifying of GCF IL-34, RANKL and OPG levels and their relative ratios were calculated.

Results: Greater values for GCF IL-34 and RANKL levels were found in the both of periodontitis groups than in CTRL group at baseline, whereas GCF OPG levels were statistically lower at baseline ($P < 0.05$). GCF IL-34 and RANKL levels decreased in the 6th week after NSPT in the both periodontitis groups, while the concentration OPG levels statistically increased ($P < 0.05$). Significantly positive correlations among the IL-34 with RANKL, sampled-site clinical attachment level (CAL), and gingival index (GI), whereas negative correlation with OPG were reported ($P < 0.05$).

Conclusions: GCF IL-34 levels was high in patients with periodontitis and decreased after NSPT and its levels showed positive correlations with RANKL/OPG ratio levels CAL and GI. Determining of IL-34 levels together with RANKL/OPG ratio in GCF may therefore be valuable in detecting high risk individuals with periodontitis patients.

1. Introduction

Periodontitis is an inflammatory condition that leads to damage of supporting periodontal tissues around the teeth. An important aspect of tissue damage is loss of alveolar bone that ultimately results in the tooth loss [1]. The osteoclasts are multinucleated cells that are crucial in bone development and regeneration. Bone resorption mediated by the osteoclasts might be a potential curative target to treat erosive bone diseases, such as periodontitis [2].

Receptor activator of nuclear factor -kB (RANK) and its ligand (RANKL) and macrophage colony-stimulating factor (M−CSF, also known as CSF-1) are the essential cytokines in differentiation of osteoclasts. RANK and RANKL interaction activates formation of osteoclasts in the presence of CSF-1 [3]. This leads to differentiation of osteoclast progenitors and activation of the mature osteoclasts that mediates bone resorption [4]. Activation of osteoclast that triggers bone destruction is regulated by RANKL, RANK and osteoprotegerin (OPG). The effects of RANKL is modified by OPG by blocking RANKL/RANK interaction [5]. RANKL and OPG can be discovered in gingival tissue[6], gingival crevicular fluid (GCF) [5], saliva [7], serum [8]. Studies [5–7] demonstrated significantly rising RANKL/OPG ratio in periodontitis patients compared with healthy individuals. Any observe the alterations in the

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RANKL/OPG ratio might ensure reliable information about the state of periodontal disease [5,9]. CSF-1 plays essential role in the progress of macrophage lineage from hemopoietic stem cells. It induces as a primary regulator the survival, differentiation and proliferation of monocytes, macrophages, myeloid and osteoclast progenitor cells [2]. CSF-1 has been associated with inflammatory diseases, such as: inflammatory bowel disease [10], rheumatoid arthritis [11], and cancer [12]. It has also been attributed to periodontal disease [13,14]; the blockage of the CSF-1 receptor (CSF-1R) leads to reduced alveolar bone loss [2]. The lack of CSF-1R results in osteopetrosis, diminished mononuclear phagocyte and reproductive defect indicating the function of CSF-1 is through CSF-1R [15]. Furthermore, gene expression of CSF-1 polymorphism is related with aggressive periodontitis patients [16], and CSF-1 deficiency is useful in the inhibition of inflammation in experimental periodontitis [14].

A novel cytokine interleukin 34 (IL-34) is a second possible functional ligand of CSF-1R. IL-34 is secreted in different tissues including: heart, brain, lung, liver, kidney, thymus, testes, ovary, small intestine, prostate, colon and spleen [17]. IL-34 shares vital functions of CSF-1, and manage myeloid cell survival, differentiation and proliferation. It is produced by synovial fluid, gingival fibroblasts [1] and human adipose tissue [17], and is controlled by the transcription factor RANK and activation of c-Jun N-terminal kinase (CJNK) [18]. Additionally, the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1beta (IL-1β) arrange IL-34 release from gingival fibroblasts, by a mechanism including nuclear factor -kB (NF-kB) and mitogen activated protein kinase (MAPK) [1].

Several studies [17–21] evaluated the role of IL-34 in the pathogenesis of chronic periodontitis. The concept of targeting CSF-1/CSF-1R and RANK signaling pathway may need to be considered in the regulation of IL-34. The interaction between IL-34 levels and RANKL/OPG ratio in GCF might disclose a new bone devastation pathway in periodontitis. To the best of our knowledge, any of these studies reported the relationship between GCF IL-34 level and GCF RANKL/OPG ratio in periodontitis before and after non-surgical periodontal therapy (NSPT). The authors theorize that elevated levels of IL-34 are associated with periodontal diseases and NSPT might bring favourable effect on IL-34 levels. The objectives of this study were to 1) explore the effect of NSPT on GCF IL-34 levels in periodontitis patients with stage 3-grade B and with stage 3-grade C, 2) analyze the relationship among IL-34, RANKL, OPG, and RANKL/OPG ratio levels in GCF on periodontally healthy subjects and patients with different periodontitis and 3) to correlate between biochemical markers and clinical recordings.

2. Materıal and methods

2.1. Study population and study design

Participants of present research were selected from volunteers who were planned to experience either dental treatment or dental check at the Department of Periodontology, Faculty of Dentistry, Bülent Ecevit University, Zonguldak, Turkey, between January 2015 and February 2016. The study design was perused and approved by the Ethics Committee of the Faculty of Medicine, Bülent Ecevit University, Zonguldak, Turkey, accordance with the Helsinki Declaration of 1975, as revisited in 2013. (Protocol ID: 2014-106-03/06, Clinical Trial. org-NCT04223869). The study consisted of 60 participants: 1) 20 healthy controls (CTRL), 2) 20 patients with stage 3-grade B peridontitis 3) 20 patients with stage 3-grade C periodontitis. The purpose of the study was clarified to each participant and a consent form was obtained from every participant before starting the study.

At the beginning of the study, the periodontal disease status was diagnosed by a specialist (ŞBD) in periodontology accordance with clinical and radiographic guidelines from the 1999 (Armitage, 1999) classification [22]. However, since the classification of the periodontal diseases revised, study groups were later adapted according to the consensus report of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions [23].

The stage 3-grade B periodontitis patients exhibited clinical attachment loss (CAL) ≥5 mm and pocket probing depth (PPD) ≥6 mm on at least two non-adjacent teeth, bone loss involving the middle or apical third of the root radiographically, moderate ridge defect and ≥30% of the teeth involved [24]. Additionally, periodontitis grade of patients has been determined by radiographic bone loss/age [25]. Radiographic bone loss was determined from the tooth showing the most severe bone loss as a percentage of root length. Grade B was evaluated the values of bone loss/age were 0.25–1.00. The inclusion criteria for stage 3-grade C periodontitis patients were interdental CAL ≥ 5 mm and PD ≥ 6 mm on at least 6 teeth and at least three of these six teeth were not molars or incisors. These patients showed severe periodontal tissue destruction and loss of periodontal support that were inconsistent with age and plaque levels. They were under 35 years of age. Grade C was assessed the values of % bone loss/ age were >1.0. The control group presented PPD ≤ 3 mm with absence of alveolar bone and without clinical attachment loss and individuals having <%10 BOP [26]. There were at least 20 teeth in all participants and all of them were systemically healthy.

Exclusion criteria included diagnoses of unrelated systematic condition: 1) lactation; 2) pregnancy; 3) current and ex-smoking habits; 4) undergone NSPT and prescription of antibiotics or non-steroidal anti-inflammatory medication within the previous 6 months or surgical periodontal treatment within the preceding year; 5) postmenopause, 6) systemic conditions such as; diabetes mellitus, cancer, cardiovascular and respiratory diseases, or immunologic disorders, that might cause progress of periodontal disease

3. Clinical measurements and periodontal treatment

Periodontal status of participants was assessed by evaluating plaque indices (PI) [27], gingival indices (GI) [28] PPD, clinical attachment level (CAL) and BOP [29]. The periodontal bone loss was determined by taking full-mouth periapical radiographs. A single calibrated examiner (ŞBD), who was blinded to the whole study, recorded clinical parameters in millimeters by using a periodontal probe (Hu-Friedy, Chicago, IL, USA) from six sites of per tooth (mesio-buccal, disto-buccal, mid-buccal, mesiolingual, disto-lingual, and mid-lingual).

Before the actual readings, intra-examiner calibration was conducted. To be able to standardize the investigator (ŞBD) ten individuals were randomly selected and used. All clinical measurements were analyzed by the investigator at two specific times set 48 h apart. If two reading sets were >90% identical in terms of millimeter level, the calibration of the clinician was considered as completed [30]. All patients offered instructions related to everyday plaque control. Treatment of patients with periodontitis included a rigorous hygiene phase and full-mouth scaling and root planning (SRP), via use of manual scalers, curettes (Hu-Friedy, Chicago, IL, USA) and ultrasonic instruments (Cavitron select, DENTSPLY, York, PA). NSPT was performed twice a week for two weeks independently from adjunctive therapy, and each appointment lasted around 45–60 min. Clinical data were obtained from the both of periodontitis groups again after six weeks SRP.

3.1. Site selection and GCF collection

GCF samples were collected from two sites and mesiobuccal or distobuccal sites of single-rooted teeth from each participant in the all groups. The samples were taken the day after patients were clinically evaluated to prevent contamination with blood related with the probing of inflamed fields. The collection of samples were performed at baseline from whole groups and 6 weeks after NSPT from patients with periodontitis. The sites, PPD < 3 mm and absence of CAL and BOP were choosen for CTRL group samples. For periodontitis groups, the samples were collected from sites with PPD ≥ 6 mm, CAL ≥ 5 mm and 30% bone loss. The sample areas were insulated with cotton rolls, saliva

contamination was prevented, all supragingival plaque was eliminated by using curette and it was slightly airdried. The paper strips were placed into the groove until resistance was felt and then permitted to remain for 30 s [31]. The GCF amount on the paper was measured by weighing the collected liquid. The strips were placed into closed and labelled eppendorf tubes. The liquid was weighed again to prevent vaporization immediately after collection [32]. The samples consisting of saliva and blood were discarded from the study. Two available for use samples from per individual were merged to form one sample, and instantly insert in a singular Eppendorf tube, and freezed at -80°C until aftertime evaluation.

3.2. Biochemical analysis

On the same day that the assay was scheduled, 300 μl of phosphate-buffered saline (pH 7.4) were put into the tubes with sample strips and subjected to vortexing and homogenization for 60 s followed by centrifugation at 3.000 × *g* for a quarter of an hour at a temperature of 4 °C. Collection of supernatants was undertaken. Kits readily available on the market were employed to apply the sandwich enzyme-linked immunosorbent (ELISA) assay to examine the total quantities of IL-34 (R&D Systems, Inc. 614 McKinley Place NE, Minneapolis, MN 55413), OPG (Boster Biological Technology Co., Ltd., Pleasanton, CA) and RANKL (Biotek Instruments Inc., Winooski, VT) present in the samples. In keeping with the manufacturer's recommendation, the assay of every sample and standard was conducted twice. The measurement of color density was done at 450 nm and the standard curves available in every assay kit were employed to calculate the results.

Picograms unit was for all readings (IL-34, RANKL, and OPG). Measurements were performed using ELISA plate reader Bio-Tek Synergy HT*. Intra-assay CV and interassay CV were 6.2% and 7.4%, respectively with sensitivity <10 pg/mL for RANKL, intra-assay CV and inter-assay CV were 7.9% and 8.9%, respectively with sensitivity <5 pg/mL for OPG, while intra-assay CV and inter-assay CV were 7.3% and 6%, respectively with sensitivity <3.06 pg/mL for IL-34. Intensity of colour was recgistered at 450 nm, and the results were defined by utilizing the suggested curves that were ensured with the assay kits. The total amount of IL-34 (pg), RANKL (pg), and OPG (pg) divided to the volume of GCF (μL) for determining the concentrations of GCF IL/34, RANKL and OPG. The readings for concentrations are offered as picograms per microliter.

3.3. Statistical analysis

Primary outcome variable (change in IL-34 levels) was used for determining the power analysis of the study. But there were no exact information, therefore calculation of sample size could not be performed. Thus, we based our estimates on the preliminary study (unpublished) of authours, that including 10 patients in every group. It was judged that a sample number of 17 individuals, in every group would enable a type II error level of $\beta = 0.20$ (80% power) and a type I error level of $\alpha = 0.05$ (5% odds). The study involved 20 patients in per group to eliminate the account for possible dropouts. Since sample size calculation could not be performed at starting the study, a retrospective calculation was carried out later. A posteriori power calculation yielded a power of 86% to recognize differences in outcomes before and after treatment.

The data were tested for typically spread applying the Shapiro–Wilk test. The comparision of the biochemical and clinical parameters were tested by using Kruskal–Wallis nonparametric test, followed by post-hoc group comparisons with the Bonferroni-adjusted Mann–Whitney *U* test. The comparision of baseline values with values gathered after therapy were determined by carrying Wilcoxon signed-rank test (paired observations). BOP percentage between groups was compared by applying χ^2 analysis. The interactions between IL-34, OPG, RANKL with the CAL and GI detected by using Spearman's rank correlation test. The SPSS 19.0 software program (SPSS Inc., version 19.0, Chicago, IL, USA) was utilized in the analysis process of the data. The value of $P < 0.05$ was considered as statistically significant.

4. Results

4.1. Clinical findings

Stage 3-grade C patients' s age (31.50, min–max; [22.00–35.00]) were statistically lower than from stage 3-grade B group (45.50, min–max; (38.00–56.00)) and CTRL group (41.50, min–max; (25.00–55.00) ($P < 0.05$), but there were no diffrence between stage 3-grade B group and CTRL group. No notable disparity of gender was obserdved among the groups (9 females, 11 males for CTRL group; 10 females, 10 males for stage 3-grade B group; 9 females, 11 males for stage 3-grade C group) ($P > 0.05$). Clinical findings are summarized Table 1. Full-mouth and sampled sites PPD, CAL, PI, GI, BOP were statistically significantly higher in the both of periodontitis groups than CTRL group ($P < 0.05$). No differences was found between stage 3-grade B group and stage 3-grade C group in the whole mouth and at sampled sites clinical parameters ($P > 0.05$) Clinical parameters in the full-mouth and sampled sites were statistically decreased in the periodontitis groups after NSPT ($P < 0.05$). GCF volumes were significantly greater in the both of the periodontitis groups than the CTRL group ($P < 0.05$). GCF volumes were not showed significant differences between the periodontitis groups ($P > 0.05$). GCF volumes significantly decreased after NSPT in the both of periodontitis groups ($P < 0.05$) (Table 1).

4.2. Biochemical findings

Total amounts and concentration levels of GCF IL-34 are summarized in Fig. 1. The total amount and concentration GCF IL-34 levels were statistically significantly higher in the both of periodontitis group than CTRL group at baseline ($P < 0.05$). Total amounts and concentration GCF IL-34 levels statistically significantly reduced after NSPT in the both of periodontitis group ($P < 0.05$). Additionally, GCF total and concentration IL-34 levels were found greater in the stage 3-grade C group than in the stage 3-grade B group at baseline and after NSPT ($P < 0.05$).

Total amounts and concentraion RANKL levels in GCF are denoted in Fig. 2. The total amount RANKL levels in GCF were found statistically greater in the both of periodontitis group than CTRL group and it's levels dropped after NSPT in the both of periodontitis groups ($P < 0.05$). Although, concentration levels of RANKL were statistically significantly greater in the both of periodontitis group than CTRL group ($P < 0.05$), the concentration RANKL level in patients with stage 3-grade B had higher after NSPT compared to with baseline level ($P < 0.05$), but there were no difference in stage 3-grade C group after NSPT compared to with baseline level ($P > 0.05$).

Fig. 3 presented the total amounts and concentration GCF OPG levels. The total amount and concentration levels of OPG were significantly lower in the both of periodontitis groups than CTRL group at baseline ($P < 0.05$). The concentration levels of OPG in the both periodontitis groups statistically increased following NSPT ($P < 0.05$). But there were no differences in the total GCF OPG levels after NSPT in the both of periodontitis group compared to baseline values ($P > 0.05$). Additionally, stage 3-grade C group had lower total and concentration OPG level in GCF than in stage 3-grade B group after NSPT.

RANKL/OPG ratio levels in GCF were significantly higher in the both of periodontitis groups than CTRL group at baseline ($P < 0.05$). This ratio statistically decreased in periodontitis groups after NSPT ($P < 0.05$). Additionally, RANKL/OPG ratio was greater in stage 3-grade C group compared to with stage 3-grade B group at baseline and after NSPT ($P < 0.05$) (Fig. 4).

4.3. Correlations

Correlation coefficients are presented in Table 2. The correlation

Table 1
Clinical Parameters Before and After Treatment (full-mouth and sampled-site periodontal examination) in Study Groups.

Groups	PPD	CAL	PI	GI	BOP	GCF Volume
CTRL						
Full-mouth	2.40 (1.65–2.98)	2.43 (1.65–3.64)	0.09 (0.02–0.21)	0.17 (0.01–0.38)	7.15 (4.15–9.42)	0.28 (0.23–0.35)
Sampled sites	2 (1–2)	2 (1–2)	0 (0–1)	0 (0)	0 (0)	
Periodontitis						
Stage 3-grade B						
Full-Mouth						
Before Treatment	4.92§ (3.18–5.88)	5.45§ (3.98–6.76)	2.15 § (1.65–2.89)	2.30§ (1.59–2.72)	85.20 § (56.40–100)	
After Treatment	2.78¶ (2.08–3.32)	3.32 ¶ (2.18–4.46)	0.46¶ (0.12–1.21)	0.79 (¶ 0.13–1.34)	20.49¶ (11.46–32.81)	
Sampled Sites						
Before Treatment	6§ (5–8)	6§ (5–8)	2§ (2–3)	2§ (2–3)	100§ (100)	0.84§ (0.75–0.95)
After Teatment	2¶ (2–3)	3¶ (3–4)	0¶ (0–1)	0¶ (0–1)0¶ (0–1)	0¶ (0)	0.54¶ (0.48–0.63)
Periodontitis				,		
Stage 3-grade C						
Full-Mouth						
Before Treatment	4.13§ (3.35–6.97)	4.53§ (3.42–7.83)	2.21§ (1.19–3.00)	2.20§ (1.23–3.00)	71.57§ (40.97–100)	
After Treatment	3.58¶ (2.85–5.64)	4.21¶ (3.33–7.92)	1.26¶ (0.35–2.71)	1.55¶ (0.64–2.54)	14.47¶ (6.80–31.76)	
Sampled Sites						
Before Treatment	7.5§ (6–9)	7.5§ (6–9)	2§ (2–3)	2§ (2–3)	100§ (100)	0.89§ (0.77–0.96)
After Teatment	4¶ (3–6)	5¶ (4–7)	0¶ (0–1)	0¶ (0–1)	20¶ (0–100)	0.57¶ (0.48–0.69)

Data are expressed as the median (min–max). Kruskal-Wallis/Bonferroni-adjusted Mann–Whitney *U* (unpaired observations). *Wilcoxon* signed-rank test (paired observation).

§ Statistically significant difference from CTRL ($P < 0.05$).

¶ Statistically significant difference from before treatment ($P < 0.05$).

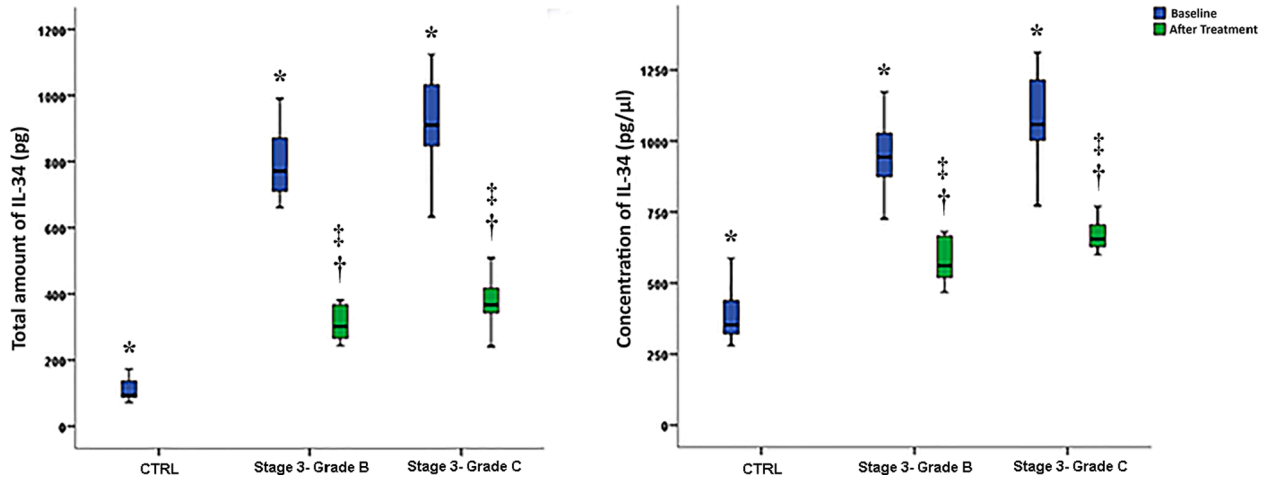

Fig. 1. Total amount and concentration levels of IL-34 in GCF. *Statistically significant difference among groups (Kruskal-Wallis/Bonferroni-adjusted Mann–Whitney *U*). †Statistically significant difference between groups (Mann–Whitney *U*). ‡Statistically significant difference from baseline (Wilcoxon signed-rank test) Data are presented as box and whisker plots. The median value is indicated by the line within the box plot. The box extends from the 25th to the 75th percentiles. Whiskers extend to show the highest and lowest values.

analyses were performed by using the total amounts for the elimination of GCF volume. A significantly positive correlation was demonstrated among the total of IL-34 level, RANKL, CAL and GI ($P < 0.05$), whereas IL-34 levels was negative correlated with OPG. RANKL levels with also negative correlated with OPG and OPG levels with negative correlated with sampled site CAL and GI ($p < 0.05$) when every group were examined together. IL-34 levels also positive correlated with RANKL/OPG ratio when all groups were examined together ($P < 0.05$).

5. Discussion

The clinical speciality of periodontitis is alveolar bone loss. The sensitive balance between osteoblasts and osteoclasts controls bone metabolism [33] and cytokines have essential role in maintaining bone homeostasis. CSF-1 and RANKL play a central role in osteoclastogenesis [1]. And, the functional similarity of IL-34 and CSF-1 is reported by their role in osteoclastogenesis [2]. Additionally, RANKL and OPG have been demonstrated to play role in the regulation of bone metabolism and the change in RANKL / OPG ratio or OPG / RANKL ratio better reflects the amount of bone resorption or turnover compared to the change in the level of one of these factors alone [33,34]. Therefore this study is purposed to evalaute the changes in GCF IL-34 levels after NSPT and at the same time, to investigate the interactions among RANKL, OPG, RANKL/OPG ratio and IL-34 levels in GCF in patients with periodontitis.

On the molecular level, interaction between RANKL and OPG regulates bone destruction [5]. The local production of increased RANKL or diminished OPG can induce bone resorption at several region of the human skeleton. Contrary, diminished RANKL or increased OPG production could lead to increased bone formation and result in osteopetrotic conditions [9]. The present study is based total amount of a components in the GCF rather than its concentration because the total amount of biomarkers are more suitable when investigating the interactions between the GCF components and periodontal diseases [33,35]. In the present study, the total RANKL levels in GCF were higher in the both of periodontitis group than CTRL group and this levels were decreased after NSPT. The total amount of OPG were statistically lower

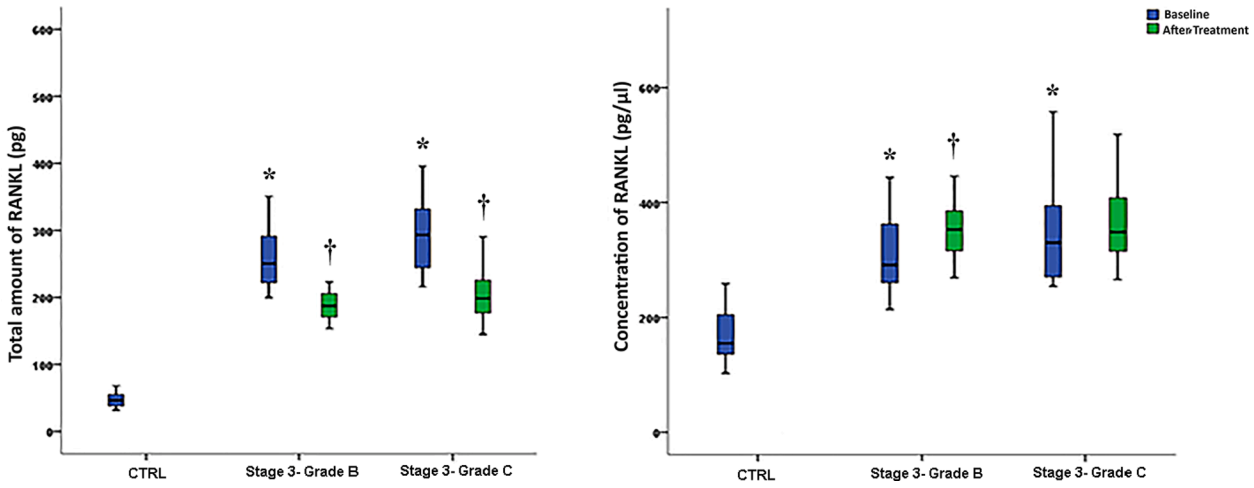

Fig. 2. Total amount and concentration levels of RANKL in GCF. *Statistically significant difference from CTRL (Kruskal-Wallis/Bonferroni-adjusted Mann–Whitney *U*). †Statistically significant difference from baseline (Wilcoxon signed-rank test) Data are presented as box and whisker plots. The median value is indicated by the line within the box plot. The box extends from the 25th to the 75th percentiles. Whiskers extend to show the highest and lowest values.

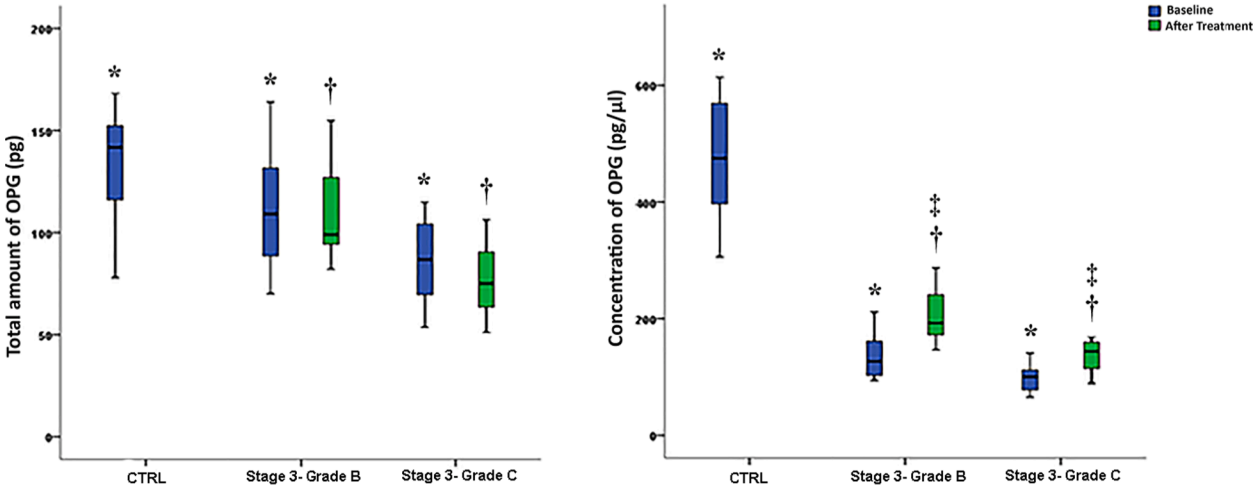

Fig. 3. Total amount and concentration levels of OPG in GCF. *Statistically significant difference among groups (Kruskal-Wallis/Bonferroni-adjusted Mann–Whitney *U*). †Statistically significant difference between groups (Mann–Whitney *U*). ‡Statistically significant difference from baseline (Wilcoxon signed-rank test) Data are presented as box and whisker plots. The median value is indicated by the line within the box plot. The box extends from the 25th to the 75th percentiles. Whiskers extend to show the highest and lowest values.

in the both of periodontitis groups than CTRL group at baseline. But there were no diffrences in the total GCF OPG levels after NSPT in patients with periodontitis. A higher RANKL/OPG ratio is addressed symptomatic of the presence of untreated periodontitis. It is proposed that this ratio might potentially serve as a molecular diagnostic mediator for the disease [5]. Bostanci et al. [36] investigated an establish role of GCF RANKL and OPG levels in the pathogenesis of periodontitis. They reported unchanged RANKL/OPG ratio after NSPT and their result did not associate with clinical parameters, despite the improved clinical outcome in the patients with chronic periodontitis and aggressive periodontitis. The other hand, Branco-de-Almeida et al. [37] showed positive correlation between RANK/OPG ratio and sampled sites PPD and CAL parameters in localized aggressive perioodontitis patients. Balli et al. [33] demonstrated higher RANKL/OPG ratio in patients with periodontitis before therapy and the RANKL/OPG ratio diminished after NSPT, but this decrease was not significant in their study. Paralell with Balli's study [33] the present study showed higher RANKL/OPG ratio levels in GCF in the both of periodontitis groups than CTRL group at baseline and this ratio significantly decreased together with a significant improvement in clinical parameters in periodontitis groups after NSPT. There were conflict results [33,38,39] in the literature about the effects of NSPT on RANKL/OPG ratios and the prognostic significance of RANKL/OPG. According to present study results, it can be concluded that a clinically achieved therapy result may presumably outcome in decrease of the RANKL/OPG ratio.

IL-34 and CSF-1 share the same receptor (CSF-1R), The targeting CSF-1 merely is not adequate to inhibit the effect via CSF-1R. IL-34 in combination with RANKL caused osteoclasts differentiation and bone resorption in CSF-1 deficient mouse bone marrow cells, and bone mass decreased when IL-34 is administered systemically [2]. IL-34 plays a crucial role in RANKL-induced osteoclastogenesis; the osteoclasts differentiation is mediated by the same way with CSF-1 [2]. However, neither RANKL nor IL-34 solely can cause osteoclast formation suggesting IL-34 is required but not enough [2]. Recently, the role of IL-34 in periodontitis is investigated. Martinez et al. [18] explored the existence of CSF-1 and IL-34 in whole saliva in relation to periodontal disease. They showed lower IL-34 levels and higher CSF-1 levels in saliva in patients with periodontitis and reported negative correlations between saliva IL-34 levels and clinical parameters. Clark et al. [40] examined the release of CSF-1 and IL-34 in gingival tissue and gingival fibroblasts

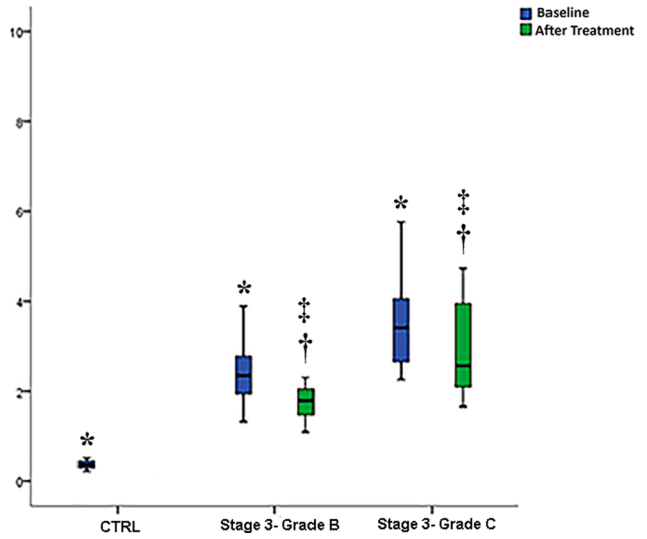

Fig. 4. The relative ratios of RANKL/OPG in GCF. *Statistically significant difference among groups (Kruskal-Wallis/Bonferroni-adjusted Mann–Whitney *U*). †Statistically significant difference between groups (Mann–Whitney *U*). ‡Statistically significant difference from baseline (Wilcoxon signed-rank test) Data are presented as box and whisker plots. The median value is indicated by the line within the box plot. The box extends from the 25th to the 75th percentiles. Whiskers extend to show the highest and lowest values.

(GF) from patients with periodontitis. They displayed increased CSF-1 levels in gingival tissue from periodontitis patients compared to controls, whereas IL-34 expression was similar, their finding was in line with previous reports of Martinez's study. [18] Additionally, CSF-1 and IL-34 levels were increased by pro- inflammatory stimuli and these levels were comparable levels in periodontitis patients and controls [40]. Contrary to these studies results [18,40] Guruprasad et al. evaluated GCF and plasma IL-34 levels in periodontal health and periodontal disease with presence or absence of obesity [17] and in patients with periodontal disease with and without diabetes mellitus [20]. They reported that obese and diabetic individuals with periodontitis had higher GCF and plasma IL-34 levels than non-obese and non diabetic individulas with healthy periodontium in their studies. In their other study [19] they examined the influence of smoking on IL-34 levels in GCF and plasma in periodontal health and and disease. Guruprasad et al. [19] demonstrated highest plasma and GCF IL-34 levels in smokers with chronic periodontitis in this study. There is only one study [21] about the effect of periodontal therapy on GCF IL-34 level in periodontitis. Guruprasad et al. [21] evaluated the effect of NSPT on the GCF and plasma IL-34 levels. They reported GCF and plasma IL-34 concentration levels dropped after NSPT. As a final study, Batra et al. [41] examined GCF IL-34 levels in patients with chronic and aggressive periodontitis. They reported that levels of IL-34 in GCF showed a rising level from healthy followed by chronic periodontitis group and aggressive periodontitis group and this levels positive correlated with PI, GI, PPD and CAL. Similiar to these studies [17,19–21,41] the present study found significantly greater IL-34 levels in GCF in the those of periodontitis groups than CTRL group. Additionally, total and concentration GCF IL-34 levels were greater in patients with stage 3-grade C than stage 3-grade B patients. This might recommend an eventual cellular hyperactivity that may support periodontal damage in grade C periodontitis and this may cause more destruction of periodontal tissues in stage 3-grade C than in stage 3-grade B. Ertugrul et al. [42] reported that aggressive periodontitis patients showed greater GCF cytokine levels than patients with chronic periodontitis and they explained these result as follows that this increase was owing to gene polymorphisms cod in for the produce of inflammatory markers. As a different from the above studies, the present study evaluated effects of NSPT on GCF IL-34 levels in patients with stage 3-grade B and stage 3-grade C and compared to these results with GCF RANKL and OPG levels. Total amounts and concentration GCF IL-34 levels significantly decreased after NSPT and GCF IL-34 levels positive correlated with GI, CAL and RANKL/OPG ratio in GCF in our study. Total IL-34 GCF levels also showed statistically a positive correlation with RANKL GCF levels, while a negative correlation with OPG GCF levels when all groups were examined together. The results demonstrate that periodontal treatment can notably lower IL-34 levels and RANKL/OPG ratio in GCF, indicating a significant difference in all clinical periodontal parameters among both periodontitis groups after treatment. Hence, it can be argued that IL-34 exhibits a pro-inflammatory property and plays pivotal role in the development of periodontitis. Considering the GCF IL-34 results of the present, IL-34 may be a valuable detection marker in individuals at high risk of developing periodontal disease and may be a potential indicator for better efficient treatment. Additionally, the present study GCF IL-34 levels in patients with periodontitis were considered by comparing with RANKL and OPG levels, which are known to play an important role in bone resorption. The production of IL-34 could be provoked by raising RANKL/OPG ratio in individuals suffering from periodontitis. It is probable to suggest that IL-34 may be take a role in alveolar bone loss in patients with periodontitis. However, alternative approaches are necessary to support the this suggestion. In particular, the level of IL-34 in GCF should be evaluated by comparing it with GCF osteoclastgenic cytokine levels (such as; IL-1β, IL-17A and TNF-α) to clarify of the role of IL-34 in periodontitis pathogenesis. The other limitation of present study is, it did not assess CSF-1 and CSF-1R levels in GCF. The potential association of among IL-34, CSF-1, CSF-1R, TNF-α and RANKL deserves further investigation. Additionally, short term evaluation (at 6th week) of results after NSPT is not sufficient to assess the effects on the alveolar bone. In this regard, longer follow-up studies are needed.

6. CrediT authorship contribution statement

The study was mainly planned by Assoc Prof. Şeyma Bozkurt Doğan. Dr. Doğan was responsible for the clinical measurement recordings and gingival crevicular fluid sampling. The patient selection of the study was done by Assoc Prof. Figen Öngöz Dede. ELISA analyses were performed by Assoc Prof. Erdim Sertoğlu. Statistical analyses were made by Assoc

Table 2
Spearman's Rank Correlation (r) Among Groups With Respect to IL-34, RANKL, OPG and Sampled-Site CAL and GI.

	IL-34 to RANKL	IL-34 to OPG	RANKL to OPG	IL-34 to CAL	RANKL to CAL	OPG to CAL	IL-34 to GI	RANKL to GI	OPG to GI	IL-34 to RANKL/OPG
CTRL	−0.113	−0.329	0.083	−0.145	−0.184	−0.044	0.228	−0.236	0.033	−0.036
Periodontitis Stage 3-grade B	−0.200	−0.274	0.041	0.246	−0.116	−0.089	0.021	0.143	0.099	0.200
Periodontitis Stage 3-grade C	0.066	−0.356	0.003	0.383	−0.152	−0.080	0.063	−0.022	0.196	0.212
All groups	0.666**	−0.627**	−0.440**	0.694**	0.582**	−0.402**	0.683**	0.590**	−0.355**	0.779**

** Statistically significant ($P < 0.05$).

Prof. Umut Ballı. Assoc Prof. Seyma Bozkurt Doğan has contributed to writing of this manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This study was funded by the researchers.

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