ORIGINAL ARTICLE

JOURNAL OF
Periodontology

Baseline interleukin-10, CD163 and tumor necrosis factor-like weak inducer of apoptosis gingival tissue levels in relation to clinical periodontal treatment outcomes: A 12-week follow-up study

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Abstract

Background: The aim of this study was to examine the relationship between healing response after non-surgical periodontal treatment and baseline gingival tissue levels of M2 macrophage activation-related proteins CD163, interleukin (IL)-10, interferon (IFN)-γ, and tumor necrosis factor-like weak inducer of apoptosis (TWEAK), and the CD163/TWEAK ratio.

Methods: Eighty-eight gingival tissue samples from 44 Stage III/IV, Grade C periodontitis patients (18 smokers) and 41 tissue samples from 41 periodontally healthy participants (18 smokers) were evaluated. Clinical parameters were recorded in periodontally healthy individuals at baseline and in periodontitis patients at pre-treatment and 2, 6, and 12 weeks following therapy. IL-10, IFN-γ, CD163, and TWEAK levels were analyzed with Luminex technique.

Results: Tissue levels (median, 1st–3rd quartile) of IL-10 (pg/ng protein), CD163 (pg/μg protein) and TWEAK (pg/μg protein) were as follows: IL-10 periodontitis: 2.08, 0.86–5.32 and periodontally healthy: 5.22, 3.20–10.25; CD163 periodontitis: 8.85, 4.92–14.06 and periodontally healthy: 18.36, 12.51-34.02; TWEAK periodontitis: 0.08, 0.05–0.11 and periodontally healthy: 0.16, 0.12–0.21. IL-10, CD163, and TWEAK levels were higher ($P < 0.001$) in periodontally healthy tissues than in periodontitis tissues. Pocket closure at 12 weeks was associated with elevated baseline gingival CD163 levels ($P = 0.047$) and CD163/TWEAK ratio ($P = 0.001$). Elevated baseline gingival CD163/TWEAK ratio was associated with pocket reduction at 6 ($P = 0.022$) and 12 weeks ($P = 0.002$).

Conclusion: Associations of pocket closure with pre-treatment gingival tissue CD163 levels and CD163/TWEAK ratio indicate that baseline M2 macrophage activation profile may play a role in periodontal wound healing.

1 | INTRODUCTION

The aim of periodontal treatment is to re-establish tissue homeostasis by disrupting the microbial biofilm and, thereby, to ease the prolonged, destructive inflammatory burden.[1] Inflammation initiates as a natural reaction to pathogens with a concurrent counter reaction to confine infection's deteriorating effects and restore tissue integrity.[2] The pathogenic activity of the biofilm and the balance between pro- and anti-inflammatory signals determine whether the disease persists or resolves.[2] These signals are formed by a complex network of molecules, such as cytokines and complement proteins, and cells like neutrophils, T-lymphocytes, and macrophages.[3] Macrophages can be roughly categorized as classically activated pro-inflammatory M1 and alternatively activated M2 phenotypes. M2 macrophages have anti-inflammatory, pro-angiogenetic, and proliferative functions.[4] The inability to switch between macrophage phenotypes has been considered to cause persistent inflammation in various systemic conditions.[5,6]

CD163, the hemoglobin scavenger receptor exclusive to the monocyte/macrophage lineage, is a marker of the M2 subset (CD163$^+$) with anti-oxidative and anti-inflammatory effects.[7] CD163 is up-regulated by anti-inflammatory cytokines, such as interleukin (IL)-10, and suppressed by pro-inflammatory stimuli, such as interferon (IFN)-γ.[8,9] CD163 overexpression improves epithelial healing by interacting with keratinocytes and fibroblasts, and CD163$^+$ derived IL-10 is considered as a potential propellant in enhanced epithelial-mucosal healing.[10-13] In addition, CD163 acts as a scavenger or decoy receptor for tumor necrosis factor-like weak inducer of apoptosis (TWEAK),[14,15] which is a member of the tumor necrosis factor (TNF) superfamily and is associated with autoimmune and inflammatory disorders.[16] It has been shown that CD163 allows cell differentiation rather than prolonged myogenic progenitor cell proliferation by blocking TWEAK signaling at ischemic injury areas.[17] This suggests a potential role of CD163-TWEAK interactions in tissue repair and regeneration. CD163/TWEAK ratio has been priorly used as a prognostic marker in cardiovascular events and digital ulcer formation.[18-20]

Higher mRNA expressions of CD163 and lower CD163-positive cell counts,[21,22] and higher TWEAK-positive cell numbers[23] have been observed in gingival biopsies taken from periodontitis patients. While these findings indicate the potential role of M2 macrophage activation-related proteins CD163 and TWEAK in periodontal disease pathogenesis, their association with periodontal healing has not been investigated previously. Moreover, although smokers exhibit lower IL-10 and higher IFN-γ mRNA levels in periodontal tissues,[24,25] the impact of smoking on gingival CD163 and TWEAK levels are unknown. Considering particularly the regulatory roles of IL-10 and CD163 in epithelial-mucosal healing, we hypothesized that periodontitis lesions with elevated IL-10 and CD163 levels and CD163/TWEAK ratio as well as decreased IFN-γ levels have a tendency to enhanced pocket healing following non-surgical periodontal treatment. Therefore, the aims of this study were (1) to reveal and compare M2 macrophage activation-related IL-10, IFN-γ, CD163, TWEAK levels and CD163/TWEAK ratio in periodontitis lesions and periodontally healthy gingiva, and (2) to relate their baseline periodontitis lesion levels to indicators of gingival healing (pocket reduction and pocket closure) following non-surgical periodontal treatment, in smokers and non-smokers.

2 | MATERIALS AND METHODS

2.1 | Ethics and sample size

The study was registered on ClinicalTrials.gov with the number NCT04792372 in February, 2021. The accordance of the study to the ethical guidelines of the Helsinki Declaration was approved by the Clinical Research Ethics Committee of Biruni University Medical Faculty (Istanbul, Turkey) with the number 2015-KAEK-43-19-27. The study protocol was explained to all participants and their written informed consent was obtained.

A statistical power analysis program[*] was used for the sample size calculation. Since there were no prior studies evaluating CD163/TWEAK protein levels in gingival tissues, the effect size was defined based on previous research reporting significantly different gingival crevicular fluid levels of TWEAK between periodontitis and health.[26] The sample size was estimated with the effect size being measured at 1.594 for a two-tailed hypothesis ($\alpha = 0.05$; 1-$\beta = 0.95$). These analyses resulted in the minimum required sample size for each group (smokers with periodontitis, non-smokers with periodontitis, periodontally healthy smokers, and periodontally healthy
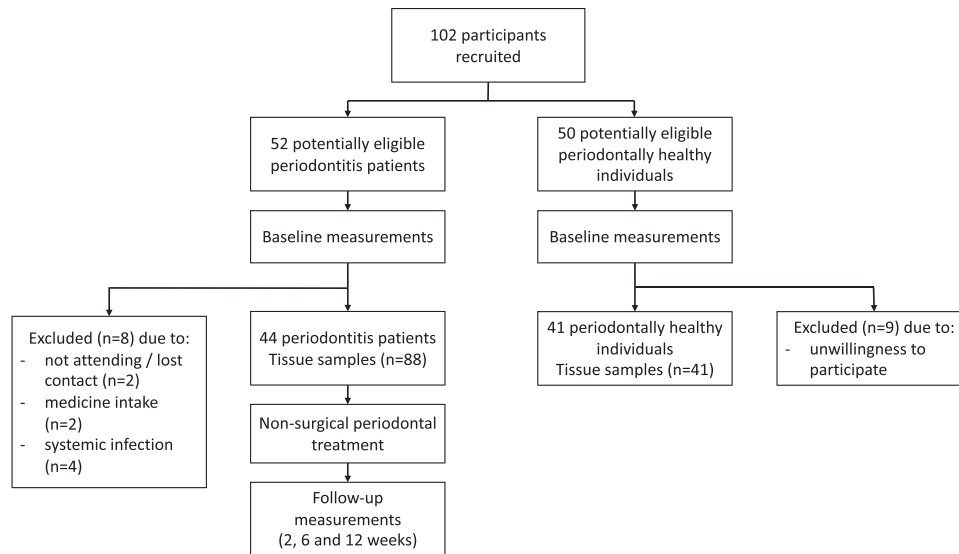
FIGURE 1 Flow diagram of the study protocol

non-smokers) being 12 participants. Considering potential losses during the follow-up and difficulty in estimating the percentage of closed pockets following treatment, to maintain the statistical power, 52 periodontitis patients and 50 periodontally healthy individuals were initially recruited. Following drop-outs due to various reasons (Figure 1), 44 periodontitis patients and 41 periodontally healthy controls concluded the study.

2.2 | Study population and eligibility criteria

The study participants were recruited among patients, who were referred to the Faculty of Dentistry, Biruni University, Istanbul, Turkey, between March 2021 and August 2021 for their dental treatment. Patients diagnosed with generalized Stage III or IV, Grade C periodontitis[27] with at least two bleeding sites with pocket depths (PDs) between 6 mm and 10 mm were included in the study. The grade was determined based on indirect evidence of disease progression (% radiographic bone loss/age > 1.0) and the impact of smoking (≥10 cigarettes daily) on clinical status. In addition, periodontally healthy subjects, who had full-mouth bleeding on probing (BoP) < 10% and no sites with PD > 3 mm, were included in the study as controls. The exclusion criteria were as follows: (1) < 15 teeth, (2) diagnosed systemic disorders such as rheumatoid arthritis, diabetes mellitus, and chronic medicine use, which are known to influence the inflammatory status, (3) occasional or former smokers, (4) use of any antimicrobial or anti-inflammatory medicine within the 3 months prior to the study, and (5) contraceptive use, pregnancy, or

lactation. Smoking status was classified as smokers and non-smokers; that is, people, who consumed ≥5 cigarettes per day for at least 1 year were considered smokers, whereas non-smokers had never smoked before.

2.3 | Periodontal treatment, tissue sampling, and clinical monitoring

Clinical measurements at baseline and at all follow-up sessions were performed by a single calibrated examiner (E.D.; Kappa values for pocket depth were 0.72–0.80 with 85.7% agreement). At baseline, plaque index (PI), PD, indirect clinical attachment level (CAL), and BoP scores of each site were recorded with a stainless-steel UNC15 probe.[†] Thereafter, full-mouth periodontal non-surgical treatment was performed and oral hygiene measures were instructed. The non-surgical periodontal treatment consisted of supragingival scaling and root debridement conducted with a combination of a piezoelectric ultrasonic device[‡] in periodontology mode and gentle use of hand instruments[§] under local anesthesia, which was completed in two consecutive sessions during the same day.

Tissue sampling and treatments were conducted by a single periodontist (M.Y.). Two gingival tissue samples were collected from each periodontitis patient (not pooled, analyzed individually), while one tissue sample was collected from each periodontally healthy participant. Healthy gingival tissue samples (PD ≤3 mm; BoP-)

[†] 54B XSI, LM-Dental, Parainen, Finland.
[‡] VarioSurg, NSK, Tokyo, Japan.
[§] Gracey 5/6, 7/8, 11/12, 13/14; American Eagle Instruments, Missoula, MT.

of periodontally healthy participants in need of crown lengthening or extraction due to orthodontic or restorative reasons, were obtained with a crestal incision reaching the bottom of the crevice at the beginning of their corresponding treatment. Gingival tissue samples (PD ≥6 mm and ≤10 mm; BoP+) of periodontitis patients were obtained during the non-surgical treatment at the beginning of the procedure. Effort was given to choose the sampling sites from different quadrants and/or zones. The samples were collected from the pocket wall with a stroke from the bottom to the margin with a hand curette. Each sample was immediately put into cryotubes containing 100 μl phosphate buffer saline (PBS, pH 7.2) and stored at -80°C. Samples were sent to the Institute of Dentistry, University of Turku, on dry ice for the analyses. Clinical measurements were repeated at 2, 6, and 12 weeks following therapy. At 6 and 12 weeks, supragingival biofilm was removed without disturbing the root surface, and oral hygiene was reinforced.

2.4 | Quantification of CD163, TWEAK, IL-10, and IFN-γ

The samples were ground and homogenized with a high-speed tissue homogenizer[**] in 2 ml tubes each containing one 0.5 mm sterile metal bead and 100 μl of PBS, and ultrasonicated thereafter. CD163, TWEAK, IL-10 and IFN-γ levels were determined with a bead-based immunoassay technique[††] with commercial kits[‡‡] according to manufacturer's instructions. The detection range of the assays were as follows: CD163 (1338.7 – 975916.6 pg/ml); TWEAK (3.1 – 6772.8 pg/ml); IL-10 (1.7 – 3781.2 pg/ml); IFN-γ (6.3 – 13694.9 pg/ml). Total protein level of each sample was determined with a commercially available protein determination method[§§] as specified by the manufacturer. IL-10, CD163, and TWEAK levels were normalized to the total protein levels of each sample. The gingival tissue levels of CD163 and TWEAK were presented as pg/μg protein, and IL-10 as pg/ng protein due to the low quantity of IL-10 levels.

2.5 | Definitions of the healing response

Two site-specific clinical endpoints of periodontal treatment, *pocket closure* and *pocket depth reduction > 30%*,

were used as parameters of healing responses. The main endpoint, pocket closure, was defined as sites with PD ≤3 mm (regardless of BoP) or PD ≤4 mm (BoP-) after non-surgical periodontal therapy.[28] Pocket depth reduction > 30% was defined as the secondary clinical endpoint. The threshold of > 30% was decided based on a recent systematic review, which indicated that usually a PD reduction of 1.5 mm in shallow pockets (4–6 mm) and a PD reduction of 2.6 mm in deep pockets (≥7 mm) is observed at 3–6 months following treatment.[29] Therefore, the sites met pocket depth reduction > 30% criteria when a site with a PD of 6 mm at baseline demonstrated a PD reduction of ≥2 mm, a site with a PD of 7–9 mm at baseline demonstrated a PD reduction of ≥3 mm, and a site with a PD ≥10 mm at baseline demonstrated a PD reduction of ≥4 mm.

2.6 | Data analysis

The data were analyzed with statistical software.[***] Shapiro-Wilk test and Q-Q plots were used to evaluate the normality of the data. For equality of means of quantitative variables, independent t-test was conducted, while chi-squared was used for testing qualitative variables. The mean changes of clinical values at different time points were tested using repeated measures ANOVA test with Bonferroni correction. Kruskal-Wallis with Mann Whitney U as post-hoc or chi-square test were used to compare linear measurements with skewed distributions and frequencies between groups, respectively. A binary logistic regression analysis with smoking, sex, and age adjustment was conducted to assess the association between the analytes' levels and the healing response in the whole population. $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | Demographics and smoking habits

Among the periodontitis group, there were 18 smokers (mean age: 42.4 years; seven female) and 26 non-smokers (mean age: 42.9 years; 17 female). The periodontally healthy group consisted of 18 smokers (mean age: 37.4 years; nine female) and 23 non-smokers (mean age: 41.3 years; 15 female). Age, sex, and smoking status were equally distributed in the periodontitis and the healthy groups ($P = 0.086$, $P = 0.711$, and $P = 0.078$, respectively). In addition, age and sex distribution were similar in smokers and non-smokers ($P = 0.465$ and

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[**] The TissueLyser LT, Qiagen, Hilden, Germany.

[††] Luminex xMAP, Luminex Corporation, Austin, TX.

[‡‡] Bio-Plex Pro Human Inflammation Panel 1, Bio-Rad Laboratories, Hercules, CA.

[§§] Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA.

[***] SPSS 27, IBM, Armonk, NY.

$P = 0.055$, respectively). None of the participants reported post-treatment complications.

The mean cigarette consumption of the smoking participants of our study was 14.66 ±6.75 cigarettes per day, and the average years of smoking was 16.46 ±8.68. Among the periodontitis patients, three participants were smoking five cigarettes per day (5–10 years), while the rest were smoking at least 10 cigarettes per day (5–45 years). The mean exposure to smoking, calculated as pack-years, of periodontitis patients and of periodontally healthy individuals was 16.06 ±11.02 and 10.18 ±9.3, respectively.

3.2 | Clinical parameters

By design, baseline percentage of PI-positive sites (86.4%), PD (7.40 ±0.95 mm) and CAL (7.59 ±1.08 mm) scores were higher in periodontitis group in comparison to those [% of PI-positive sites (17.1%), PD (1.76 ±0.77 mm) and CAL (1.76 ±0.77 mm)] of the healthy sites ($P < 0.001$). Within both periodontitis and healthy groups, baseline clinical parameters did not exhibit any difference between smokers and non-smokers (Table 1).

Baseline and post-treatment 2-, 6-, and 12-week clinical measurements of periodontitis sites are shown in Table 2. While initial clinical measurements did not differ between smokers and non-smokers ($P > 0.05$), at 2, 6, and 12 weeks the smokers demonstrated higher PD, lower PD reduction (mm, %), fewer sites with PD reduction > 30%, and fewer closed pockets in comparison to non-smokers. PI and BoP scores did not differ between the sites of smokers and non-smokers at any time point ($P > 0.05$).

3.3 | Levels of IL-10, CD163, and TWEAK and CD163/TWEAK ratio

CD163 and TWEAK were detected in all samples. IL-10 levels of 22 periodontitis samples (25%) and one healthy sample (2.44%) were below the limit of detection (LOD). IL-10 levels below the LOD were substituted with 0.85 pg/ml, which was half of the LOD. Since IFN-γ levels were below the LOD in all samples, they were not included to the statistical analysis.

IL-10, CD163, TWEAK levels, and CD163/TWEAK ratio according to periodontal status and smoking are presented in Table 1. Elevated IL-10 ($P < 0.001$), CD163 ($P < 0.001$), and TWEAK ($P < 0.001$) levels were observed in the periodontally healthy group in comparison to periodontitis patients. The CD163/TWEAK ratio did not show a statistically significant difference between the periodontitis and periodontally healthy groups ($P = 0.086$). When non-smokers were evaluated separately, IL-10 ($P < 0.001$),

CD163 ($P < 0.001$), and TWEAK ($P < 0.001$) levels of the healthy samples were higher than those of periodontitis patients, while CD163/TWEAK did not differ among the groups ($P = 0.520$). Periodontitis tissues of smokers exhibited lower CD163 ($P < 0.001$) and TWEAK ($P = 0.009$) levels compared to healthy sites of smokers; IL-10 ($P = 0.474$) and CD163/TWEAK ($P = 0.067$) levels were similar across groups in smokers.

Baseline IL-10, CD163, TWEAK and CD163/TWEAK levels in relation to pocket closure and pocket reduction at different time points according to smoking status are given in Table 3 and Table 4, respectively. In non-smokers, sites demonstrating pocket closure ($P = 0.023$) and pocket depth reduction > 30% ($P = 0.041$) at 12 weeks had elevated baseline CD163 levels. Logistic regression analysis (Table 5) revealed that pocket closure at 12 weeks ($P = 0.001$) and pocket depth reduction > 30% at 6 ($P = 0.022$) and 12 weeks ($P = 0.002$) were associated with elevated pre-treatment CD163/TWEAK ratio, and sites showing pocket closure at 12 weeks were associated with elevated CD163 levels ($P = 0.047$).

4 | DISCUSSION

To the best of our knowledge, this study is the first to reveal that sites with a tendency to enhanced pocket healing response after non-surgical periodontal treatment, as defined by pocket closure and pocket depth reduction, have elevated baseline CD163 and CD163/TWEAK ratios. Protein and mRNA levels of CD163 and TWEAK in periodontitis were analyzed in cross-sectional studies[21-23]; however, IL-10/IFN-γ/CD163/TWEAK cascade in relation to treatment results were not evaluated previously.

The main strength of the present study is the simultaneous evaluation of M2 macrophage activation-associated IL-10, CD163, TWEAK levels, and CD163/TWEAK ratio in gingival tissues. CD163/TWEAK ratio was previously used as a prognostic/diagnostic marker in atherosclerosis, cardiovascular events, and skin pathologies[18,20,30,31]; however, its relation to periodontal disease pathogenesis is unknown. A second strength is the implementation of smoking in our study design. In order to assess the influence of smoking more prominently and to avoid grey zones, former smokers and casual smokers were not included, since prior research demonstrated that past, current, light or heavy smoking can have diverse effects on the clinical parameters.[32,33] Finally, periodontal measurements at post-treatment 2, 6, and 12 weeks allowed us to detect early and late healing responses in relation to M2 macrophage activation-associated IL-10, CD163, and TWEAK levels. Junctional epithelium is re-established at 2 weeks and connective tissue maturation is completed

TABLE 1 Sample size, PI, PD, IL-10, CD163, TWEAK, and CD163/TWEAK levels according to periodontal status and smoking

Variable	Whole population			Periodontitis			Periodontally healthy		
	Periodontitis	Periodontally healthy	P-Value	Smokers	Non-smokers	P-Value	Smokers	Non-smokers	P-Value
Sites n	88	41		36	52		18	23	
PI+ sites n (%)	76 (86.4)	7 (5.6)	**<0.001**	32 (88.9)	44 (84.6)	0.566	4 (22.2)	3 (13.0)	0.438
PD (mm) Mean ± SD	7.40 ± 0.95	1.76 ± 0.77	**<0.001**	7.42 ± 0.97	7.38 ± 0.95	0.923	1.89 ± 0.76	1.65 ± 0.78	0.294
IL-10 (pg/ng protein) Median (Q1–Q3)	2.08 (0.86–5.32)	5.22 (3.20–10.25)	**<0.001**	4.23 (0.62–10.54)	1.29 (0.49–3.06)	**<0.001**	4.97 (2.93–10.80)	5.98 (3.42–10.09)	0.834
CD163 (pg/μg protein) Median (Q1–Q3)	8.85 (4.92–14.06)	18.36 (12.51–34.02)	**<0.001**	10.14 (4.48–15.51)	8.72 (5.27–12.61)	0.445	17.48 (13.80–43.63)	21.78 (12.18–31.27)	0.854
TWEAK (pg/μg protein) Median (Q1–Q3)	0.079 (0.052–0.114)	0.159 (0.119–0.214)	<0.001	0.085 (0.060–0.166)	0.073 (0.048–0.096)	0.073	0.149 (0.106–0.268)	0.160 (0.120–0.199)	0.793
CD163/TWEAK ratio Median (Q1–Q3)	121.70 (78.98–165.75)	132.12 (98.61–201.76)	0.086	110.12 (77.58–161.94)	124.89 (80.96–177.91)	0.333	134.45 (102.14–283.2)	129.63 (94.57–189.09)	0.462

Note: P statistical significance comparing smokers and non-smokers within periodontitis and periodontally healthy groups. P values in bold indicate statistically significant difference (< 0.05).
Abbreviations: SD, standard deviation; PI, plaque index; Q1, lower quartile; Q3, upper quartile.

TABLE 2 Clinical parameters of periodontitis sites at baseline and at 2, 6, and 12 weeks following non-surgical periodontal treatment

Variable	Baseline				2 Weeks				6 Weeks				12 Weeks			
	Total (n = 88)	Smokers (n = 36)	Non-smokers (n = 52)	P-Value	Total (n = 88)	Smokers (n = 36)	Non-smokers (n = 52)	P-Value	Total (n = 88)	Smokers (n = 36)	Non-smokers (n = 52)	P-Value	Total (n = 88)	Smokers (n = 36)	Non-smokers (n = 52)	P-Value
PD (mm) mean± SD	7.40 ± 0.95	7.42 ± 0.97	7.38 ± 0.95	0.923	5.45 ± 1.42[b]	5.86 ± 1.42[b]	5.17 ± 1.37[b]	0.023	4.59 ± 1.62[b]	5.25 ± 1.66[b]	4.13 ± 1.43[b]	0.001	4.27 ± 1.74[b]	5.11 ± 1.74[b]	3.69 ± 1.50[b]	< 0.001
CAL (mm) mean± SD	7.59 ± 1.08	7.64 ± 1.15	7.56 ± 1.04	0.957	5.86 ± 1.70[b]	6.19 ± 1.69[b]	5.63 ± 1.68[b]	0.125	5.10 ± 1.83[b]	5.64 ± 1.69[b]	4.73 ± 1.85[b]	0.006	4.80 ± 1.89[b]	5.56 ± 1.78[b]	4.27 ± 1.79[b]	< 0.001
BoP (%)	100	100	100	.	59.1[b]	58.3[b]	59.6[b]	0.904	43.2[b]	47.2[b]	40.4[b]	0.524	36.4[b]	38.9[b]	34.6[b]	0.682
PI (%)	86.4	88.9	84.6	0.566	38.6[b]	44.4[b]	34.6[b]	0.352	45.5[b]	50.0[a]	42.3[b]	0.476	48.9[b]	58.3[a]	42.3[b]	0.139
PD ≥4 mm n (%)	88 (100)	36 (100)	52 (100)	.	81 (92.0)	34 (94.4)	47 (90.4)	0.489	61[b] (69.3)	29[a] (80.6)	32[b] (61.5)	0.057	51[b] (58.0)	29[a] (80.6)	22 (42.3)	< 0.001
PD ≥6 mm n (%)	88 (100)	36 (100)	52 (100)	.	41[b] (46.6)	21[b] (58.3)	20[b] (38.5)	0.066	26[b] (29.5)	16[b] (44.4)	10[b] (19.2)	0.011	21[b] (23.9)	16[b] (44.4)	5[b] (9.6)	< 0.001
Closed pockets n (%)	.	.	.	.	15 (17.0)	2 (5.6)	13 (25.0)	0.017	37 (42.0)	9 (25.0)	28 (53.8)	0.007	48 (54.5)	12 (33.3)	36 (69.2)	< 0.001
PD red (mm) mean± SD	.	.	.	.	1.94 ± 1.08	1.56 ± 1.08	2.21 ± 1.00	0.007	2.81 ± 1.50	2.17 ± 1.46	3.25 ± 1.37	< 0.001	3.13 ± 1.52	2.31 ± 1.26	3.69 ± 1.44	< 0.001
PD red % mean± SD	.	.	.	.	26.64 ± 14.73	21.20 ± 14.80	30.40 ± 13.59	0.006	38.10 ± 19.07	29.44 ± 18.90	44.10 ± 16.90	< 0.001	42.72 ± 19.86	31.97 ± 17.94	50.16 ± 17.72	< 0.001
PD red > 30% n (%)	.	.	.	.	29 (33.0)	6 (16.7)	23 (44.2)	0.007	51 (58.0)	13 (36.1)	38 (73.1)	< 0.001	60 (68.2)	17 (47.2)	43 (82.7)	< 0.001

Note: P values define the statistical difference between smokers and non-smokers within each time point. Pocket depth reduction (PD red) in comparison to baseline pocket depth (PD) values are given in mm and in percentages.

Abbreviations: SD, standard deviation; CAL, clinical attachment level; BoP, bleeding on probing; PI, plaque index.

[a] (P < 0.05) Statistically significant difference with the corresponding baseline value.

[b] (P < 0.001) Statistically significant difference with the corresponding baseline value.

TABLE 3 Baseline IL-10, CD163, TWEAK, and CD163/TWEAK levels in relation to pocket closure at 2, 6, and 12 weeks following non-surgical periodontal treatment

Smoking status	Healing time	Pocket closure	%	IL-10 (pg/ng protein) Median (Q1–Q3)	CD163 (pg/μg protein) Median (Q1–Q3)	TWEAK (pg/μg protein) Median (Q1–Q3)	CD163/TWEAK ratio Median (Q1–Q3)
Smokers	2 weeks	Closed pocket	5.56	7.77 (5.17–7.77)	26.10 (13.38–26.10)	0.165 (0.110–0.165)	149.08 (122.07–149.08)
		Residual pocket	94.44	4.11 (1.50–11.17)	9.35 (4.37–9.35)	0.081 (0.056–0.143)	104.82 (76.47–160.99)
		P		0.447	0.203	0.229	0.384
	6 weeks	Closed pocket	25	5.17 (4.10–9.67)	13.47 (10.14–28.62)	0.132 (0.085–0.210)	122.07 (84.81–196.90)
		Residual pocket	75	3.39 (0.25–13.62)	7.50 (3.92–15.11)	0.078 (0.052–0.120)	99.91 (73.73–158.08)
		P		0.407	**0.047**	0.067	0.218
	12 weeks	Closed pocket	33.33	4.96 (2.34–11.89)	13.43 (6.38–20.56)	0.100 (0.050–0.190)	142.24 (102.36–188.58)
		Residual pocket	66.67	3.74 (1.34–10.54)	8.07 (3.96–14.85)	0.081 (0.060–0.124)	92.67 (72.83–150.34)
		P		0.585	0.177	0.631	**0.038**
Non-smokers	2 weeks	Closed pocket	25	0.95 (0.38–1.97)	7.92 (4.88–11.08)	0.083 (0.058–0.093)	108.18 (74.76–159.80)
		Residual pocket	75	1.63 (0.59–3.44)	8.89 (5.14–13.97)	0.069 (0.044–0.114)	134.30 (92.95–178.84)
		P		0.194	0.441	0.775	0.216
	6 weeks	Closed pocket	53.85	0.96 (0.46–1.86)	8.97 (6.30–11.82)	0.070 (0.055–0.088)	137.92 (87.03–181.50)
		Residual pocket	46.15	2.08 (0.69–3.36)	7.92 (4.92–14.06)	0.079 (0.042–0.124)	119.85 (67.54–162.03)
		P		0.066	0.673	0.521	0.331
	12 weeks	Closed pocket	69.23	1.39 (0.60–4.06)	9.17 (7.22–13.80)	0.075 (0.054–0.090)	139.14 (100.60–184.71)
		Residual pocket	30.77	0.84 (0.46–2.13)	5.85 (4.17–9.10)	0.063 (0.036–0.116)	107.14 (50.21–132.05)
		P		0.212	**0.023**	0.766	**0.032**

Note: Pockets with probing depth (PD) of ≤ 3 mm (regardless of BoP) or = 4 mm (BoP-) were defined as closed pockets. Q1, lower quartile; Q3, upper quartile. *P* values in bold indicate statistically significant difference (< 0.05).

TABLE 4 Baseline IL-10, CD163, TWEAK, and CD163/TWEAK levels in relation to pocket depth reduction (PD red) at 2, 6, and 12 weeks following non-surgical periodontal treatment

Smoking status	Healing time	Pocket depth reduction	%	IL-10 (pg/ng protein)	CD163 (pg/μg protein)	TWEAK (pg/μg protein)	CD163/TWEAK ratio
Smokers	2 weeks	PD red > 30%	16.67	4.41 (0.52–6.47)	11.24 (3.49–19.74)	0.084 (0.045–0.137)	116.29 (85.32–166.07)
		PD red ≤30%	83.33	4.23 (1.72–13.05)	9.59 (4.41–15.82)	0.085 (0.063–0.177)	98.09 (76.47–160.99)
		P		0.308	0.852	0.725	0.788
	6 weeks	PD red > 30%	36.11	4.32 (2.73–7.63)	13.38 (7.44–18.27)	0.090 (0.063–0.189)	162.41 (84.81–196.90)
		PD red ≤30%	63.89	4.09 (0.98–14.17)	7.50 (3.92–15.56)	0.082 (0.052–0.126)	96.28 (72.53–138.09)
		P		0.987	0.214	0.721	**0.040**
	12 weeks	PD red > 30%	47.22	4.32 (2.18–9.67)	13.38 (4.87–19.94)	0.090 (0.049–0.189)	122.07 (93.53–186.62)
		PD red ≤30%	52.78	4.09 (1.26–14.17)	7.50 (3.89–15.11)	0.082 (0.065–0.120)	91.67 (67.33–138.09)
		P		0.912	0.175	0.778	**0.023**
Non-smokers	2 weeks	PD red > 30%	44.23	1.58 (0.52–4.26)	9.33 (7.22–15.32)	0.083 (0.054–0.099)	118.42 (80.17–164.32)
		PD red ≤30%	55.77	0.99 (0.47–2.70)	8.70 (4.58–12.20)	0.064 (0.042–0.093)	125.28 (76.37–183.93)
		P		0.315	0.315	0.214	0.733
	6 weeks	PD red > 30%	73.08	1.22 (0.57–3.19)	8.83 (5.43–13.45)	0.070 (0.050–0.096)	137.28 (82.55–180.19)
		PD red ≤30%	26.92	1.89 (0.46–2.69)	8.22 (5.07–12.49)	0.079 (0.042–0.115)	118.79 (69.25–146.66)
		P		0.934	0.578	0.680	0.312
	12 weeks	PD red > 30%	82.69	1.32 (0.62–3.11)	9.02 (5.90–13.97)	0.073 (0.052–0.090)	137.71 (98.08–182.39)
		PD red ≤30%	17.31	0.47 (0.40–2.51)	5.80 (4.21–8.22)	0.090 (0.038–0.130)	86.06 (46.23–121.71)
		P		0.261	**0.041**	0.634	**0.017**

Note: Values are given as median (lower quartile–upper quartile). P values in bold indicate statistically significant difference (< 0.05).

TABLE 5 Unadjusted and adjusted (age, sex, and smoking) associations between explanatory (IL-10, CD163, TWEAK, and CD163/TWEAK levels) and outcome (pocket reduction and pocket closure) variables

Clinical outcome	Healing time	IL-10 Unadjusted OR (95%CI)	IL-10 Adjusted OR (95%CI)	CD163 Unadjusted OR (95%CI)	CD163 Adjusted OR (95%CI)	TWEAK Unadjusted OR (95%CI)	TWEAK Adjusted OR (95%CI)	CD163/TWEAK Unadjusted OR (95%CI)	CD163/TWEAK Adjusted OR (95%CI)
Pocket reduction > 30%	2 weeks	0.974 (0.906–1.046) P = 0.467	1.008 (0.935–1.086) P = 0.842	1.000 (0.965–1.036) P = 0.983	1.002 (0.966–1.040) P = 0.905	0.647 (0.00–854.316) P = 0.906	4.097 (0.001–13006.32) P = 0.732	0.999 (0.992–1.005) P = 0.690	0.998 (0.992–1.005) P = 0.623
	6 weeks	0.949 (0.888–1.013) P = 0.118	0.983 (0.917–1.054) P = 0.628	1.020 (0.980–1.063) P = 0.334	1.035 (0.983–1.090) P = 0.185	0.194 (0.00–165.378) P = 0.634	2.589 (0.001–6726.98) P = 0.813	1.009 (1.001–1.017) **P = 0.020**	1.010 (1.001–1.019) **P = 0.022**
	12 weeks	0.968 (0.91–1.03) P = 0.307	1.002 (0.933–1.977) P = 0.949	1.037 (0.891–1.097) P = 0.198	1.057 (0.994–1.124) P = 0.079	0.118 (0.00–128.845) P = 0.550	1.999 (0.001–4115.95) P = 0.859	1.015 (1.006–1.025) **P = 0.002**	1.018 (1.007–1.030) **P = 0.002**
Pocket closure	2 weeks	0.891 (0.752–1.055) P = 0.181	0.945 (0.809–1.103) P = 0.473	0.985 (0.931–1.042) P = 0.598	0.994 (0.946–1.044) P = 0.807	0.082 (0.00–1446.361) P = 0.616	0.457 (0–23514.37) P = 0.887	0.997 (0.988–1.006) P = 0.478	0.998 (0.989–1.006) P = 0.587
	6 weeks	0.954 (0.887–1.026) P = 0.201	0.981 (0.911–1.055) P = 0.602	0.999 (0.966–1.033) P = 0.947	1.002 (0.968–1.038) P = 0.9	0.084 (0.00–96.519) P = 0.491	0.658 (0–1225.81) P = 0.913	1.005 (0.999–1.012) P = 0.094	1.005 (0.998–1.012) P = 0.161
	12 weeks	0.996 (0.938–1.05) P = 0.909	1.035 (0.962–1.113) P = 0.359	1.035 (0.987–1.084) P = 0.152	1.059 (1.001–1.120) **P = 0.047**	0.786 (0.001–652.649) P = 0.944	9.245 (0.004–19409) P = 0.569	1.013 (1.005–1.022) **P = 0.002**	1.019 (1.008–1.031) **P = 0.001**

Note: Data are presented as odds ratios (OR) and 95% confidence intervals (95%CI). *P* values in bold indicate statistically significant difference (< 0.05).

at 6–8 weeks after instrumentation, while the greatest pocket depth reduction and clinical attachment gains are observed within 4–12 weeks.[34-36] For research purposes and in accordance with the above-mentioned healing intervals, we recorded periodontal clinical parameters at 2, 6, and 12 weeks following periodontal treatment. This can be deemed unconventional and too early to make any solid assumptions, since, in a clinical setting, the re-evaluation following non-surgical periodontal therapy is recommended to be performed at 6–8 weeks.[37] However, these time points were decided on to monitor early healing outcomes and keep track of any exceptional results, considering the CD163/IL-10 signaling pathway has been associated with enhanced epithelial healing.[11,12,38] A limitation of our study is that gingival tissue samples were collected only before the non-surgical periodontal treatment, due to ethical considerations. Thus, analyzing post-treatment alterations in tissue levels of IL-10, IFN-γ, CD163, and TWEAK was not possible. In addition, sampled tissue size and morphology differed between the periodontitis and periodontally healthy groups; in the periodontitis group, tissue samples were formed of granulation tissues collected from periodontal pockets, while in the control group, tissue samples were formed of structurally intact gingiva. To eliminate the effect of harvesting method and tissue volume on our results, the analytes' levels were normalized to the total protein of each site. In the present study, the role of IFN-γ could not be investigated, since IFN-γ levels were below the limit of detection in all samples. Considering the sensitivity of the applied IFN-γ detection method (6.3 pg/ml), the failure of detection can be related to the enzymatic degradation or the short half-life of IFN-γ. Finally, grouping sampling sites based on their healing responses at each time point created small group sizes and limited the statistical power. A regression analysis was performed in order to minimize the effect of this limitation.

According to our results, an elevated baseline CD163/TWEAK ratio in gingival tissues of periodontitis patients is related to pocket closure at 12 weeks and pocket depth reduction at 6 and 12 weeks following treatment. Moreover, elevated baseline CD163 levels were related to pocket closure at 12 weeks. M2 macrophages are known to regulate re-vascularization, fibroblastic activity, and collagen production, leading to efficient wound closure or scar formation.[4] It has been shown that M2 polarization characterized with CD163 expression at the early stages of wound healing can enhance periodontal tissue regeneration,[39] and CD163 overexpression results in faster and regular epithelial growth.[12] Thus, greater pocket depth reduction observed in sites with a high CD163/TWEAK ratio is possibly associated with enhanced re-epithelization and connective tissue remodeling stimulated by the pro-repair M2 activities. TWEAK takes part in epithelial healing, fibroblastic functions, and connective tissue remodeling as well,[19,40,41] although it is considered mainly a pro-inflammatory mediator.[16] CD163 and TWEAK have specific and functional interactions; CD163$^+$ cells have the capability of scavenging and internalizing TWEAK, and preventing its pro-inflammatory functions such as NF-kB signaling and cytokine or metalloproteinase expression.[14,15,41] It has been shown that CD163 macrophages can regulate regeneration of ischemic injury areas through controlling TWEAK signaling.[17] However, it is worth noting that CD163/TWEAK interactions are complex and can have distinctive functions depending on the context. High soluble CD163/TWEAK ratios were associated with severe fibrotic skin involvement in systemic sclerosis but also with fewer digital ulcer formations.[19] Given these facts, our results indicate potential interactions between baseline CD163/TWEAK levels and the healing events following periodontal non-surgical therapy. Consecutive research can be beneficial to clarify their action mechanisms in periodontal wound healing.

Based on our results, the levels of IL-10, CD163, and TWEAK are decreased in tissues affected by periodontitis when compared to healthy gingiva. Górska et al. demonstrated very low or undetectable IL-10 levels in severe periodontitis lesions, although this cytokine was more frequently detected in normal tissue biopsies.[42] There are also contradicting studies reporting elevated IL-10 levels in inflamed gingival samples.[43,44] Considering that IL-10 is a critical molecule for the resolution of inflammation,[45] our results indicate that reduced IL-10 levels may lead to exacerbated inflammation during periodontitis. However, it must also be noted that active IL-10 is an unstable molecule and has a short half-life.[46] Considering the elevated proteolytic activity in periodontitis lesions, IL-10 can undergo fast degradation in granulation tissues, which may explain the differences between the studies. Regarding the gingival tissue levels of CD163 and TWEAK, the available information is limited and contradictory. According to one of the two previous studies assessing TWEAK levels in tissues affected by periodontitis, TWEAK mRNA is more frequently expressed in periodontitis when compared to healthy samples.[47] Kataria et al., on the other hand, demonstrated increased TWEAK-expressing cells in periodontitis, while TWEAK mRNA levels did not differ between healthy and inflamed tissues in the same study.[23] Additionally, high levels of TWEAK have been detected in gingival crevicular fluid of sites with peri-implantitis and periodontitis in comparison to health.[26] To the best of our knowledge, two studies evaluated CD163 in gingival tissues obtained from periodontitis patients priorly. While in the first study, increased CD163 mRNA levels have been reported,[22] according to the other one, the

number of CD206$^+$CD163$^+$ macrophages were decreased in periodontitis.[21] These inconsistencies can be related to variations in immune response activation or the intensity of proteolytic activity of the sampled periodontitis sites. As recently demonstrated, CD163 response during development of experimental gingivitis show inter-individual differences,[48] indicating that not only the presence of inflammation but also the extent of host response can influence the levels of these molecules.

Finally, while a significantly deteriorated healing response was observed in smokers, the only difference in tissue levels of M2 macrophage activation related proteins between smokers and non-smokers was observed in tissue IL-10 levels of periodontitis patients. Reduced gingival crevicular fluid IL-10 levels were shown in both healthy and diseased sites of smoker patients in comparison with non-smokers,[49] however, gingival tissue levels of IL-10 were previously evaluated only in one study, in which the effect of smoking was not assessed.[42] Higher numbers of CD163$^+$ alveolar macrophages were shown in smokers in comparison to non-smokers.[50] We could not compare our present findings with the literature as the effect of smoking on periodontal tissue IL-10, CD163, and TWEAK protein levels has not been demonstrated previously.

5 | CONCLUSION

The results of the present study suggest that baseline gingival tissue CD163 levels and CD163/TWEAK ratio, but not IL-10, are related to pocket closure and pocket depth reduction up to 12 weeks following periodontal treatment. Profiling the macrophage activation cascades in periodontitis-affected tissues can reveal the contribution of macrophage phenotypes to periodontal healing and disease remission.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS
Authors Ulvi Kahragman Gürsoy, Erhan Firatli, and Mustafa Yilmaz conceived the concept and the protocol of the study. Mustafa Yilmaz and Esra Demir carried out the data collection. Mustafa Yilmaz, Ulvi Kahraman Gürsoy and Mervi Gürsoy conducted the analyses. Mustafa Yilmaz wrote the draft, while all authors contributed to the critical revision of the manuscript.

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