Effects of antimicrobial photodynamic therapy and local administration of minocycline on clinical, microbiological, and inflammatory markers of periodontal pockets

Takahiro Hokari[1], Toshiya Morozumi[1], Yasutaka Komatsu[1], Taro Shimizu[1,2], Toshiaki Yoshino[3], Maki Tanaka[3], Yoshie Tanaka[3], Kaname Nohno[4], Takehiko Kubota[1], and Hiromasa Yoshie[1]

[1] Division of Periodontology, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

[2] Oral Implant Clinic, Niigata University Medical & Dental Hospital, Niigata, Japan

[3] Seikeikai Hospital, Seikeikai Group, Yokohama, Japan

[4] Division of Preventive Dentistry, Department of Oral Health Science, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Correspondence: Dr. Toshiya Morozumi, Division of Periodontology, Department of Oral Biological Science, Niigata University Graduate School of Medical and Dental Sciences,

2-5274 Gakkocho-Dori, Chuo-ku, Niigata 951-8514, Japan

Phone: +81 25 227 2871, Fax: +81 25 227 0808

E-mail: moro@dent.niigata-u.ac.jp

E-mail addresses

Takahiro Hokari: hokari@dent.niigata-u.ac.jp

Toshiya Morozumi: moro@dent.niigata-u.ac.jp

Yasutaka Komatsu: komatsu@dent.niigata-u.ac.jp

Taro Shimizu: taro-taro@dent.niigata-u.ac.jp

Toshiaki Yoshino: yoshino-do@nifty.com

Maki Tanaka: true-pleasure@able.ocn.ne.jp

Yoshie Tanaka: tanaka-yoshie@yoshino-do.jp

Kaname Nohno: no2@dent.niigata-u.ac.jp

Takehiko Kubota: kubota@dent.niigata-u.ac.jp

Hiromasa Yoshie: yoshie@dent.niigata-u.ac.jp

Abstract

Objective. We evaluated the efficacies of antimicrobial photodynamic therapy (aPDT) and minocycline ointment (MO) on clinical and bacteriological markers and on the local host immune-inflammatory response. *Materials and Methods.* Thirty patients with chronic periodontitis were randomly assigned to two groups. Selected periodontal pockets (probing depth 5–7 mm with bleeding on probing) were treated with aPDT or MO as monotherapy. Measurement of clinical parameters and collection of gingival crevicular fluid (GCF) and subgingival plaque were performed at baseline, and at 1 and 4 weeks post-treatment. Quantification of periodontopathic bacteria in the sulcus and multiplex bead immunoassay of ten inflammatory cytokines in the GCF were performed. *Results.* Local MO administration resulted in substantial improvement in clinical parameters and a significant reduction in bacterial count ($P < 0.01$) and interleukin-1β and interferon-γ levels at 1 and 4 weeks after treatment ($P < 0.01$). There was no significant change in the aPDT group, except in clinical parameters. *Conclusions.* While local administration of MO exhibited beneficial effects on clinical and microbiological markers and crevicular cytokine levels in periodontal pockets, aPDT did not show any additional benefits within the limitations of this study.

This trial is registered with UMIN Clinical Trials Registry UMIN000013376.

1. Introduction

Chronic periodontitis is an inflammatory and multifactorial disease caused by periodontopathic bacteria and host immune response [1]. Mechanical removal of the etiologic agents that cause inflammation, which include dental plaque, bacterial products, and calculus, is essential for healing in periodontitis. However, invasive periodontal procedures, including scaling and root planing (SRP), frequently induce bacteremia [2, 3]. Repeated entry of oral bacteria into the bloodstream could be implicated in the development of infective endocarditis [4], prosthetic joint infection [5], and a variety of other distant-site infections in high-risk patients. Thus, reducing subgingival periodontal pathogen populations and attendant inflammation by effective bactericidal therapy prior to mechanical debridement is necessary.

Several clinical and bacteriological studies have indicated the possibility of various anti-infectious therapies, including the use of disinfectants and antibiotics [6]. We have also previously reported the antimicrobial effects of essential- oil-containing antiseptics [3, 7], minocycline ointment (MO) as a local drug-delivery system [8, 9], and oral administration of azithromycin (AZM) [3]. The effectiveness of systemic antimicrobial administration in the treatment of periodontal disease has been demonstrated [10]. However, side effects resulting from overdose or systemic appearance of drug-resistant bacteria have also been reported [10, 11]. Similarly, laser therapy also might cause irreversible thermal damage to the surrounding periodontal tissue if used at high power [12]. Thus, there is a need to assess the safety and

efficacy of local antimicrobial therapy for local inflammation resulting from subgingival periodontal pathogens in chronic periodontitis patients that have not yet received mechanical debridement.

Antimicrobial photodynamic therapy (aPDT), which involves low-intensity diode laser irradiation in conjunction with photosensitizers, is a new method of antimicrobial treatment. Activation of the photosensitizer by irradiation at a suitable wavelength results in the release of singlet oxygen, which interacts with and is toxic to the cells or microorganisms [13]. Minocycline is a semisynthetic derivative of tetracycline with a broad antibacterial spectrum [14]. It is known that MO possesses features such as marked substantivity, slow-release, and superior lipophilicity [14]. Furthermore, in addition to its antibacterial activity, MO exhibits a therapeutic effect in periodontitis by directly inhibiting collagenase activity [15]. Thus, MO is one of the more suitable antibiotics for periodontal disease control, especially for local therapy.

Most previous studies in this field have mainly focused on the clinical and microbiological effects of these modalities, and knowledge regarding the local inflammatory response is limited [6, 16]. Furthermore, few studies have evaluated changes in gingival crevicular fluid (GCF) cytokine levels due to aPDT or MO monotherapy, which reflect the inflammatory state in periodontal tissues. Therefore, it is worth investigating the effectiveness of these antibacterial treatments.

Taking all these aspects into consideration, one might hypothesize that both aPDT and MO might contribute to improve the subgingival bacterial and local immunological markers. The objectives of the present study were to evaluate the effects of aPDT and MO on clinical and bacteriological markers and on local primary inflammatory cytokine levels in GCF from periodontal pockets in patients with chronic periodontitis.

2. Materials and Methods

2.1. Subjects

This study was a clinical intervention trial with a 4-week follow up. Thirty patients with moderate to severe chronic periodontitis were recruited from two facilities (Niigata University Medical & Dental Hospital and Seikeikai Hospital) in Japan, between March 2014 and October 2015. Periodontal diagnosis was based on the Guidelines of the American Academy of Periodontology [17]. This study received approval from the Ethics Committee of Niigata University Medical & Dental Hospital (NH25-010, NH25-010N). Written consent was obtained from all participants involved. All individuals were above the age of 30 years, systemically healthy, non-smokers and possessed at least 20 teeth. Subjects with the following conditions were excluded: pregnancy or breast feeding; acatalasia; glucose-6-phosphate dehydrogenase deficiency; photosensitivity disorders; allergy to tetracycline or methylene blue; and use of systemic photosensitizing agents, antibiotics or anti-inflammatory drugs within 3 months prior to enrollment. Individuals who had received periodontal therapy within the previous 6 months were also excluded.

2.2. Clinical protocol

To evaluate the efficacies of the two therapeutic procedures for periodontal pockets, the 30

enrolled subjects were randomly assigned to two groups (aPDT and MO group; n = 15, each) on the basis of the treatment protocol, using random tables prepared by one of the authors (TK). Each subject was assigned a code number, which was then used to identify the subject throughout the study. Experimental procedures and data collections were performed in the two facilities between May 2014 and December 2015. A flowchart of the clinical procedure is shown in Figure 1. In brief, for over a month before commencement of the study, each subject received standard oral hygiene instructions over the course of several visits as well as full-mouth supragingival scaling with an ultrasonic device. All subjects eventually demonstrated a plaque control record of under 20%. After the subjects underwent the periodontal examination, two periodontal pockets (probing depth [PD] 5–7 mm, with bleeding on probing [BOP]) were selected from the data for evaluation, such that the pockets were in different, single-rooted teeth, and each tooth was in a different quadrant. For baseline parameters, GCF or subgingival plaque samples were taken from each pocket. Subsequently, the selected pockets—specifically, each selected pocket and its diagonal sulcus—were treated by aPDT or MO. This treatment was repeated a week later. Subsequent sample collection and periodontal examination at the two selected sites were performed 1 and 4 weeks after treatment. Treatments were performed by one of two periodontists (TM, TY), who underwent sufficient training to minimize technical differences as much as possible. Clinical data was collected by independent periodontists (YK, MT). Calibration sessions for the examiners were

held at the beginning and middle of the study period. The two therapeutic procedures are described below.

2.3. Antimicrobial photodynamic therapy

The periodontal pocket was filled with the 0.01% methylene blue photosensitizer using a blunt needle in a coronal direction starting in the most apical portion. After a minute, the pocket was irradiated for 60 s using a 670-nm wavelength laser (Periowave^{TM} therapy, Ondine Bioharma Corporation, Canada) with an energy dose of 21 J/cm^2 and power output of 140 mW, in accordance with the manufacturer's instructions [18].

2.4. Treatment with minocycline ointment

Two percent minocycline gel (PERIOCLINE, Sunstar, Osaka, Japan) was gently inserted into the base of the periodontal pocket and then slowly pulled out in a zig-zag motion while continuing the injection, as described previously [19].

2.5. Clinical assessment

Five clinical parameters were recorded based on periodontal examination: BOP, PD, and

clinical attachment level (CAL) at six sites per tooth; plaque index (PlI) and gingival index (GI) at four sites per tooth. The rate of bone resorption was calculated on the basis of alveolar bone-defect depth measured using dental X-ray radiographs.

2.6. Sample collection

After removing the supragingival plaque on the targeted teeth, GCF collection was performed at one of the sites by consecutively inserting four sterile Perio-paper strips (Harco Electronics, Winnipeg, MB, Canada) into the orifice of the gingival crevice until mild resistance was felt; it was then left in place for 30 s per strip. At the second site, two sterile #40 paper points (Zipperer Absorbent Paper Points, VDW GmbH, Munich, Germany) were inserted consecutively into the periodontal pocket for 10 s per point to collect subgingival plaque samples. All strips absorbed GCF were stirred in 200 μL of phosphate buffer supplemented with 0.5% bovine serum albumin for 15 min at room temperature, which was then centrifuged at 12,000 × *g* for 10 min after removing the strips. The supernatant was collected, and immediately sent to a medical laboratory (Filgen Inc., Nagoya, Japan) for multiplex array analysis [20]. Meanwhile, the subgingival plaque samples were sent to BML Corporation (Tokyo, Japan) for bacterial analysis [7].

2.7. Quantification of periodontal bacteria from subgingival plaques

Quantitative analysis of total and periodontopathic bacterial counts, including *Porphyromonas gingivalis* and *Tannerella fosythia*, were performed using a modified Invader PLUS assay, as described previously [21, 22]. The proportions of the two pathogens compared to total bacterial counts were calculated [23]; the ratio (%) of each species was used for various comparisons as well as for bacterial count (log_{10}).

2.8. Terminal restriction fragment length polymorphism analysis

Two subjects—one from each group—were randomly selected for this experiment. Bacterial DNA samples from these subjects were submitted for terminal restriction fragment length polymorphism (T-RFLP) analysis at TechnoSuruga Laboratory Co. Ltd (Shizuoka, Japan), as previously described [24, 25]. In brief, PCR amplicons of the 16S rRNA gene were digested with 10 U of either *Hha*I or *Msp*I (Takara Bio, Shiga, Japan), and the resultant terminal restriction fragments (T-RFs) were analyzed by capillary electrophoresis using an ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems). GeneMapper® software (Applied Biosystems) was used to estimate the fragment sizes. Similarities in microbial patterns among the samples were elucidated with cluster analyses (GeneMaths; Applied Maths, Sint-Martens-Latem, Belgium). The results were arranged to produce a dendrogram. The dendrogram type was established by Pearson's similarity coefficient analysis and the

unweighted pair group method with the arithmetic mean [26].

2.9. Analysis of inflammatory mediators

GCF levels of ten inflammatory mediators were assayed by multiplex assay technique (ProcartaPlex multiplex immunoassays human Th1/Th2 cytokine panel; Affymetrix eBioscience, Santa Clara, CA, USA) according to the manufacturers' instruction manual. The assay was read by Bio-Plex 200 System (Bio-Rad) with the Bio-Plex Manager software v6.0 (Bio-Rad) [27]. To demonstrate a high level of correlation between measurements, duplicate measurements were performed with a subset of samples, for which the intraclass correlation coefficients varied from 0.95 to 1.0 ($P < 0.001$). The following cytokines were measured: interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF).

2.10. Statistical analysis

Data were subjected to descriptive analysis, and the results are presented as means ± standard deviation. All intergroup comparisons were performed using the Mann–Whitney U test, except gender distribution, which was conducted by Fisher's exact test. A P value < 0.05 was considered statistically significant. Intragroup comparison of clinical, bacterial, and GCF

markers at three time points was performed using the Wilcoxon signed–rank test with the Bonferroni correction for which the accepted significance threshold was $P < 0.017$. All analyses were performed using IBM SPSS Statistics V19 (IBM Japan, Tokyo, Japan). Sample size calculation was performed before the study was initiated. According to Cohen's suggestion [28], setting the effect size = 0.80, $\alpha = 0.05$ and the power at 80%, the sample size calculation yielded 12 subjects per group. Thus, we recruited 15 subjects per group.

3. Results

All participants successfully completed the study protocol, and postoperative healing was uneventful in all cases. None of the subjects reported any general or oral health problems during the study period. Table 1 shows the clinical and demographic data of the study subjects. No statistically significant difference were observed between the two groups in all characteristics.

Table 2 shows the intra- and intergroup comparisons of the clinical parameters and subgingival bacterial levels at sites treated with aPDT or MO. Relative to baseline values, the mean scores for PD (mm), CAL (mm) and BOP (% positive) had significantly improved at 4 weeks after aPDT ($P < 0.017$); the corresponding scores in the MO group had also significantly improved at 1 and 4 weeks post-treatment ($P < 0.017$). Further, the MO group exhibited a significantly lower PD score than the aPDT group at 1 week post-treatment ($P < 0.05$). In the aPDT group, no significant difference was apparent in bacterial count or ratio (bacterial count of each species/total bacterial count) of either of the bacterial species among the various time points. In contrast, the bacterial counts and ratios of both *P. gingivalis* ($P < 0.01$) and *T. forsythia* ($P < 0.01$) had significantly decreased a week after application of MO; a significant reduction in *P. gingivalis* count was still observed even 4 weeks after MO treatment ($P < 0.01$). At 1 week post-treatment, the bacterial counts and ratios of both *P. gingivalis* ($P < 0.001$) and *T. forsythia* ($P < 0.01$) in the MO group were significantly lower

than those in the aPDT group.

Figure 2 presents a representative case from each group, indicating the relative abundance of T-RFs of the 16S rRNA gene after restriction digestion by *Hha*I (Fig. 2a) or *Msp*I (Fig. 2b) at three time points. The comparison of the peak area ratio (PAR) of T-RFs at 1 week post-treatment to those at baseline among various genera showed a tendency of increase in the PAR of *Porphyromonas*, *Prevotella* in the aPDT group. On the other hand, the PAR of T-RFs of some genera, including *Porphyromonas*, *Prevotella*, and *Fusobacterium*, tended to decrease a week after subgingival application of MO.

Figure 3a presents the levels of GCF inflammatory mediators at baseline and 1 and 4 weeks after aPDT. The levels of all markers, except those of IL-1β and IL-13, decreased gradually from baseline, although the decrease was not statistically significant. Figure 3b shows the levels of GCF inflammatory mediators at baseline and 1 and 4 weeks after local administration of MO. Relative to the baseline values, there was a marked decrease in the levels of IL-1β (P = 0.0022) and IFN-γ (P = 0.0032) a week after treatment. The levels of IL-1β (P = 0.0076) and IFN-γ (P = 0.0076) had significantly decreased relative to the baseline at 4 weeks after treatment. There were no significant changes in the levels of IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, TNF-α and GM-CSF among the three time points. No significant intergroup difference was observed in the level of any of the cytokines.

4. Discussion

In this study, we examined the clinical, microbiological, and local host immune-inflammatory responses in patients with chronic periodontitis to the treatment of periodontal pockets with two different modalities (i.e., aPDT and MO). We demonstrated that MO provides effective clinical and anti-bacterial outcomes as well as positive modulation of local cytokine levels. In contrast, aPDT did not provide any additional benefits within the limitations of this study.

We found significant differences in the levels of several GCF cytokines after local application of MO. Interleukin-1β and IFN-γ were the only cytokines that had decreased in concentration, relative to the baseline, a week after treatment and remained at significantly lower levels until the end of the observation. Interleukin-1β is known as a representative pro-inflammatory cytokine which initiate and regulate the inflammatory response, and mediate the periodontal tissue destruction; its production and cellular response is partly affected by IFN-γ [29]. To our knowledge, no study to date has reported the effects of monotherapy by MO on GCF cytokine levels. Therefore, the present study is the first report of changes in GCF cytokine levels in periodontal pockets due to monotherapy by MO before mechanical therapy. It was interesting to observe a similar tendency of decrease after therapy in IL-1β, IFN-γ, TNF-α, and GM-CSF; as with IL-4 and IL-6. It was somewhat surprising that the levels of IL-1β were markedly higher than those of other cytokines. Direct comparison of the present results with those of other studies might be difficult because of

differences in evaluation techniques for selected mediators in GCF between the present and previous studies [30]. A recent study of 14 different immunoassays reported important inter-assay disparities as well as marked variations among laboratories, suggesting the results obtained using a specific assay cannot be compared with those obtained with another type of assay [31].

Although MO administration promoted significant changes in bacterial and GCF markers, those inflammatory markers hardly exhibited any changes in the aPDT group. This difference might be attributable to the presence of subgingival calculus. Patients not yet subjected to subgingival scaling and SRP were the target subjects of this study. Before commencement of the study, we removed only supragingival plaque and tartar because these bacterial deposits interfere with insertion of the applicator tip for MO administration, application of the photosensitizer, and light illumination into periodontal pockets. Nevertheless, it is possible that, during insertion of the laser probe into the base of the periodontal pocket, the tip was obstructed by subgingival calculus, and, consequently, laser irradiation failed to adequately produce oxygen radicals. However, it appears that, owing to their physical properties, both MO and the photosensitizer could reach the deeper part of the periodontal pocket. Recently, Kolbe et al. [32] reported monotherapy by aPDT to be advantageous in terms of modulation of cytokines; however, in contrast to the present study, the previous study targeted periodontal pockets that were already treated by SRP. de Oliveira et al. [33], too, reported the effects of

aPDT on crevicular inflammatory mediators in patients with aggressive periodontitis. Aggressive periodontitis exhibits features such as phagocyte abnormalities and a self-limiting disease pattern and is, therefore, different from chronic periodontitis, which was the disease target in the present study [34]. In terms of methodology, it appears to be challenging to achieve an adequate therapeutic effect with aPDT before subgingival mechanical treatment for periodontal pockets. This method might be better suited for combination therapy with SRP, or monotherapy of periodontal pockets with relatively small amounts of dental calculus during supportive periodontal therapy [32].

A week after MO administration in the present study, subgingival bacterial counts of *P. gingivalis* and *T. forsythia* had reduced by approximately $1/100^{th}$ and $1/10^{th}$ from baseline levels, respectively. It is interesting that, despite their comparable bacterial counts at baseline, there was a substantial difference in decrement between the two species; other studies involving local application of MO in patients undergoing supportive periodontal therapy [35] or combination therapy with MO and SRP [36] have also reported a similar tendency. Furthermore, in the present study, only the *P. gingivalis* population continued to remain significantly low at 4 weeks post-treatment. Therefore, we believe that *P. gingivalis* might be more susceptible to minocycline than *T. forsythia*. In a previous study, using the same detection method as the present study, we demonstrated a reduction in *P. gingivalis* count in periodontal pockets by $1/100^{th}$ of the baseline level after oral medication with AZM [3]. This

may mean that local application of MO exhibits the same degree of microbicidal efficacy as systemic administration of AZM. Curiously, our results showed no significant change in total bacterial count in the MO group. Okuda et al. [37] reported that local delivery of MO causes a decrease in the proportions of periodontal pathogen such as spirochetes, motile rods, dark-pigmented *Bacteroidens* spp., and *Prevotella intermedia* and, contradictorily, an increase in the proportions of cocci and *Streptococcus* spp., which are considered favorable for periodontal health. This differential effect of MO might also have occurred in the periodontal pockets treated in the present trial. Indeed, the present results of T-RFLP analysis showed a sharp decrease in the PAR of the *Porphyromonas, Prevotella* genera, but an increase in the PAR of *Streptococcus* a week after MO injection.

In the MO group, we observed significant improvements in PD, CAL and BOP at the treated sites; the BOP scores, especially, had improved drastically. This might have been due to an increase in tissue resistance to periodontal probing force brought about by a post-treatment improvement in bacterial flora and the subsequent decrease in inflammation in the periodontal pockets [38]. It is somewhat strange that the aPDT group, too, exhibited improvements in clinical parameters. Considering the lack of significant changes in bacterial markers in this group, it is difficult to determine the most probable reason for the improvement in clinical parameters; one of the possible causes could be the Hawthorne effect.

Most previous studies have reported the adjunctive effects of MO to SRP, with the aim of

thoroughly eliminating bacterial populations remaining in the periodontal pockets after mechanical treatment [39, 40]. In terms of the healing effect, combination treatment might be superior to monotherapy; however, we purposely designed the study to reliably establish the efficacies of these treatments as monotherapies. In addition, we designed this study keeping in mind the possibility of future application of these treatments for the prevention of bacteremia after mechanical treatment. A notable finding in the present study is that the inflammatory cytokine levels in GCF were significantly decreased after MO administration without SRP. Therefore, this approach may have potential as a prophylaxis for bacteremia secondary to SRP, though its application should be carefully managed to prevent the local appearance of drug-resistant organisms.

5. Conclusion

In conclusion, we have demonstrated that local MO administration as monotherapy provides clinical and anti-bacterial benefits as well as effective local cytokine modulation. However, there was no additional benefit with aPDT as monotherapy within the limits of this study.

Conflicts of interest

The authors have no conflict of interest to declare.

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Figure legends

FIGURE 1: Flowchart of the study from enrollment to completion of the trial.

FIGURE 2: A representative case indicating the relative abundance of terminal restriction fragments of the 16S ribosomal RNA gene after restriction digestion by *Hha*I (a) or *Msp*I (b) in the antimicrobial photodynamic therapy (a-PDT) and minocycline ointment (MO) groups at three time points.

FIGURE 3: Changes in IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, IFN-γ, TNF-α, and GM-CSF levels in gingival crevicular fluid in the aPDT (a) and MO (b) groups. Results are shown as mean ± standard deviation. Data were adjusted for multiple comparison. *Significant changes at $p < 0.017$. IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; aPDT, antimicrobial photodynamic therapy; MO, minocycline ointment.

Table 1. Characteristics of the study population

	aPDT group (n = 15)	MO group (n = 15)
Gender (male/female)	7/8	6/9
Age (years)	61.4 ± 10.2	66.7 ± 9.5
Number of teeth (n)	23.3 ± 3.7	24.5 ± 3.6
Bone resorption (%)	24.3 ± 7.0	28.3 ± 9.6
PlI	0.4 ± 0.7	0.5 ± 0.6
GI	0.9 ± 0.7	1.0 ± 0.6
PD (mm)	3.0 ± 0.9	2.9 ± 0.8
CAL (mm)	3.8 ± 1.1	3.7 ± 1.2
BOP (% positive)	27.1 ± 16.5	28.6 ± 15.7

Values are represented as mean ± standard deviation. Gender: Fisher's exact test ($P < 0.05$).

Other parameters: Mann–Whitney U test ($P < 0.05$).

Table 2. Changes of clinical parameters and bacterial levels after aPDT or local minocycline administration

	(a) Baseline		(b) 1 week post-treatment		(c) 4 weeks post-treatment		*p*-value						
							between (a) and (b)		between (a) and (c)		between aPDT and MO groups		
	aPDT group	MO group	aPDT group	MO group	aPDT group	MO group	aPDT group	MO group	aPDT group	MO group	(a)	(b)	(c)
PD-treated sites (mm)	5.8 ± 1.0	5.4 ± 1.1	5.4 ± 1.3	4.7 ± 1.3	4.8 ± 1.4	4.3 ± 1.4	0.0569	0.0024†	0.0003†	0.0001†	0.1417	0.045*	0.1581
CAL-treated sites (mm)	6.8 ± 1.6	6.1 ± 1.8	6.2 ± 1.8	5.4 ± 1.6	5.8 ± 1.8	5.1 ± 1.7	0.0254	0.004†	0.0005†	0.0022†	0.1809	0.1275	0.2443
BOP-treated sites (% positive)	76.7 ± 43.0	80.0 ± 40.7	66.7 ± 47.9	43.3 ± 50.4	40.0 ± 49.8	46.7 ± 50.7	0.4054	0.0023†	0.0116†	0.0039†	0.756	0.0717	0.6054
Total bacteria (log_{10})	4.72 ± 1.05	4.53 ± 0.87	4.68 ± 0.76	4.16 ± 0.53	4.57 ± 1.11	4.17 ± 0.67	0.8015	0.1228	0.4771	0.2101	0.3826	0.0531	0.0805
P. gingivalis (log_{10})	2.63 ± 1.62	2.68 ± 1.44	2.42 ± 1.68	0.61 ± 0.41	2.28 ± 1.79	1.12 ± 1.31	0.8753	0.0033†	0.3574	0.0053†	0.9331	0.0006*	0.0548
P. gingivalis ratio (%)	2.70 ± 3.47	5.80 ± 12.11	3.59 ± 4.39	0.01 ± 0.02	3.20 ± 4.48	1.20 ± 3.49	0.4328	0.0022†	0.7213	0.0414	0.6153	0.0006*	0.0518
T.forsythia (log_{10})	3.01 ± 1.50	2.89 ± 1.31	2.89 ± 1.54	1.45 ± 0.87	2.87 ± 1.51	2.03 ± 1.23	0.7986	0.0032†	0.5627	0.064	0.5883	0.0088*	0.0915
T.forsythia ratio (%)	4.08 ± 3.58	4.27 ± 4.60	4.75 ± 4.62	0.77 ± 1.03	4.51 ± 4.30	2.62 ± 4.70	0.7537	0.0019†	0.7532	0.2719	0.9834	0.0083*	0.1694

Data are presented as mean ± standard deviation. Ratio was defined as individual bacteria count/total bacterial count. Intergroup comparison between groups: Mann-Whitney U test (*: $P < 0.05$). Intragroup comparisons: Wiloxon signed-rank test with the Bonferroni correction for multiple comparisons (†: $P < 0.017$).

FIGURE 1

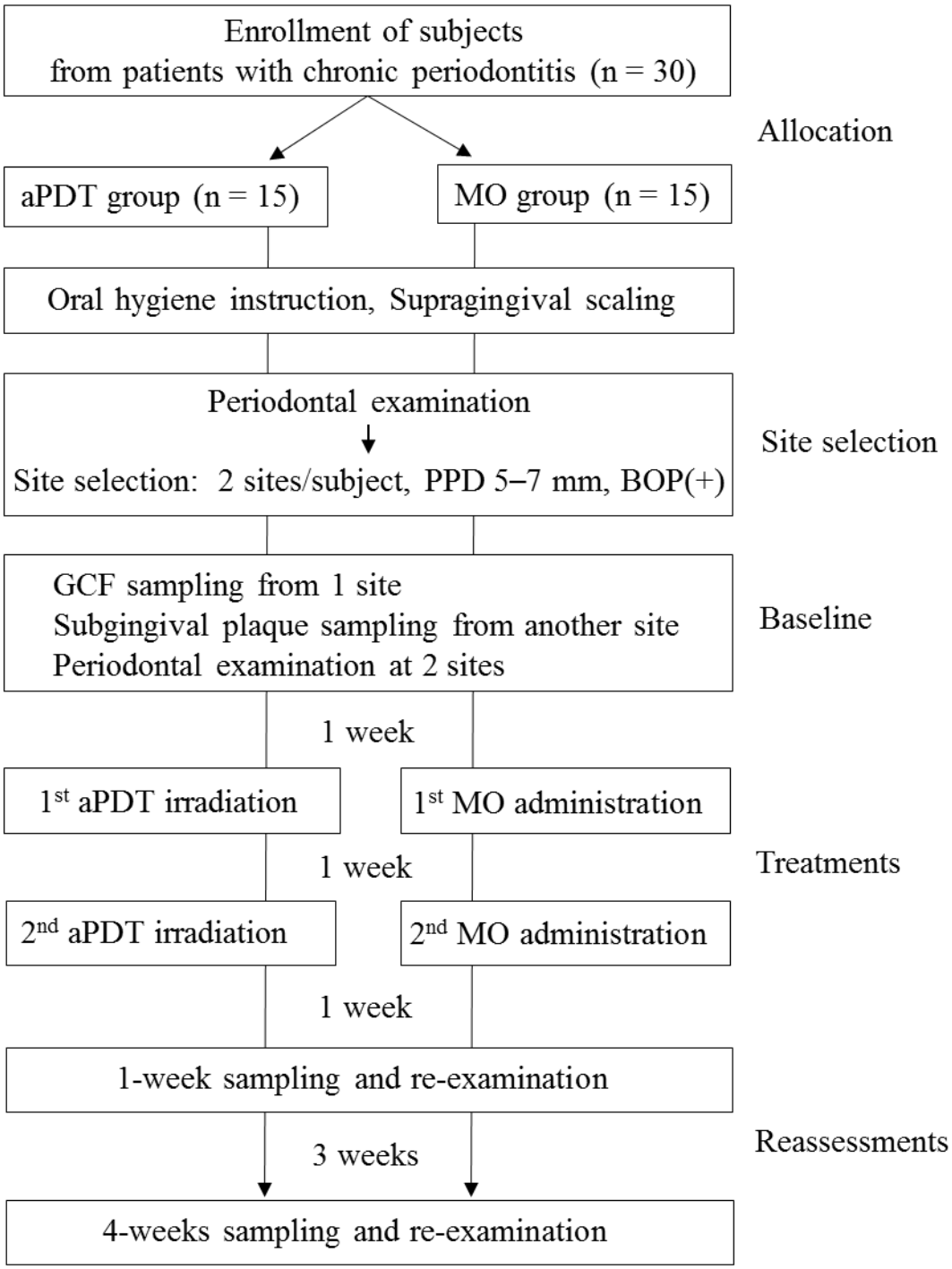
Enrollment of subjects
from patients with chronic periodontitis (n = 30)
Allocation
aPDT group (n = 15)
MO group (n = 15)
Oral hygiene instruction, Supragingival scaling
Periodontal examination
Site selection: 2 sites/subject, PPD 5–7 mm, BOP(+)
Site selection
GCF sampling from 1 site
Subgingival plaque sampling from another site
Periodontal examination at 2 sites
Baseline
1 week
1st aPDT irradiation
1st MO administration
1 week
Treatments
2nd aPDT irradiation
2nd MO administration
1 week
1-week sampling and re-examination
3 weeks
Reassessments
4-weeks sampling and re-examination

FIGURE 2

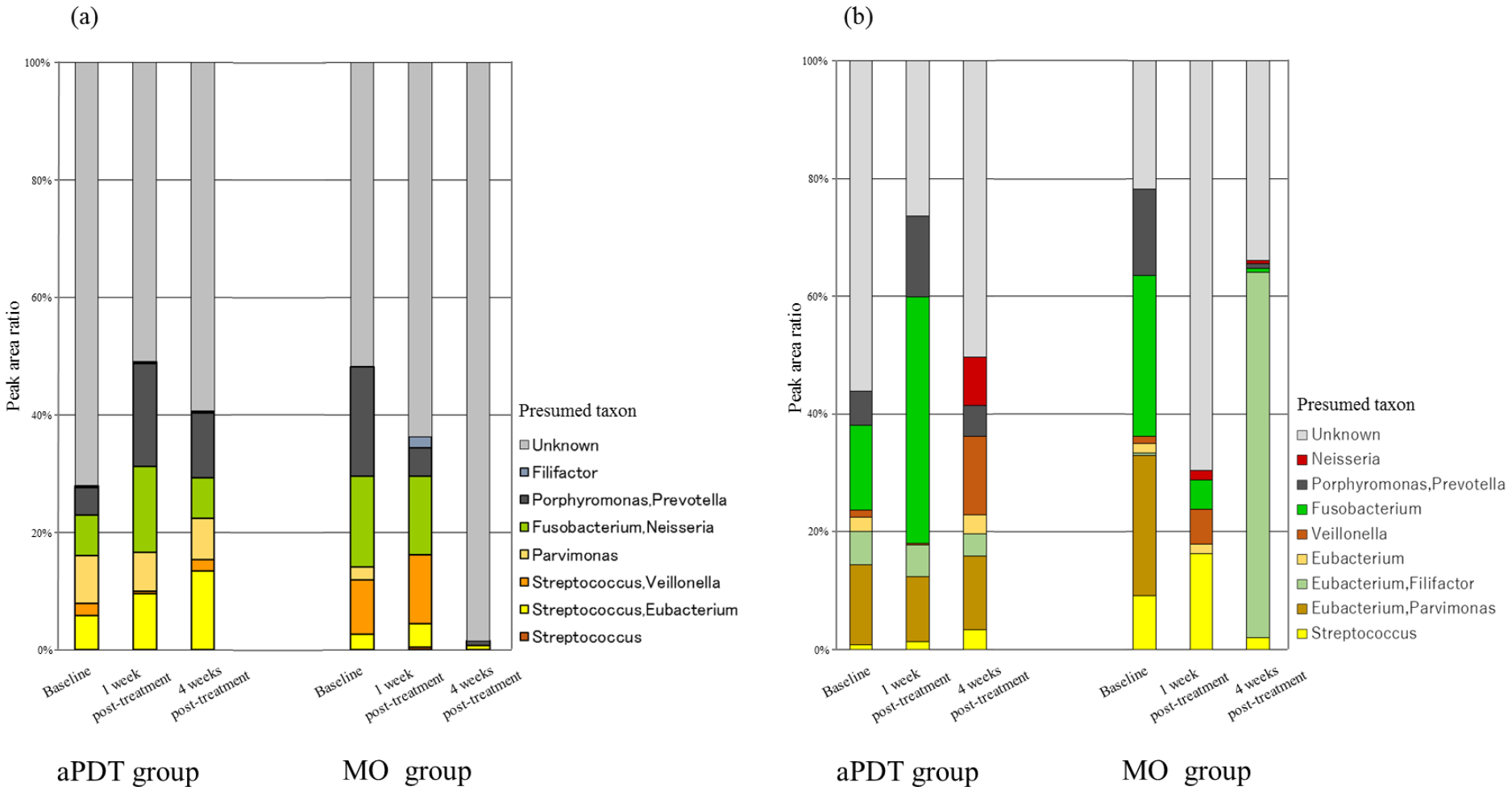
(a)
(b)
Peak area ratio
100%
80%
60%
40%
20%
0%
Baseline
1 week post-treatment
4 weeks post-treatment
Presumed taxon
Unknown
Filifactor
Porphyromonas,Prevotella
Fusobacterium,Neisseria
Parvimonas
Streptococcus,Veillonella
Streptococcus,Eubacterium
Streptococcus
Neisseria
Fusobacterium
Veillonella
Eubacterium
Eubacterium,Filifactor
Eubacterium,Parvimonas
aPDT group
MO group

FIGURE 3 (a)

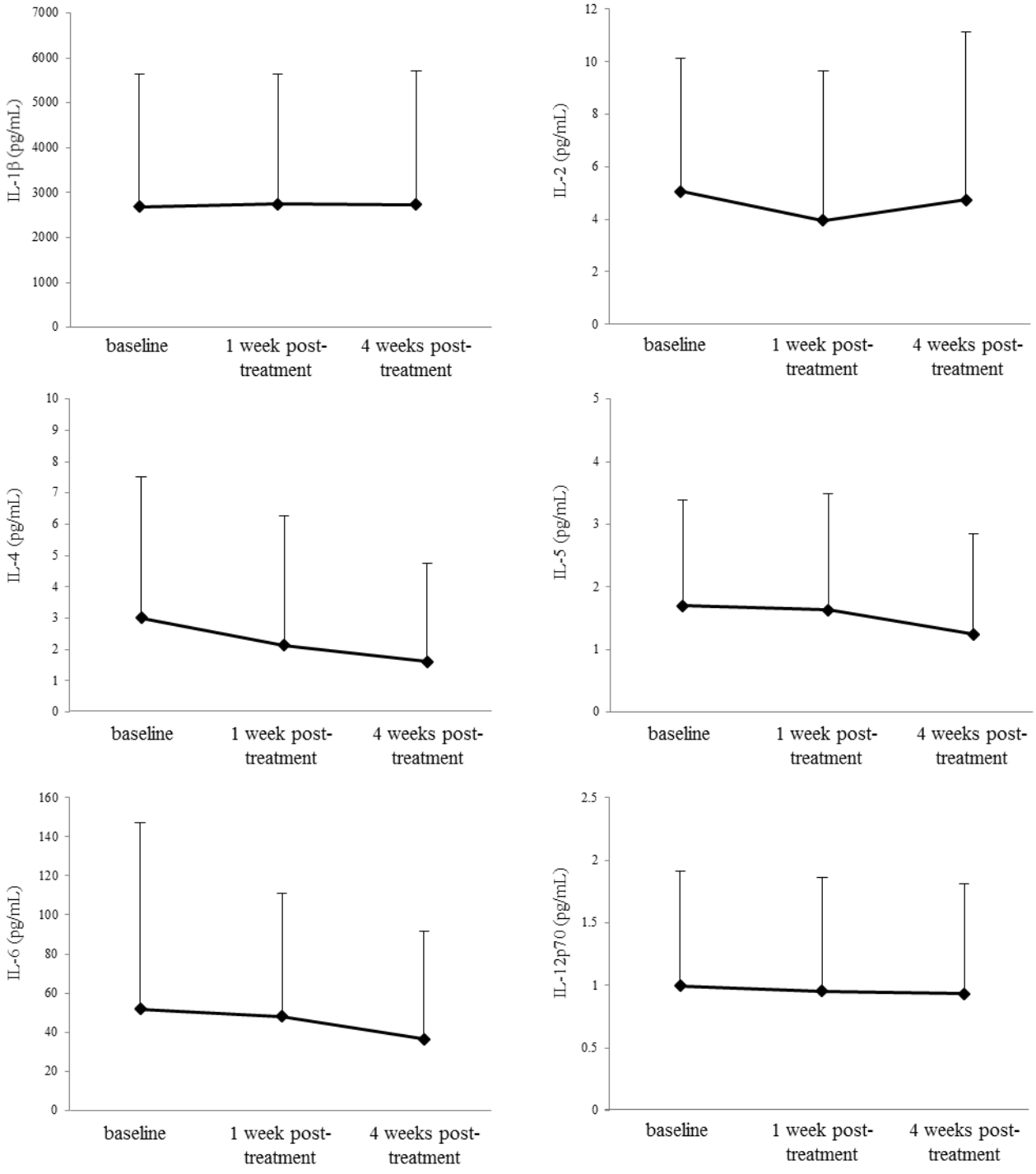
IL-1β (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-2 (pg/mL)
12
10
8
6
4
2
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-4 (pg/mL)
10
9
8
7
6
5
4
3
2
1
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-5 (pg/mL)
5
4
3
2
1
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-6 (pg/mL)
160
140
120
100
80
60
40
20
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-12p70 (pg/mL)
2.5
2
1.5
1
0.5
0
baseline
1 week post-treatment
4 weeks post-treatment

FIGURE 3 (a) (continued)

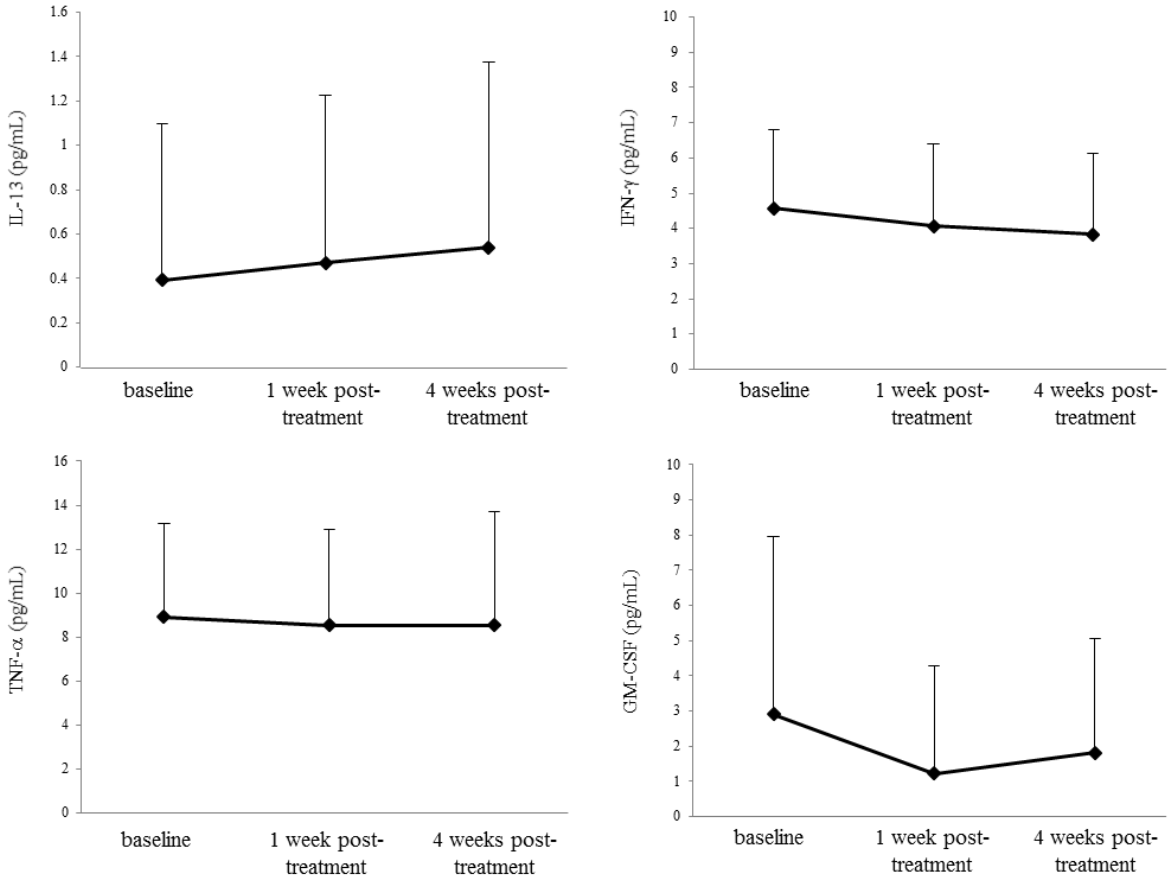

FIGURE 3 (b)

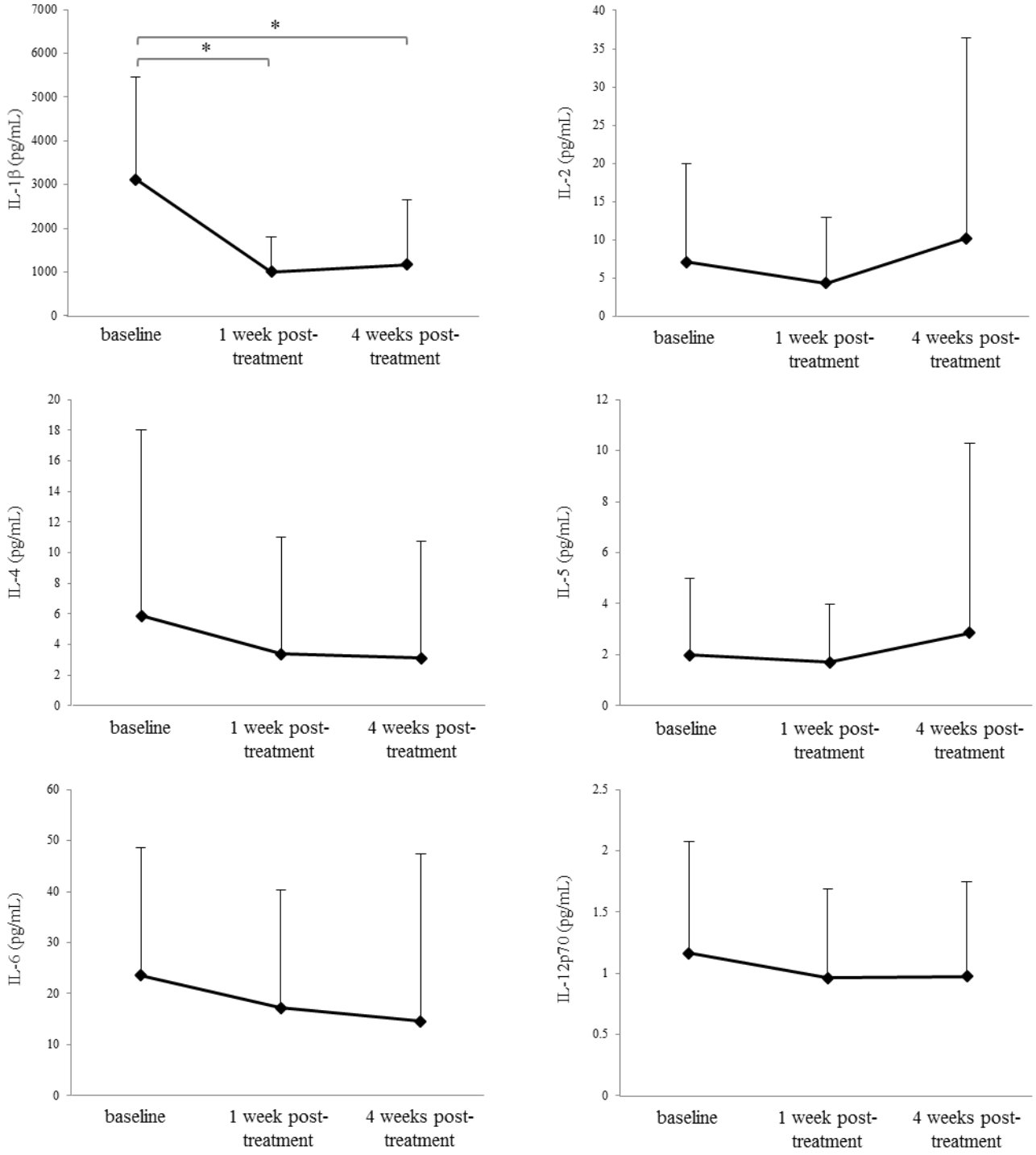
IL-1β (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
*
*
baseline
1 week post-treatment
4 weeks post-treatment
IL-2 (pg/mL)
40
35
30
25
20
15
10
5
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-4 (pg/mL)
20
18
16
14
12
10
8
6
4
2
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-5 (pg/mL)
12
10
8
6
4
2
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-6 (pg/mL)
60
50
40
30
20
10
0
baseline
1 week post-treatment
4 weeks post-treatment
IL-12p70 (pg/mL)
2.5
2
1.5
1
0.5
0
baseline
1 week post-treatment
4 weeks post-treatment

FIGURE 3 (b) (continued)