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Biomarker levels in gingival crevicular fluid of generalized aggressive periodontitis patients after non-surgical periodontal treatment.

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ABSTRACT

Objectives: The aim of this study was to assess the effects of non-surgical periodontal treatment on gingival crevicular fluid (GCF) cytokines in patients with generalized aggressive periodontitis (GAgP), in relation to clinical parameters.

Materials and Methods: Data were obtained from 16 GAgP patients and 15 periodontally healthy controls. Periodontal parameters and GCF biomarker levels were evaluated at baseline, and repeated 3 and 6 months after treatment for GAgP subjects. Moderate and deep pocket sites were analysed separately. The amount of interleukin (IL)-1β, IL-9, tumor necrosis factor (TNF)-α, platelet-derived growth factor (PDGF-bb) and vascular endothelial growth factor (VEGF) were measured using a highly specific and sensitive multiplex bead immunoassay.

Results: At baseline cytokine levels in the moderate and deep pocket sites of GAgP patients were higher than those of the healthy control sites. In GAgP group periodontal treatment led to improvement in all examined clinical parameters and resulted in a statistically significant reduction in the total amounts of IL-1β, VEGF, and TNF-α, in comparison to baseline, already 3 months after therapy in both moderate and deep pocket sites and of PDGF-bb in deep sites ($p < 0.01$). At the concentration level, only IL-1β and VEGF were affected.

Conclusion: Non-surgical treatment of GAgP provided significant clinical benefits leading to a marked decrease in the GCF levels of some pro-inflammatory and pro-angiogenic cytokines, but not of IL-9 and PDGF-bb.

Clinical relevance: Although the periodontal therapy successfully decreased clinical signs of inflammation, the GCF levels of some inflammatory cytokines were still elevated.

Keywords: aggressive periodontitis; gingival crevicular fluid; inflammatory mediators; periodontal/therapy.

Introduction

Generalized aggressive periodontitis (GAgP) has a complex pathogenesis in which a particular host response to the long term microbial challenge has been proposed to contribute to its clinical manifestations in terms of disease onset and progression rate [1]. As such, beyond genetic characteristics [2] and functional defects of polymorphonuclear leukocytes [3], previous studies have considered the periodontal levels of inflammatory mediators that may be involved in protective and damaging reactions to periodontal pathogens [4, 5]. A noninvasive approach to monitor the local host response involves analysis of such biomarkers in the gingival crevicular fluid (GCF) [6]. There is increasing evidence that patients with GAgP have higher GCF concentrations of inflammatory markers compared to healthy control subjects [7-9], but it is not clear whether they share similar cytokine profile to chronic periodontitis patients [10]. Unlike data for classic markers such as interleukin (IL)-1ß, IL-6, IL-8 and tumor necrosis factor (TNF)-α, there is no or little evidence regarding the GCF levels of mediators involved in angiogenesis and in modulation of inflammatory/immune response before and after periodontal treatment [11-14]. Angiogenesis is a prominent feature of inflammation and healing, but its role in promoting the progression of periodontal lesions is not yet clear [15]. Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that plays a pivotal role during inflammation by mediating neovascularization [16]. There is considerable evidence that VEGF increases micro-vascular permeability, stimulates endothelial cell proliferation, and induces proteolytic enzymes and migration of endothelial cells and monocytes [17]. In recent years, the possible role of VEGF in the pathogenesis of chronic periodontitis has been investigated in some clinical studies with conflicting results [18-20]. Platelet-derived growth factor (PDGF) is another key mediator for vascular development and is one of the most potent serum mitogens. It promotes cell migration and proliferation and stimulates fibroblasts to synthetize collagen and proteoglycans [21]. While VEGF initiates endothelial cell proliferation and blood vessel formation, PDGF is an important growth factor for maturation and remodeling of newly formed blood vessels [22]. VEGF expression is regulated by IL-1 and TNF-α, which are primary mediators in the early inflammatory response and are able to induce tissue destruction and bone loss in periodontal diseases [23]. These cytokines induce vascular alterations and prompt migration to the

periodontium of different cells, e.g. neutrophils [24, 25], and are involved in T-helper type 1 (Th1) cells immune response [26].

Recently, Th9 cells were discovered and shown to interact with the Th1 subpopulation in the modulation of inflammatory/immune responses [27]. Th9 cells characteristically produce IL-9, which can exert anti-inflammatory activities by modulating IL-1 and TNF-α production [28]. Previous investigations demonstrated that IL-9 is expressed in both human granulomas and animal experimental periapical lesions as it is related to the lesion stability [29, 30]. Apart from one study analyzing the IL-9 serum levels in chronic periodontitis patients, no data are available on its role in periodontal diseases [31].

In our hypothesis, the higher GCF concentrations of inflammatory mediators observed in patients suffering from GAgP should be reduced after periodontal treatment, as well as those of angiogenic mediators. Anyhow, the relationship between cytokine profiles and clinical state of periodontal sites has not yet been clarified in patients suffering from GAgP [12,13,32]. Therefore, the aim of the present study was to determine whether non-surgical periodontal treatment for GAgP would change the GCF levels of cytokines involved in angiogenesis and inflammatory pathways. The treatment effects on GCF level of IL-1β, IL-9, TNF-α, PDGF-bb and VEGF in GAgP patients were examined in relation to the severity of initial periodontal damage and compared to periodontally healthy controls.

Material and methods

Study design

The GAgP and periodontally healthy subjects participating in the study were consecutively recruited from a pool of first time patients at the Section of Periodontology, C.I.R. Dental School, Department of Surgical Sciences, University of Turin (Italy). The study was conducted between May 2015 and February 2017 in accordance with the Helsinki Declaration of 1975, as revised in 2002 and was approved by the local Ethics Committee (Protocol n° 0119237). Informed consent was obtained from each patient before the study.

GAgP patients were Caucasians and were aged between 18 and 35 years. The diagnosis of GAgP was done according to the clinical and radiographic criteria by the 1999 classification of GAgP [33] and required familiar aggregation (during the anamneses patients were asked whether they had at least one

member of the family presenting or with a history of periodontal disease) [14]. They were also required to have at least 20 natural teeth and to demonstrate a minimum of 12 teeth with probing depth (PD) and clinical attachment level (CAL) ≥ 5 mm, and radiographic evidence of alveolar bone loss. At least six teeth apart from first molars or incisors had to be involved [14].

To be included in the study, the periodontally healthy subjects had to be systemically healthy, have no PD and CAL >3 mm, less than 15% of the sites with bleeding on probing (BoP), and no horizontal or vertical bone loss in radiographic examination.

Exclusion criteria for the two groups were: pregnancy, lactation, current or past smoking, allergy, asthma, periodontal treatment or/and antibiotic therapies in the previous 6 months, and any systemic disease that could influence the course of periodontal disease. Subjects using anti-inflammatory and immunosuppressive medications, or any other medications known to affect periodontal status were also excluded.

Sample size calculation

The change in VEGF levels after non-surgical periodontal treatment was set as the primary outcome. The sample size was calculated using variations reported for total amount of VEGF in chronic periodontitis patients [20] and an expected effect size. The minumum expected difference was assumed to be 6.0 pg/ml and the amount of variation reported was 10.9 pg. Therefore, at 0.05 two-sided alpha error and 80% power, the calculated sample size was 14 GAgP patients for this paired trial that increased to 16 for compensation of possible dropout.

Clinical Examination and Periodontal Treatment

All enrolled subjects underwent periodontal examination by an experienced periodontist (F.R.). After calibration, a 94.8% concordance within 1 mm for measurements of PD and CAL between the first and the second recording with an interval of 24 h was reached. Clinical measurements were taken at six sites per tooth of every tooth present, except third molars, with a standardized periodontal probe (PCP UNC15, Hu-Friedy, Chicago, IL, USA) and included presence of plaque (PI), BoP, gingival index (GI) [34], PD, and CAL. Full-mouth percentage of sites with PI (full-mouth plaque score, FMPS) and BoP (full-mouth bleeding score, FMBS) was also recorded. Full-mouth periapical radiographs were taken with the long cone paralleling using Rinn holders.

After baseline examination, all GAgP subjects underwent a session of supragingival scaling and polishing and received instructions in proper self-performed plaque control measures, including instructions in the Bass technique and interproximal cleaning with dental floss and interdental brushes. One week later, patients were subjected to quadrant-wise full-mouth subgingival scaling and root planing (SRP) in four sessions by two experienced dental hygienists (L.Bi. and L.Bo.) using hand instruments (Gracey curettes, Hu-Friedy, Chicago, IL, USA) and ultrasonic scalers (Cavitron Select, Dentsply, York, PA, USA). Subgingival instrumentation was performed under local anaesthesia without a time limit until the root surface felt smooth and clean to an explorer tip. The entire non-surgical periodontal treatment was completed in 28 days without the administration of local and/or systemic antimicrobials. Supportive therapy, including professional plaque control and reinstruction of oral hygiene was performed on a 2-week interval during the first 6 weeks postoperatively and every 2 months up to the 6-month evaluation. Reevaluations were performed 3 and 6 months after completion of SRP procedure.

GCF sampling

The gingival crevicular fluid (GCF) samples were collected on the following day the clinical examination to prevent their contamination with blood due to probing of inflamed sites. Two inflamed moderate (sites with redness or BoP, PD 4-5 mm) and deep sites (sites with BoP, PD ≥ 6 mm) with no endodontic involvement were selected on the mesial aspect of anterior periodontally involved teeth in contralateral quadrants in GAgP patients. The same sites were sampled at baseline, 3 and 6 months after treatment. In the healthy control group two sites with absence of plaque and inflammation were sampled on the mesiobuccal aspect of anterior teeth.

Sites to be sampled were isolated with cotton rolls and supragingival plaque was carefully removed. After the sites were gently dried with air syringe, GCF samples were collected with paper strips (PerioPaper Strips, Oraflow Inc., Plainview, NY, USA) that were inserted into the pocket until mild resistance was felt and then allowed to remain there for 30 s. Strips contaminated by bleeding were discarded. The amount of collected GCF was measured using an electronic device Periotron 8000 (Oraflow Inc., Plainview, NY, USA), which was calibrated based on a protocol described before [35]. The readings from the electronic instrument were converted to an actual volume (μl) by reference to

the standard curve. Throughout the experimental period, the reliability of the calibration of the device was checked at periodic intervals and, when necessary, it was renewed by triplicate readings. All strips with GCF were placed separately into coded sealed eppendorf microcentrifuge tubes containing 100 µl of sterile phosphate-buffered saline (PBS) and stored at -80°C until processing.

Multiplex bead immunoassay

IL-1β, TNF-α, IL-9, PDFG-bb, and VEGF concentrations were detected in biological samples by means of a high-sensibility Bio-Plex Suspension Array System (Bio-Rad Laboratories S.r.l., Segrate, Milan, Italy) according to the manufacturer's instructions. Briefly, opportune anticytokine antibody-conjugated beads were loaded into individual wells of a 96-well plate. After washing, standards and GCF undiluted samples were added into respective wells and incubated 30 min. After plates were washed, biotin-conjugated detection antibody was added. After another 30 min of incubation and consequent washing, streptavidin-conjugated PE was added for 10 min. After an additional wash, the complex was solubilized by adding the Bio-Plex assay buffer to each well. Then, plates were analyzed with the Bio-Plex Suspension Array System. Total amounts (pg) and concentrations (pg/µl) of each cytokine were determined.

Statistical analysis

A statistical software program (Graphpad Prism version 6.0e, GraphPad Software, San Diego, CA, USA) was used for data analysis. The primary outcome measure of the study was mean reduction in VEGF levels after therapy. Secondary outcomes included changes of GCF levels of IL-1β, IL-9, TNF-α, PDGF-bb as well as changes in clinical parameters. Only clinical measurements at the GCF sampling sites (experimental sites) were included in the present calculations. The mean values for each clinical and cytokine parameter was then calculated for each subject and averaged across subjects at each time points separately.

The Shapiro–Wilk test and Q-Q normality plots were applied to verify the normal distribution of the continuous variables. The significance of changes in clinical data with time in moderate and deep pocket sites was determined using the repeated measures ANOVA (PD, CAL, GCF volume) or the Friedman test (PI, GI). Pairwise multiple comparisons were performed by the Tukey test or the Bonferroni corrected Wilcoxon signed rank test.

Changes in cytokine concentration and total amount through the follow-up period in different PDs in the GAgP and healthy control groups were evaluated by means of the ANOVA test followed by the post hoc Tukey test. The statistical significance of correlations between clinical parameters and biomarkers was determined using the Pearson and Spearman correlation test as appropriate. *P* values < 0.05 were considered statistically significant.

Results

Twenty-six GAgP patients and twenty-two control subjects were consecutively screened for enrolment. Fourteen patients did not meet the inclusion criteria and three patients did not attend the baseline examination. Sixteen GAgP subjects (nine males and seven females, mean age 32.3 ± 4.3 yrs) were enrolled and completed the trial. The control group included six males and nine females with a mean age of 30.2 ± 6.1 yrs.

Clinical parameters of the experimental sites selected for GCF sampling in GAgP and healthy controls are presented in Table 1. As expected, mean values of all periodontal parameters were significantly higher in GAgP than in control sites ($p < 0.001$).

The postoperative healing was uneventful in all GAgP cases. No complications, such as abscess or infection, were observed throughout the study. Plaque level, presence and severity of inflammation, mean PD and mean CAL improved at 3 and 6 months after periodontal treatment ($p < 0.001$) in both moderate and deep pocket sites. PD reduction and CAL gain were significantly greater at deep sites at both 3- and 6-month examination ($p < 0.001$). Periodontal treatment was also associated with a statistically significant reduction in mean GCF volumes that was more pronounced in deep sites ($p < 0.001$).

As reported in Table 2, mean FMPS and FMBS decreased in GAgP group from baseline to 6 months ($p < 0.001$). At 3 and 6 months after therapy, no statistically significant differences were observed for FMPS values for the two groups.

Significant differences were detected over time in IL-1β (Fig. 1) and VEGF (Fig. 2) concentration and total amount in the GAgP group with a different trend according to the baseline PD values. Both cytokines had significantly lower concentration at deep sites already 3 months after the non-surgical treatment ($p < 0.05$), whereas at moderate sites, a statistically significant decrease was observed after 6

months ($p < 0.001$ IL-1β and $p < 0.05$ VEGF, respectively) (Fig. 1A and Fig. 2A). Furthermore, the concomitant reduction in GCF volumes induced a significant decrease ($p < 0.001$) of IL-1β (Fig. 1B) and VEGF (Fig. 2B) collectable picograms already after 3 months at both moderate and deep pocket sites.

A transient increment in IL-9 concentration was detected at moderate sites at 3 months postoperatively ($p < 0.05$), but concentration lowered until reached not significant differences with respect to baseline values at 6 months (Fig. 3A). No significant changes were detected considering the total amounts of this cytokine (Fig. 3B).

On the contrary, despite no differences were detected in GCF concentrations of PDGF-bb (Fig. 4A) and TNF-α (Fig. 5A), both cytokines were modulated in terms of total amount. PDGF-bb (Fig. 4B) was reduced already at 3 months in deep sites only, while TNF-α (Fig. 5B) also at moderate sites.

After 3 and 6 months of treatment, levels of all cytokines were still higher than those of healthy control sites ($p < 0.001$).

Correlation data between clinical parameters and inflammatory markers for which statistically significant differences were found at 6 months after non-surgical periodontal therapy in terms of total amount are shown in Table 3. IL-1β levels showed a significant and positive correlation with PD and GI at baseline ($p < 0.05$) and also at 6-month evaluation ($p < 0.01$). The amount of VEGF were significantly correlated with baseline PD and GI ($p < 0.05$) and with persistent inflammation ($p < 0.01$) at 6 months. PDGF-bb was correlated with PD and GI at baseline ($p < 0.001$), and TNF-α only with GI at baseline ($p < 0.01$).

Discussion

To the best of our knowledge this is the first study conducted in any population regarding the detection of VEGF, PDGF-bb and IL-9 in GCF of subjects with GAgP before and after non-surgical periodontal treatment. In the present investigation a multiplex bead immunoassay was employed to simultaneously measure the concentration of multiple biomarkers, whereas almost all previous studies used commercial immunoenzymatic assay (ELISA) kits to measure individual cytokine levels [10]. This new rapid and high sensitive/specific assay has so far not been used to assess the expression of these inflammatory mediators in GCF samples.

Our current understanding of factors, which may increase host susceptibility to periodontal tissue destruction by regulating individual response to chronic gingival inflammation in GAgP patients, is still incomplete [36]. Studies about periodontitis revealed that there is a relationship between increased number of blood vessels and progression of the disease [37, 38], In this scenario, VEGF and PDGF play a central role in regulating angiogenesis in inflammatory and wound healing process [16, 21]. VEGF is a multifunctional cytokine inducing proliferation of endothelial cells and increasing vascular permeability [17]. In spite of its frequent detection in periodontal tissues, data about its role in periodontal disease are limited and conflicting. In chronic periodontitis subjects the production of VEGF is upregulated in diseased sites if compared to healthy sites [18, 39, 40]. Its concentration increases proportionally with the severity of periodontal disease [18, 41] suggesting a role in the progression from gingivitis to periodontitis. On the contrary, a greater expression of VEGF was observed during the healing phase of periodontal disease [42].

In the present study, the GAgP patients were treated by quadrant-wise non-surgical periodontal treatment and strict plaque control measures were instituted. The improvements in clinical inflammatory and disease parameters were in line with those reported in the literature [43]. It is important to point out that plaque scores were maintained at a low level (<15%) through the study period, indicating both good oral hygiene performance of all patients and successful re-motivation during post-treatment controls.

These clinical outcomes were accompanied by a statistically significant decrease in VEGF detection (pg and concentration) in both moderate and deep pocket sites. These results agree with those of previous studies in chronic periodontitis [18, 19] and may support the active pathological role of VEGF even in GAgP diseased sites. An intriguing finding from this study was the most pronounced decrease in VEGF content, in terms of total amount and concentration, in deep sites 3 months after treatment when compared to moderate sites. This finding well related to that reported by Pradeep et al. [18] and to the clinical demonstration that deep pockets experienced more PD reduction than moderate sites. The density of blood vessels increases with increasing PD [15].

It is interesting that deep pocket sites experienced a statistically significant decrease in PDGF amounts after periodontal treatment. In contrast, no statistically significant changes were observed when

considering PDGF concentrations. Information in the literature on the role of PDGF in periodontal diseases is very limited and still debated. Previous studies demonstrated an enhanced expression in periodontal diseased sites in the rat model [44] and in the inflamed gingival tissue of periodontitis patients [45]. It is known that PDGF stimulates the secretion and production of collagenase by fibroblasts suggesting a potential role in the progression of periodontal disease [21, 22].

Pro-inflammatory cytokines such as IL-1β and TNF-α seem to play an important role in GAgP by controlling cellular interactions and functions [4]. When released in high concentration, they can stimulate the production of other inflammatory mediators (e.g. IL-6, prostaglandins, matrix metalloproteineases) involved in extracellular matrix connective tissue destruction and osteoclastic-mediated bone loss, as well as up-regulate the production of angiogenic mediators [23, 24]. IL-1β and TNF-α have been extensively studied in GCF of GAgP patients [8-10]. However, the effects of periodontal treatment on GCF levels of IL-1β are contraddictory. Some studies [12-14, 46] indicate that periodontal therapy reduces the GCF total amount of IL-1β, suggesting a role for this cytokine in the disease process, whereas others report no effect or an increase after periodontal treatment [47-49]. Here, IL-1β expression (pg and concentration) was markedly reduced in both moderate and deep pocket sites until 6 months following periodontal therapy and there was correlation between IL-1β and clinical parameters. Data from Engebretson et al. [47] and de Lima Oliveira et al. [14] support this observation, demonstrating that PD and CAL were each associated with increased GCF IL-1β levels. By contrast, we found an effect of mechanical periododontal therapy on GCF total amounts but not on GCF concentrations of TNF-α. In agreement with the current data, a previous study observed that TNF-α concentrations remained stable after mechanical periodontal therapy [14]. Bastos et al. [32] demonstrated that TNF-α levels were elevated in GCF of both healthy and diseased sites from GAgP individuals, indicating that this mediator can be expressed in sites with different clinical status. The present data confirm the altered inflammatory response of GAgP subjects and suggest that TNF-α may be a suitable indicator for periodontitis development.

The levels of IL-9 had not been studied yet in GCF but its serum concentrations were found to be elevated in periodontitis patients compared to healthy individuals [31]. IL-9 has been described as a

pleiotropic cytokine, whose pro- or anti-inflammatory activities may significantly differ depending on the overall cytokine milieu [28]. Interestingly, the augment of IL-9 in inactive periapical lesions and a negative correlation with TNF-α were recently described, suggesting a role of IL-9 in periapical lesion stability [29, 30]. In the present study, a different trend was observed when considering IL-9 GCF amount or concentration. While a significant elevation in IL-9 concentration was observed at moderate pocket sites 3 months after the completion of non-surgical periodontal therapy, its total amount was slightly decreased or fairly stable in moderate and deep pocket sites at 3- and 6-month evaluation. Further controlled studies with larger sample size may be useful to clarify the role of IL-9 in the etiopathogenesis of GAgP.

Conclusions

A significant decrease in the GCF levels of pro-inflammatory and angiogenic cytokines was observed in response to quadrant-wise non-surgical periodontal treatment, although they were still elevated when compared to healthy control group. Nevertheless, plaque control measures seem not to affect mediators such as IL-9 and partially PDGF-bb. These findings suggest a potential hyperreactivity of cells in these individuals that may favor periodontal tissue breakdown and a local inflammatory burden even with insignificant amount of bacterial plaque on teeth. The role of IL-9 on local periodontal destruction in these subjects requires further investigations.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Funding The authors declare that there were no financial support from any external source regarding the current study.

Ethical approval All procedures involving humans were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Table 1. Clinical parameters (mean ± SD) of GCF sampling sites over the experimental period in patients with GAgP and periodontally healthy controls

	Healthy controls (*n*=15)	GAgP subjects (*n*=16)					
		Moderate pocket sites (4-5 mm)			*Deep pocket sites (≥ 6 mm)*		
		Baseline	**3 months**	**6 months**	**Baseline**	**3 months**	**6 months**
PI (%)	0	90.6 ± 20.2	9.4 ± 27.2***	12.5 ± 28.9***	93.8 ± 17.1	15.6 ± 23.9***	21.9 ± 31.5***
GI	0	1.9 ± 0.5	0.5 ± 0.6***	0.8 ± 0.8***	2.5 ± 0.5¶	0.8 ± 0.7***	0.9 ± 0.8***
PD (mm)	1.6 ± 0.5	4.7 ± 0.5	3.3 ± 0.6***	3.1 ± 0.8***	8.8 ± 1.6§	5.3 ± 1.1***§	5.0 ± 1.8***§
CAL (mm)	1.6 ± 0.6	5.3 ± 0.8	4.2 ± 1.0***	4.0 ± 1.0***	9.4 ± 1.8§	6.6 ± 0.9***§	6.3 ± 1.1***§
GCF (µl)	0.21 ± 0.07	0.50 ± 0.35	0.27 ± 0.21***	0.32 ± 0.16**	1.03 ± 0.34¶	0.53 ± 0.36***¶	0.58 ± 0.35***¶

GCF gingival crevicular fluid volume, *GAgP* generalized aggressive periodontitis, *PI* presence of plaque, *GI* gingival index, *PD* probing depth, *CAL* clinical attachment level, *SD* standard deviation.
**P < 0.01 *versus* baseline.
***P < 0.001 *versus* baseline.
¶P < 0.01 *versus* moderate pocket sites.
§P < 0.001 *versus* moderate pocket sites.

Table 2. FMPS and FMBS over the experimental period in patients with GAgP and periodontally healthy controls

	Healthy controls (*n*=15)	GAgP subjects (*n*=16)		
		Baseline	**3 months**	**6 months**
FMPS (%)	11.3 ± 2.7	37.3 ± 11.3	13.9 ± 4.7***	14.9± 4.4***
FMBS (%)	7.9 ± 2.1	50.2 ± 13.9	18.1 ± 4.3***	20.4 ± 6.1***

FMPS full-mouth plaque score, *FMBS* full-mouth bleeding score.
***P < 0.001 *versus* baseline.

Table 3. Correlation between cytokines total amount (pg) in gingival crevicular fluid and clinical parameters at baseline and 6 months after periodontal therapy

Parameter	Baseline	6 months after treatment
IL-1ß		
GI	0.426*	0.533**
PI	0.238	0.254
PD	0.589***	0.512**
VEGF		
GI	0.584**	0.502**
PI	0.293	0.274
PD	0.419*	0.319
PDGF-bb		
GI	0.444*	0.127
PI	0.321	0.136
PD	0.635***	0.115
TNF-α		
GI	0.402*.	0.285
PI	0.212	0.324
PD	0.264	0.297

GI gingival index, *PI* presence of plaque, *PD* probing depth.
*P <0.05,**P<0.01, ***P<0.001

Figure legends

Fig. 1. Box-and-whisker plots showing the concentration (A) and total amount (B) of IL-1β in gingival crevicular fluid of periodontally healthy controls and subjects with aggressive periodontitis according to baseline PDs before and after non-surgical periodontal therapy. The box represents median, 25% and 75% percentiles, the whiskers represent data within 10% and 90% percentiles. *P < 0.05, ***P < 0.001.

Fig. 2. Box-and-whisker plots showing the concentration (A) and total amount (B) of VEGF in gingival crevicular fluid of periodontally healthy controls and subjects with aggressive periodontitis according to baseline PDs before and after non-surgical periodontal therapy. The box represents median, 25% and 75% percentiles, the whiskers represent data within 10% and 90% percentiles. *P < 0.05, ***P < 0.001.

Fig. 3. Box-and-whisker plots showing the concentration (A) and total amount (B) of IL-9 in gingival crevicular fluid of periodontally healthy controls and subjects with aggressive periodontitis according to baseline PDs before and after non-surgical periodontal therapy. The box represents median, 25% and 75% percentiles, the whiskers represent data within 10% and 90% percentiles.*P < 0.05.

Fig. 4. Box-and-whisker plots showing the concentration (A) and total amount (B) of PDGF-bb in gingival crevicular fluid of periodontally healthy controls and subjects with aggressive periodontitis according to baseline PDs before and after non-surgical periodontal therapy. The box represents median, 25% and 75% percentiles, the whiskers represent data within 10% and 90% percentiles.**P < 0.01, ***P < 0.001.

Fig. 5. Box-and-whisker plots showing the concentration (A) and total amount (B) of TNF-α in gingival crevicular fluid of periodontally healthy controls and subjects with aggressive periodontitis according to baseline PDs before and after non-surgical periodontal therapy. The box represents median, 25% and 75% percentiles, the whiskers represent data within 10% and 90% percentiles.**P < 0.01, ***P < 0.001.

Figure 1

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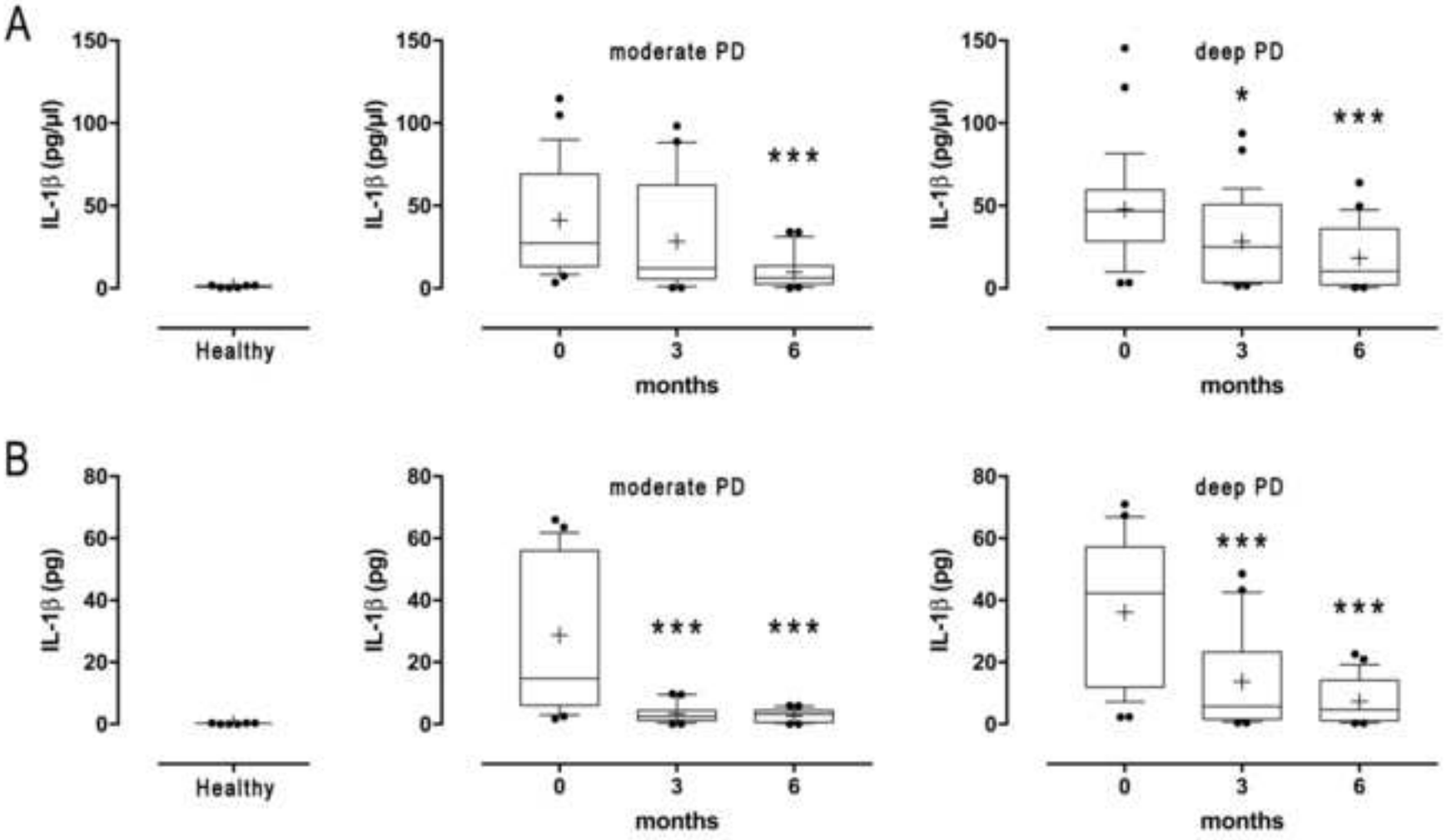

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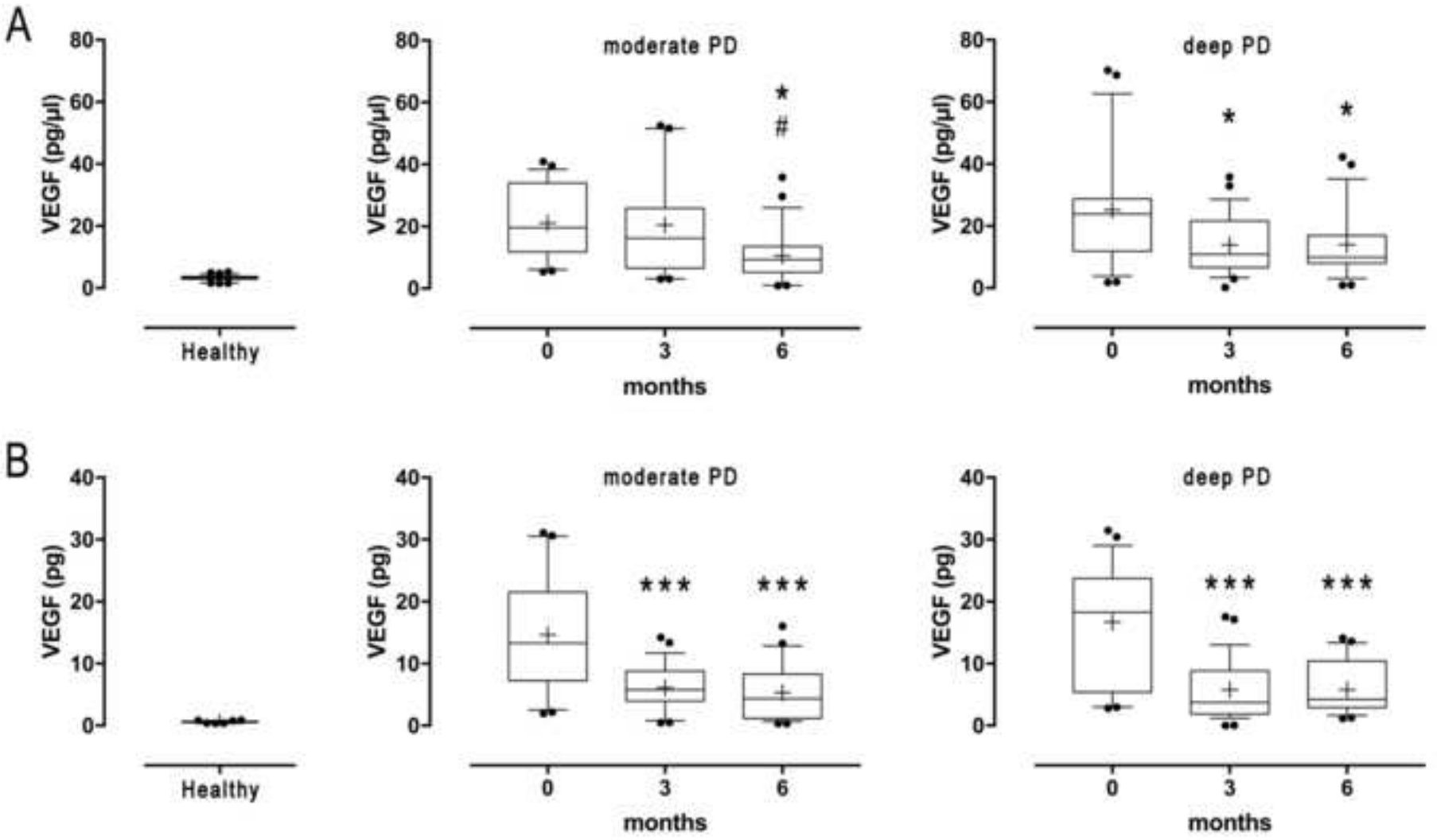

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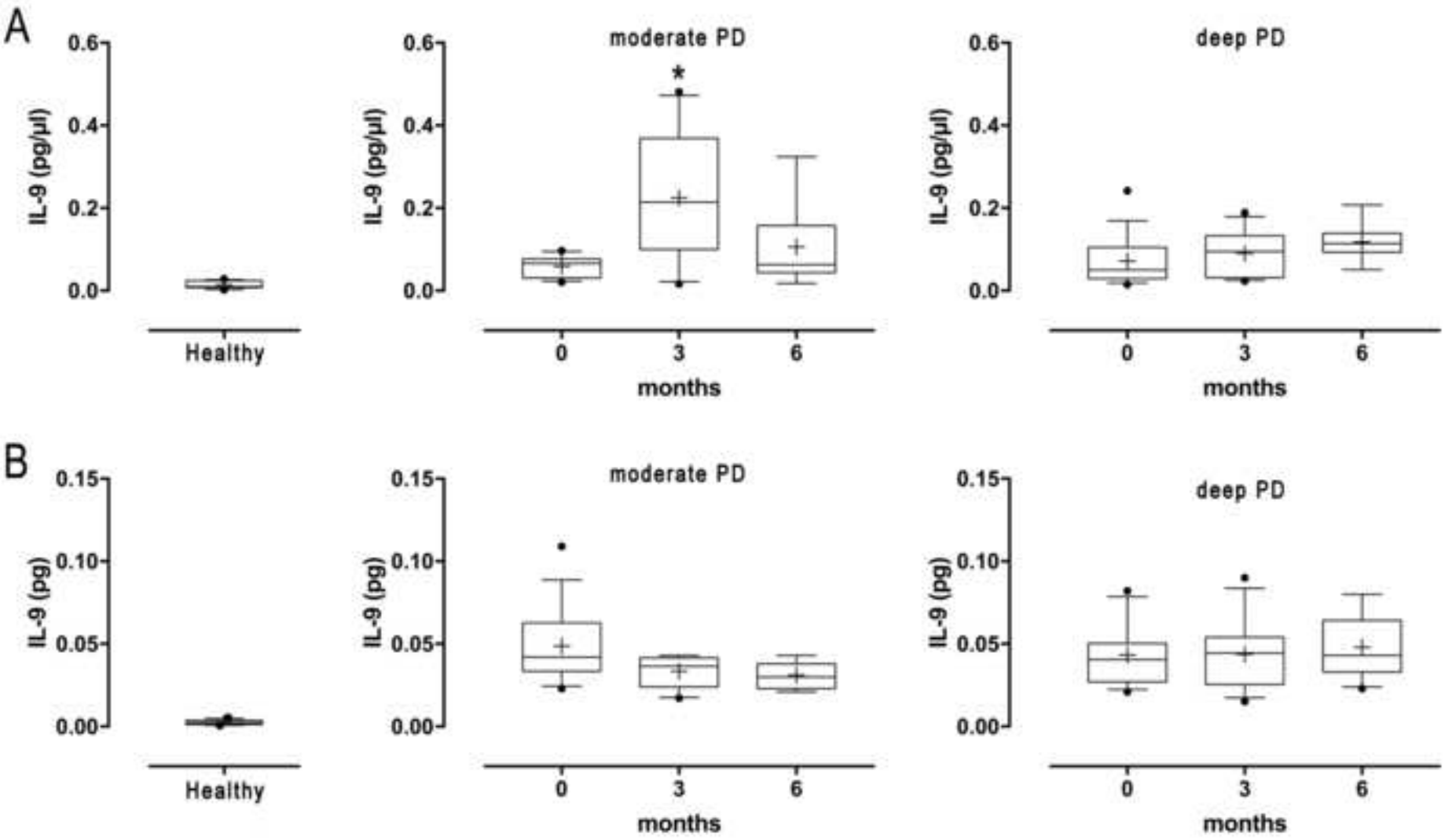

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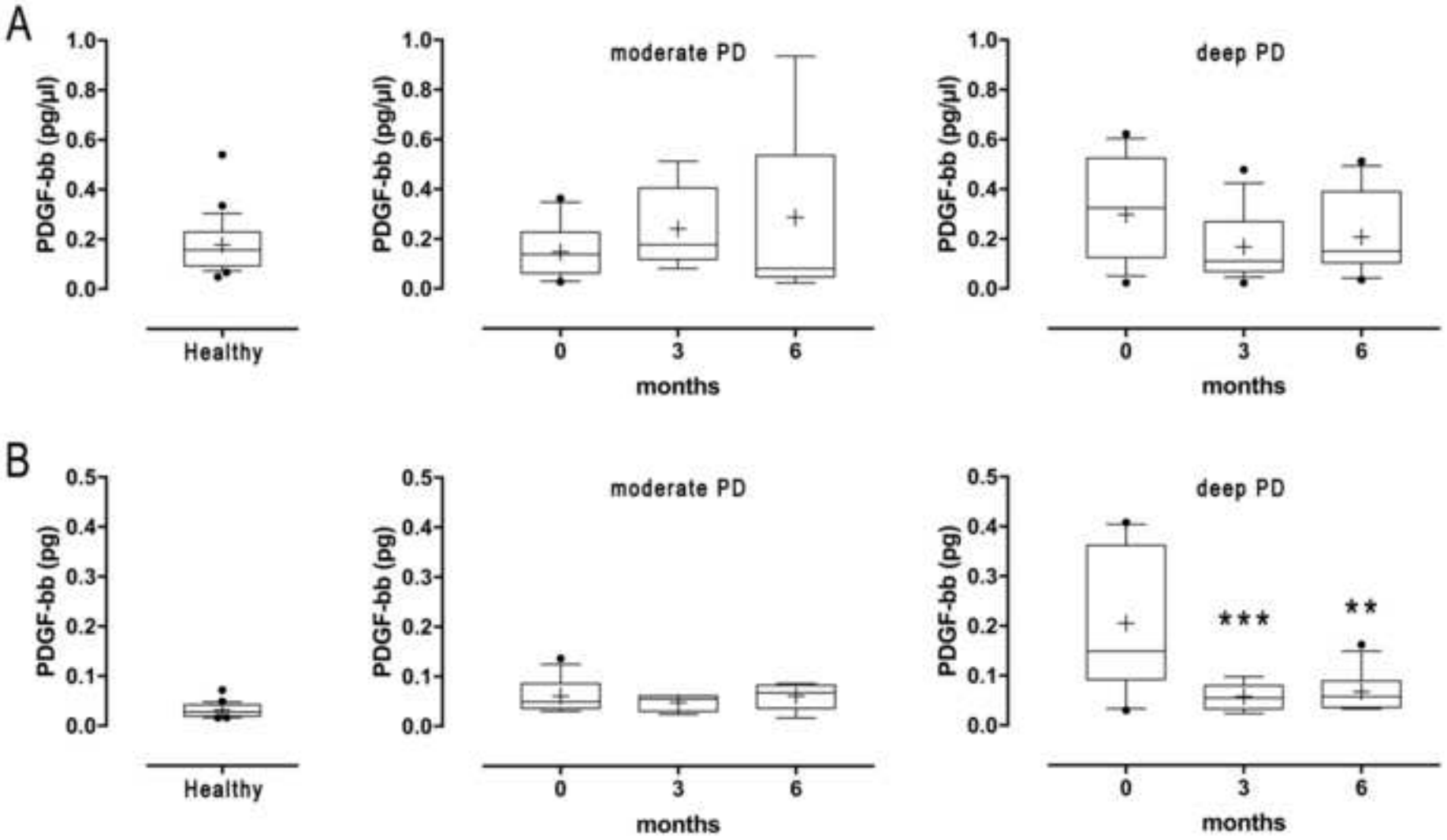

Figure 5

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