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ABSTRACT

Background and Objective: Periodontal health is mediated by supressing microorganisms inducing a local inflammatory host response. Smoking may impair this process. This study compares gingival crevicular fluid levels of inflammatory and bone remodelling markers in heavy smokers and non-smokers following active and supportive periodontal therapy in chronic periodontitis patients.

Materials and Methods: Gingival crevicular fluid (GCF) and subgingival plaque were collected from the deepest periodontal pocket in 50 patients, 25 smokers and 25 non-smokers, at baseline (T0), following active (T1) and 12 months of supportive periodontal therapy (T2). Smoking status was validated measuring serum cotinine levels. GCF levels of 27 inflammatory and two bone remodelling markers were analysed using multiplex and singleplex micro-bed immunoassays, and subgingival plaque samples using checkerboard DNA-DNA hybridization. Amounts of markers in smokers and non-smokers were compared calculating the effect size.

Results: Expression of inflammatory and bone-remodeling markers in smokers demonstrated an overall reduced effect size at T0 and T2 ($p<0.001$) More specifically, pro-inflammatory markers ($p<0.001$), chemokines ($p=0.007$) and growth-factors ($p=0.003$) at T0, osteoprotegerin ($p=0.003$) at T1, pro-inflammatory markers ($p=0.019$) and chemokines ($p=0.005$) at T2. At T2, IL-8 was detected in significantly higher levels in smokers. Ten different markers in non-smokers and none in smokers responded to periodontal therapy ($p<0.05$). An overall negative association was revealed between smoking and sub-groups of markers at sites presenting $\geq 10^5$ red complex periodontal microbial species.

Conclusion: Except for an upregulation of IL-8, smokers exhibited reduced GCF levels of several inflammatory markers at baseline and following active and supportive periodontal therapy. Only inflammatory responses in non-smokers adapted to periodontal therapy. Apparently, there seems to be an immunosuppressant effect of smoking regulating the local inflammatory response and bone remodelling markers captured in GCF following periodontal therapy.

INTRODUCTION

Cigarette smoking may affect periodontal tissues through modulating the immune response to periodontal pathogens. A dose-dependent hypo-immune inflammatory reaction has been suggested, mainly through systemic exposure following lung absorption (1-3). An additive local effect of smoking through direct exposure and absorption of toxic substances may adversely shift the periodontal equilibrium towards impaired repair and tissue breakdown (4, 5). Further, differences exist in composition of subgingival microbiota among smokers and non-smokers (6). Moreover, smoking appears to modulate composition, promote colonization of key periodontal pathogens, and influence bacterial aggregation (7, 8) rather than rate and amount of plaque accumulation (9, 10).

Periodontal pathogens induce release of inflammatory and bone remodelling markers orchestrating innate and adaptive immune responses. The interaction and balance of these markers determine whether the tissue response remains stable or leads to destruction and disease progression (11). Gingival crevicular fluid (GCF), an inflammatory exudate or transudate collected from the gingival crevice, contains components of host-derived inflammatory markers (12). Thus, GCF analysis appears a non-invasive approach to investigate site-specific inflammatory responses and assess presence of various inflammatory markers (13). In smokers, site-specific tissue responses are clinically expressed by a specific attachment loss profile (14). However, site-specific mapping of inflammatory and bone-remodelling markers in smokers with chronic periodontitis reports conflicting results (15). A majority of studies have reported decreased local expression of some pro-inflammatory cytokines and chemokines in smokers (16-18) indicating an immunosuppressant effect of smoking that may increase susceptibility to periodontitis. Conversely, elevated expressions of chemokines and pro-inflammatory cytokines have also been reported in smokers (19, 20). For bone remodelling, decreased GCF levels of osteoprotegerin (OPG) have been demonstrated following ≥20 years of smoking (21). As OPG blocks activation of receptor activator for nuclear factor kappa-B ligand (RANKL), the RANKL:OPG ratio, a surrogate marker for periodontal bone homeostasis, might increase in smokers and induce bone loss.

Compromised healing following periodontal therapy in smokers is well documented (22-24) and impaired site-specific treatment outcomes may to some extent be explained by altered inflammatory responses (25). A few prospective studies have investigated the relationship between smoking and levels of pro-inflammatory markers, chemokines, and bone markers in GCF following periodontal therapy. IL-1β and TNF-α, are mostly studied; IL-1β levels decreased in smokers and non-smokers following 6 weeks of non-surgical therapy (26), whereas at 6 months the levels were reduced in non-smokers only (27). TNF-α did not change in smokers and non-smokers following 1 month of non-surgical therapy (28). At 6 months, however, a decreased level was observed in smokers (29). Interestingly, smoking seemed to upregulate the chemokine IL-8 following therapy (30), whereas OPG decreased in both smokers and non-smokers (31).

Previous follow-up studies evaluating the local inflammatory status, have analysed a limited number of GCF markers. Multiplex immunoassay has the potential to simultaneously quantify multiple markers providing unique information necessary for a more complex understanding of the inflammatory response. By measuring several inflammatory markers over time, a site-specific tissue response to periodontal therapy can be monitored. Thus far, no prospective study has compared inflammatory responses in GCF following active and supportive periodontal therapy in smokers and non-smokers.

This prospective study was designed to test the hypothesis that smoking downregulates the expression of the inflammatory molecules in GCF during treatment of chronic periodontitis. The primary aim was to investigate the GCF levels of inflammatory markers involved in periodontal inflammation and healing following active and 12 months of supportive periodontal therapy in heavy smokers and non-smokers. The impact of smoking on the numbers of subgingival periopathogens following periodontal therapy was also studied.

MATERIAL AND METHODS

Participants and study design

From a sample of 80 patients, 40 smokers and 40 non-smokers, referred for periodontal treatment from general practitioners in a rural district of Norway, a subsample of 50 patients were enrolled in this single-arm clinical trial (for detail see Bunæs et al. 2015;(24). Briefly, criteria for inclusion were healthy subjects between 35-75 years with moderate to severe chronic periodontitis (32) having at least four non-adjacent teeth with interproximal probing depth (PD) ≥6 mm, clinical attachment loss ≥5 mm, and bleeding on probing (BoP) (33, 34). Exclusion criteria were any current medical condition or medication affecting periodontal treatment and the use of systemic antibiotic or subgingival scaling in the 6 months before initiation of the study. Demographic data were obtained from the study participants by means of health forms and questionnaires.

Based on predefined criteria, the 50 patients were allocated into two subgroups of 25 smokers reporting smoking >10 cigarettes/day for at least 5 years and with baseline pre-treatment (T0) serum cotinine level ≥300 ng/mL, and 25 non-smokers reporting never or no smoking the last 5 years and with T0 serum cotinine level <15 ng/ml. Whole blood sample obtained from each patient was coagulated and centrifuged (700 x g for 10 min) and the serum was stored in aliquots at -80°C. Serum cotinine was assessed according to the manufacturer's instructions of the serum enzyme immunoassay kit (Cotinine ELISA Kit, MyBioSource, San Diego, USA) measuring the absorbance at 450 nm using a microplate reader (FluoStar Optima V1.32 R2, BMG Labtech, Offenburg, Germany).

The study protocol and informed consent form was approved by the Institutional Medical Research Ethics Committee (2011/151-6), University of Bergen, Norway followed the Helsinki Declaration of 1975, version 2008. All patients had read and signed a written consent prior to enrolment in the study.

Clinical and microbiological examinations

Clinical assessments, group allocations, and sampling selection were performed by a calibrated examiner (DFB). Clinical and microbiological sample collection were performed at baseline (T0), at 3 months following active periodontal therapy (APT)

(T1), and following 12 months of supportive periodontal therapy (SPT) (T2). Mean time between T0 and T1 was 7.9 months and comprised non-surgical and surgical treatment. Patients presenting persistent PD >5 mm with BoP and adequate oral hygiene following non-surgical treatment were subjected to periodontal surgery. SPT was conducted every 3 months. PDs were recorded as the distance in mm from the gingival margin to the probeable base of the periodontal pocket, and clinical attachment level (CAL) as the distance in mm from the cemento-enamel junction or the margin of a dental restoration to the probeable base of the periodontal pocket. PD and CAL were measured using a periodontal probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA) at six sites per tooth rounding up to the nearest mm. Full mouth gingival bleeding was recorded as the percentage of sites showing bleeding after gentle probing (35) and full mouth dental plaque as the percentage of tooth surfaces with visible plaque following staining with disclosing solution (36). As a supplement to staining, the periodontal probe was used to discriminate between plaque and pellicle.
At T0 subgingival plaque were collected by inserting two sterile paper points into the deepest periodontal pocket in each patient, and the procedure was repeated at the same site at T1 and T2. Prior to sampling, the site was carefully cleaned of supragingival plaque and kept dry. The paper points were gently inserted towards the apex of the pocket and kept in place for 20 sec (37) before removal and immersion into a pre-reproduced sterile transport medium (PRAS Dental Transport Medium, Morgan Hill, CA,USA). The microbiological samples were analysed by DNA-DNA hybridization (checkerboard technique) at Microbiological Diagnostic Service, Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway. The analysis included detection and quantification of red (*Porphyromonas ginigvalis*, *Taneralla forsythia*, and *Treponema denticola*) and orange complex species (*Prevotella intermedia, Prevotella nigrescens, Fusobacterium nucleatum subsp polymorphum, Fusobacterium nucleatum subsp nucleatum, Fusobacterium nucleatum subsp vincentii, and Parvimonas micra*), and *Aggregatibacter actinomycetemcomitans (Aa)* (for details see Bunæs et al., 2015; (24).

Intra-examiner reliability of the examiner (DFB) was assessed using the intraclass correlation coefficients (ICC) for repeated measures. ICC ranged between

0.92 and 0.96 for PD and between 0.93 and 0.96 for CAL. A post.hoc power analysis based on 25 heavy smokers and 25 non-smokers and with the level of significance (α) set to 0.05, gave a 71% power to detect a true difference of 0.5 mm. Prior to treatment, the hypothesis that is not possible to blind an examiner towards smoking status was tested in a pre-study sample of 30 chronic periodontitis patients, 16 smokers (>10 cigarettes/day for at least 5 years) and 14 non-smokers (never or not in the last 5 years). Calculus, plaque, and staining were removed and after rinsing with 0.2% chlorhexidine gluconate (Corsodyl, GlaxoSmithKline, London, UK) for 1 min, the examiner wearing a face mask scored the smoking status as yes or no. Twenty-eight patients (93%) were correctly identified as either non-smokers or smokers. Thus, the pre-study hypothesis was accepted (p<0.001) indicating that the attempt to blind the examiner with regard to smoking status was unsuccessful.

GCF sampling and protein extraction

GCF samples were collected using Periopaper strips, (PERIOPAPER Gingival Fluid Collection Strips, Oraflow Inc, Smithtown, NY, USA). Following removal of supragingival plaque with sterile curettes and cotton pellets, air dried, and isolated with cotton rolls, the deepest periodontal pocket in each participant was sampled. The paper strips were placed 1-2 mm into the entrance of the pocket and left in place for 30 sec. Strips visually contaminated with blood or saliva were discarded. Sampled strips were immediately evaluated for GCF volume using the Periotron 8000 (Oraflow Inc, Smithtown, NY, USA). Strips were then immediately inserted into separate and dry microtubes, labelled, and stored at -80°C until further analysis.

Tris-HCl buffer (110 μL) with a final concentration of 12mM at pH 7.6 was added to each tube for protein extraction. The tubes were shaken in 3x10 min before centrifuged (1800 x g for 10 min at 4°C) and subsequently the supernatant was pipetted to new tubes for protein quantification by a commercially available kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). A plate reader (FLUOstar OPTIMA- BMG Labtech, Ortenberg, Germany) measured the absorbance at 570 nm and total protein per strip was calculated in μg.

Analysed markers

Based on inflammatory and bone remodeling molecules involved in the periodontal healing process , the following cytokines were determined (diluted 1:4) using the multiplex kit Bio-Plex Human Pro™ Assay (catalogue number LX10009222405) from Bio-Rad Inc., Hercules, CA, USA: *IL-1β, IL-2, IL-1ra, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Interferon-γ (IFN-γ), Basic Fibroblast Growth Factor (FGF), Granulocyte Colony Stimulating Factor (G-CSF), Eotaxin , Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF), Interferon Inducible Protein-10 (IP-10), Monocyte Chemo-attractive Protein-1 (MCP-1), Macrophage Inflammatory Protein-1α (MIP-1α), Macrophage Inflammatory Protein-1β (MIP-1β), Platelet-Derived Growth Factor (PDGF), Regulated Upon Activation, Normally T-Expressed, and Presumably Secreted (RANTES), Tumor Necrosis Factor-α (TNF-α) and Vascular Endothelial Growth Factor (VEGF)*. The standard curves for each marker present an overall range of 107489-0.064 pg/mL.

The level of *OPG* and *RANKL* were assessed (diluted 1:4) using Milliplex MAP Kit Human Bone Magnetic Bead Panel (catalogue number HRNKLMAG-51K-01) from EMD Millipore corp. (Billerica, MA,USA) and a range of 30367-7.28 pg/mL recombinant markers was used to establish the standard curves.

All measurements were performed using a Bio-plex 200®system (Bio-Plex Manager TM 6 software) based on the Luminex xMAP technology and the levels of all markers were reported in pg/30 sec and in pg/ml.

Statistical analysis

The Shapiro-Wilk test was applied to test for distribution of continuous variables. Descriptive statistics of clinical and demographical data were analysed using a chi-square test for categorical variables (frequencies and percentages) and by a two-sample independent t-test for continuous data (mean ± SD). As continuous data of the analysed mediators had a skewed distribution, natural logarithm transformation was employed to achieve normality prior to using the regression analysis to detect differences between smokers and non-smokers at T0, T1, and T2 and over time (T0 vs. T1, T0 vs. T2, and T1 vs. T2). Samples below detection of the standard curve were replaced by the lowest value of the standard curve (38). Regression analyses were

corrected for clustering of data within patients and the significance level of multiple comparisons were adjusted by the Sidak post-hoc test. A regression model, adjusted for age, gender, and education, and stratified by plaque (present/absent) or amount of red complex bacteria species ($<10^5$ / $\geq 10^5$) was employed to test the overall association between the subgroups of quantified markers and smoking status.

Amounts markers (pg/30 sec) in smokers and non-smokers were compared by calculating the effect size. Effect size, reported as Cohens`s coefficient, was calculated as the difference between the means of each marker in smokers and non-smokers divided by the standard deviations. Cohen`s coefficient is generally classified into small (0.2), medium (0.5), and large differences (≥0.8) and allows the size magnitude of the difference between smokers and non-smokers to be measured in a standardised scale. To present the results, forest-plots for the standardised effects sizes were used. An overall test of the difference between markers in smokers and non-smokers were analysed using fixed effects in the metan command (39).

A *p*-value ≤0.05 was considered statistically significant. All analyses were conducted using Stata 14 (Stata Statistical Software: Release 14. StataCorp LP, College Station, TX, USA).

RESULTS

A total of 50 patients were evaluated, 25 smokers and 25 non-smokers. At T0, mean pack-year consumption in the smoking group was 37.0 (range 20-108) and mean cotinine level 478 mg/mL (range 340-861 mg/mL). For each patient, GCF samples were obtained from the same site at T0, T1, and T2. Data collection started April 2012 and ended December 2014. Baseline patient related clinical and demographic characteristics according to smoking status are shown in Table 1. Compared with non-smokers, significantly lower education level, higher mean PD and CAL were found for smokers. Mean patient related clinical measures of PD, CAL, BI, and PI, decreased significantly following ACT in smokers and non-smokers ($p<0.001$) and PD, BI, and PI increased significantly following SPT ($p<0.05$). Site-specific clinical and microbiological characteristics at T0, T1, and T2 are summarised in Table 2. No significant differences between smokers and non-smokers were detected for any of the

site-specific parameter at any time point, except for significantly higher numbers of *Aa* in non-smokers compared with smokers at T0 (p=0.041).

GCF markers detected in less than 30% of the samples (IL-2, IL-5, IL-12, IL-13, IL-15, Eotaxin, FGF, MCP-1 and RANKL) were removed from the analysis. The surveyed markers at T0, T1, and T2 were stratified by smoking status and comparisons of unadjusted means of the quantities are presented as amounts per 30 sec (pg/30sec) (Table 3a) and as concentrations (pg/mL) (Table 3b). Further analyses were conducted on the amounts of markers per 30 sec (40). Compared with non-smokers, significant lower levels were detected in smokers for TNF-α, IL-9, G-CSF, GM-CSF, IFN- γ, VEGF, MIP-1α and RANTES at T0, for OPG at T1, and for IL-9, IFN- γ, PDGF, MIP-1α, MIP-1β, RANTES at T2. At T2, IL-8 was detected in significantly higher levels in smokers (p=0.034). Only non-smokers responded to treatment with significant changes in surveyed markers over time. From T0 to T1, significant reductions were revealed for IL-1β, TNF-α, IL-7, GM-CSF, IFN-γ, IL-10, VEGF and IP-10, and from T0 to T2 for IL-1β and GM-CSF. A significant upregulation of TNF-α, IL-7, IL-9, IFN-γ, IL-10, PDGF, and IP-10 was observed from T1 to T2.

Based on the biological effects, the markers were distributed into subgroups of pro-inflammatory markers (IL-1β, IL-6, IL-9, IL-12 and TNF-α), anti-inflammatory markers (IL-4 and IL-10), chemokines (IL-8, IP-10, MCP-1, MIP-1α, MIP-1β and RANTES), growth factors (PDGF and VEGF), Th-1/Th-2 (INF-γ/ (IL-4, IL-6, IL-9, IL-10), and marker of bone remodelling (OPG). Since the markers were expressed in various amounts in smokers and non-smokers and measured in different scales (range107489-0.064), the magnitude of the differences between smokers and non-smokers was calculated as effect size (Cohen`s coefficient). Figure 1a, b and c illustrate the size of the effect of smoking on the expression of marker and subgroup at T0, T1, and T2, respectively. At T0 smoking significantly reduced effect size for pro-inflammatory markers (p=0.001), chemokines (p=0.007), and growth-factors (p=0.003), at T1 for OPG (p=0.003), and at T2 for pro-inflammatory markers (p=0.019) and chemokines (p=0.005).

The subgroups were tested for overall association with smoking status after stratification by dental plaque (present/absent) and red complex microbial species

($<10^5/\geq 10^5$) (Table 4). The numbers of tested sites in the analysis are not tabulated: plaque positive sites [n=96 (45 smokers / 51 non-smokers)], plaque negative sites [n=54 (30 smokers / 24 non-smokers], red complex positive sites [n=49 (23 smokers / 26 non-smokers)], and red complex negative sites [n=101 (52 smokers / 49 non-smokers)]. At plaque positive sites, a significant negative association with smoking status was revealed for pro-inflammatory markers, chemokines, and growth factors. For each group, adjusted analyses demonstrated an overall significant negative association with smoking status for plaque positive sites and an overall positive association for plaque negative sites. Further, in both unadjusted and adjusted analyses, negative associations were revealed between smoking status and groups of pro- and anti-inflammatory markers and OPG at sites presenting $\geq 10^5$ red complex species. No significant associations were detected between smoking and groups of markers at sites presenting $< 10^5$ red complex species. The same tests were performed for orange complex species and *Aa* and a significant negative association between smoking and amounts of pro-inflammatory markers were only present for the adjusted analysis of sites $< 10^5$ orange complex species (p=0.033) (not tabulated).

DISCUSSION

Significantly smaller amounts of several inflammatory markers were detected in smokers compared with non-smokers at T0 and in the presence of increased clinical inflammation from T1 to T2. The expression of GCF markers at a site might be influenced by mean levels of PD and CAL (41). Sampling from the deepest PD in smokers and non-smokers rather from matched PD could have biased the analyses. However, the reliability of an overall reduced inflammatory response in smokers was substantiated by the fact that GCF samples were collected from sites exhibiting similar PD, CAL, BI, PI, and GCF volume in smokers and non-smokers. Another methodological concern when including smokers in clinical studies is the unsuccessful blinding of the examiner with regard to smoking status. This might in fact introduce a study bias. Further, strict sampling procedures and a trained operator intended to prevent a potential saliva contamination of the periopaper strips during the GCF collection.

An overall suppressed inflammatory response in smokers is supported by Tymkiw and suggests a local hypo-inflammatory state in smokers with chronic periodontitis (17). Reduction of important pro-inflammatory cytokines may initially alter local cytokine regulated inflammatory processes and persuade a dysfunctional response to stimuli such as bacterial lipopolysaccharides. In smokers at T0, significant reduced amounts of TNF-α, a multifunctional pro-inflammatory cytokine promoting cell migration and tissue destruction, might downregulate IL-1β and IL-6 and reduce production of chemokines (42). Though investigations of the impact of smoking on the expression of chemokines are limited, reduced amounts of chemokines in smokers have been confirmed by others (17, 43). In the present study, the slight rebound of periodontal disease during SPT coincided with increased amounts of MIP-1α, MIP-1β and RANTES at T2 in non-smokers only. As these chemokines facilitate migration and activation of specific types of leukocytes in response to periodontal pathogens (44), a downregulation of chemokines in smokers might reduce recruitment of inflammatory cells into infection sites. Chemokines also stimulate bone remodelling driving osteoblast migration (45) and reduced expression might negatively influence bone metabolism. A negative impact of smoking on bone homeostasis might be further supported by lower levels of OPG in smokers compared with non-smokers (significant at T1). RANKL was not detectable and an influence of smoking on the RANKL:OPG ratio can only be speculated upon. Nevertheless, increased bone loss in smokers with chronic periodontitis could be attributed to decrease in OPG and a subsequent increase in the RANKL:OPG ratio with a potential stimulation of osteoclasts. Another study in periodontitis patients with varying smoking status has reported reduced GCF levels of OPG in high pack-years consumption groups compared with non-smokers (21).

IL-8 was the only inflammatory marker detected in significantly higher amounts in smokers compared with non-smokers. This is in agreement with previous studies (18, 20) indicating that smoking seems to upregulate the expression of IL-8 in a dose dependent manner (46). IL-8 is a chemokine associated with subclinical inflammation of initial periodontal lesions through migration of polymorphonuclear neutrophils (PMNs) to the infection sites (47, 48). Modulated inflammatory responses in smokers are strongly associated with impaired PMN functions (49) and higher amounts of IL-8

in smokers may increase chemotaxis and migration of dysfunctional PMN cells. Moreover, IL-8 has an important role in bone metabolism with direct actions on osteoclast activity and differentiation (50). Since IL-8 is suggested to play a crucial role in the pathogenesis of chronic periodontitis, an upregulation of IL-8 production in smokers might be a detrimental factor for impaired treatment outcome and recurrence of periodontal disease, especially in heavy smokers.

None of the analysed inflammatory and bone remodelling markers responded significantly to treatment in smokers. This may strengthen the perception that smoking has a capacity to overwhelm and suppress local inflammatory response to periodontal pathogens (17, 51). Non-smokers responded to treatment with changes in the amounts of inflammatory markers reflecting positive treatment responses and for several markers a significant reduction was observed from T0 to T1 followed by an increase from T1 to T2. The amounts of three principal pro-inflammatory markers, IL-1β, TNF-α, and IFN-γ, reduced significantly as a response to therapy. INF-γ is related to Th-1 response and inhibition containment of periodontal infection by enhancing phagocytic activity of macrophages and neutrophils(52), whereas IL-1β and TNF-α are the first markers emerging during the periodontal inflammation processes. IL-1β is a major mediator for periodontal disease and involved in inflammatory cell migration and osteoclastogenesis (53). Another longitudinal study reported significant reduction of IL-1β at 4 months following non-surgical periodontal therapy, supporting present findings of a sustained decrease in IL-1β following treatment in non-smokers (47, 54). A persistent release of pro-inflammatory cytokines in smokers following active and supportive periodontal therapy may partially be explained by impaired resolution of inflammation and recurrence of periodontal disease following therapy.

As a response to active periodontal therapy, all clinical parameters improved significantly in smokers and non-smokers whereas the total numbers of red complex species, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, were significantly reduced in non-smokers only. The non-significant reduction of red complex species observed between T0 and T1 in smokers, could stimulate local inflammatory responses maintaining elevated amounts of GCF markers at T1. Otherwise, upregulated expression of inflammatory markers in GCF might favour

aggravation of the biofilm and delayed reduction of red complex species in smokers following ATP. Nevertheless, following SPT, a significant reduction of total red complex species took place in smokers without significant changes in the amounts of inflammatory markers. A dysregulated inflammatory response to periopathogenic bacteria in smokers was further supported by negative associations between smoking and groups of inflammatory markers at plaque positive sites and at sites harbouring $\geq 10^5$ of red complex species. It would be of interest to investigate the plaque with high-through put techniques, whereas DNA-DNA checkerboard hybridization technique has a rather crude accuracy. Keystone pathogens other than *P Gingivalis* may be determined for host response in smokers and *Parvimonas Mirca* is considered a keystone pathogen associated with deep pockets in smokers (7, 55). Nevertheless, the majority of immune responses to periopathogens are known to occur locally within the periodontal tissues, in GCF, and between cells conjugated to another, rather than to systemic responses (56, 57). However, in smokers, the amounts of inflammatory markers appeared to be modulated not only by site-specific factors as presence of plaque and a high number of red-complex species, but also by the complex systemic influence of cigarette smoke.

In general, expressed markers in GCF using multiplex assays and ELISA`s are detected with similar trends, but not directly comparable due to methodological variations (58), varying amounts of markers, and measurements at different scales. Two recent reviews support reporting of total marker content per 30 sec, especially for longitudinal studies, together with smoking status, and clinical parameters at collection sites (40, 53). They argue that low levels of gingival crevicular fluid volumes following periodontal therapy can negatively influence calculation of the concentration. Reduced GCF and BoP in smokers compared with non-smokers (59, 60) , could be an explanation for the suppressed response and downregulation of markers collected per 30 sec in smokers. Related to progression of chronic periodontitis, it appears to be an association between BoP in both smokers and non-smokers (3, 61). Nevertheless, GCF volume did not vary significantly between smokers and non-smokers and analysis with concentrations of markers reported the same trends as for the total amounts. By calculating the effect size of each marker, the

differences in the amounts among markers are standardized and forest plots illustrate the magnitude of the difference between smokers and non-smokers. Further, non-pooled samples and an objective validation of smoking status, reduced the number of confounding factors in the comparisons of GCF markers following periodontal therapy in smokers and non-smokers and to some extent weight up for the relatively low sample size.

The results herein confirmed the study hypothesis by a suppressed inflammatory reaction presented as reduced expression of GCF markers in heavy smokers, especially for pro-inflammatory markers and chemokines. Furthermore, smokers did not respond to periodontal therapy with significant changes in the amounts of any marker in GCF and a negative association was detected between smoking and expression of markers at plaque positive sites. Future prospective studies should contextualize the local tissue responses in smokers within the influence of a larger systemic effect of smoking.

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Figure legends

Figure 1

a. Forest plot for overall standardized effect of smoking at T0 for markers in GCF.

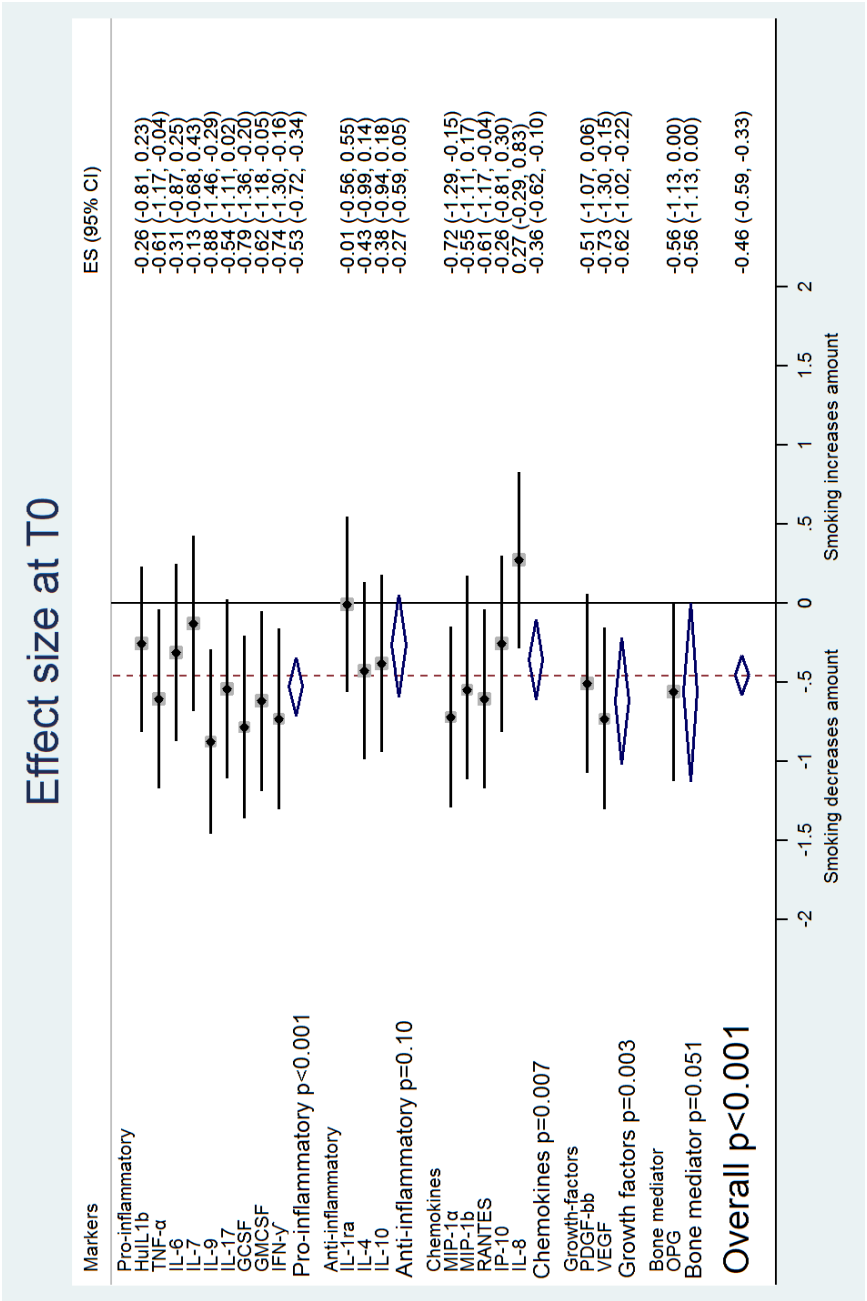
b. Forest plot for overall standardized effect of smoking at T1 for markers in GCF.

c. Forest plot for overall standardized effect of smoking at T2 for markers in GCF.

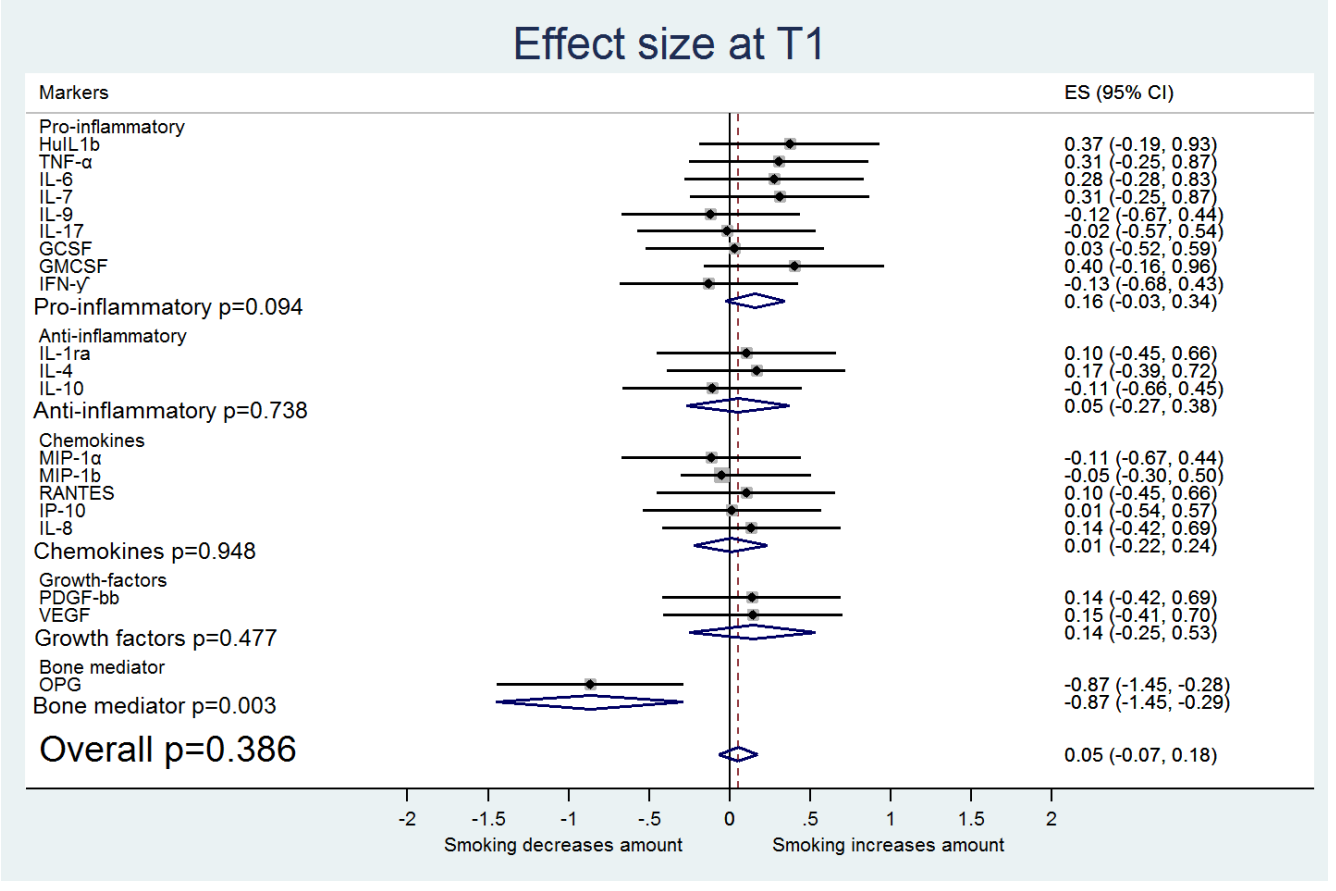
Figures

Figure 1

a)



b)


Effect size at T1
Markers
ES (95% CI)
Pro-inflammatory
HuIL1b
0.37 (-0.19, 0.93)
TNF-α
0.31 (-0.25, 0.87)
IL-6
0.28 (-0.28, 0.83)
IL-7
0.31 (-0.25, 0.87)
IL-9
-0.12 (-0.67, 0.44)
IL-17
-0.02 (-0.57, 0.54)
GCSF
0.03 (-0.52, 0.59)
GMCSF
0.40 (-0.16, 0.96)
IFN-γ
-0.13 (-0.68, 0.43)
Pro-inflammatory p=0.094
0.16 (-0.03, 0.34)
Anti-inflammatory
IL-1ra
0.10 (-0.45, 0.66)
IL-4
0.17 (-0.39, 0.72)
IL-10
-0.11 (-0.66, 0.45)
Anti-inflammatory p=0.738
0.05 (-0.27, 0.38)
Chemokines
MIP-1α
-0.11 (-0.67, 0.44)
MIP-1b
-0.05 (-0.30, 0.50)
RANTES
0.10 (-0.45, 0.66)
IP-10
0.01 (-0.54, 0.57)
IL-8
0.14 (-0.42, 0.69)
Chemokines p=0.948
0.01 (-0.22, 0.24)
Growth-factors
PDGF-bb
0.14 (-0.42, 0.69)
VEGF
0.15 (-0.41, 0.70)
Growth factors p=0.477
0.14 (-0.25, 0.53)
Bone mediator
OPG
-0.87 (-1.45, -0.28)
Bone mediator p=0.003
-0.87 (-1.45, -0.29)
Overall p=0.386
0.05 (-0.07, 0.18)
-2
-1.5
-1
-.5
0
.5
1
1.5
2
Smoking decreases amount
Smoking increases amount

c)

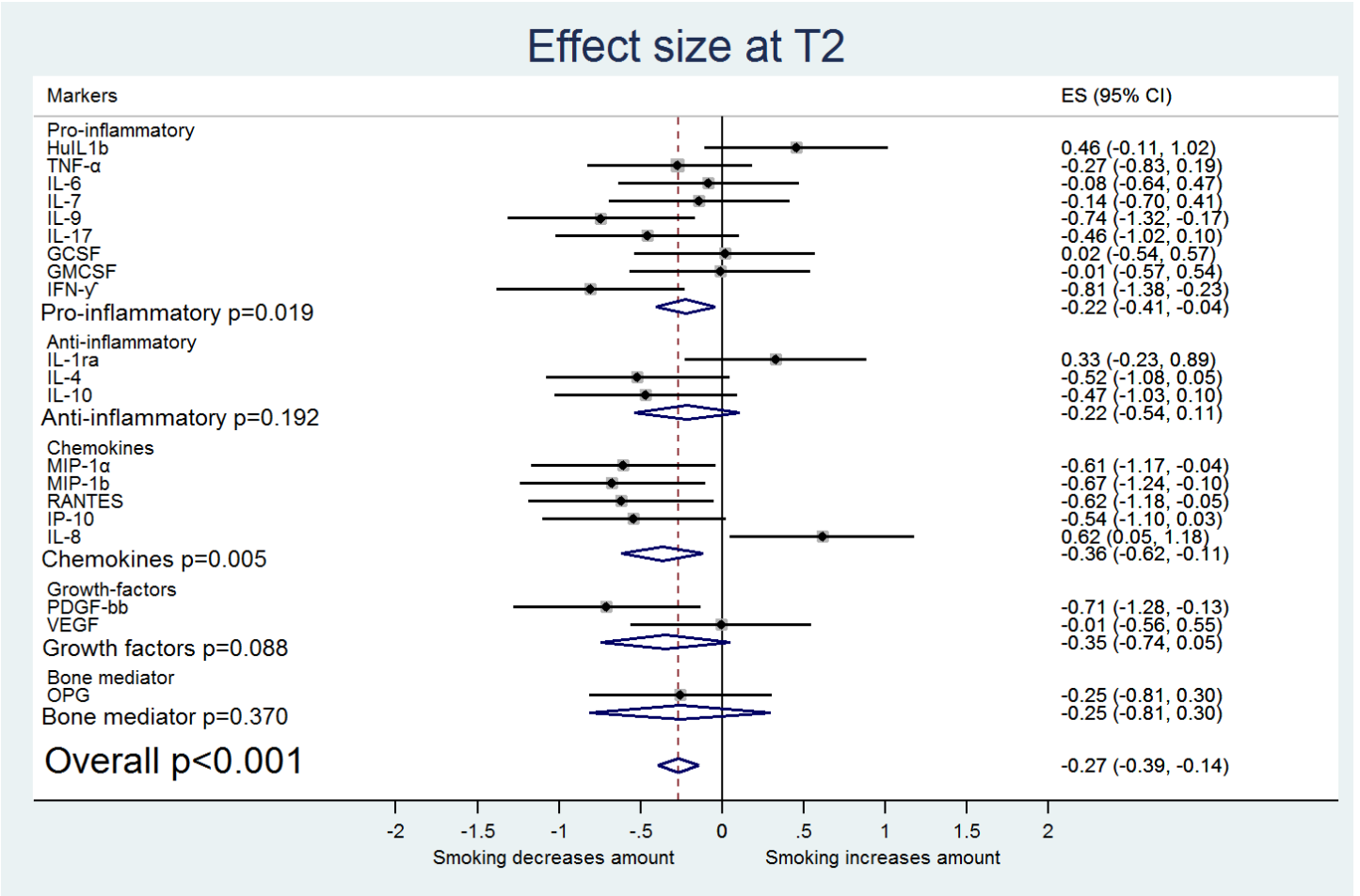

Effect size at T2
Markers
ES (95% CI)
Pro-inflammatory
HuIL1b
0.46 (-0.11, 1.02)
TNF-α
-0.27 (-0.83, 0.19)
IL-6
-0.08 (-0.64, 0.47)
IL-7
-0.14 (-0.70, 0.41)
IL-9
-0.74 (-1.32, -0.17)
IL-17
-0.46 (-1.02, 0.10)
GCSF
0.02 (-0.54, 0.57)
GMCSF
-0.01 (-0.57, 0.54)
IFN-γ
-0.81 (-1.38, -0.23)
Pro-inflammatory p=0.019
-0.22 (-0.41, -0.04)
Anti-inflammatory
IL-1ra
0.33 (-0.23, 0.89)
IL-4
-0.52 (-1.08, 0.05)
IL-10
-0.47 (-1.03, 0.10)
Anti-inflammatory p=0.192
-0.22 (-0.54, 0.11)
Chemokines
MIP-1α
-0.61 (-1.17, -0.04)
MIP-1b
-0.67 (-1.24, -0.10)
RANTES
-0.62 (-1.18, -0.05)
IP-10
-0.54 (-1.10, 0.03)
IL-8
0.62 (0.05, 1.18)
Chemokines p=0.005
-0.36 (-0.62, -0.11)
Growth-factors
PDGF-bb
-0.71 (-1.28, -0.13)
VEGF
-0.01 (-0.56, 0.55)
Growth factors p=0.088
-0.35 (-0.74, 0.05)
Bone mediator
OPG
-0.25 (-0.81, 0.30)
Bone mediator p=0.370
-0.25 (-0.81, 0.30)
Overall p<0.001
-0.27 (-0.39, -0.14)
-2
-1.5
-1
-.5
0
.5
1
1.5
2
Smoking decreases amount
Smoking increases amount

Table 1. Baseline patient related characteristics, presented as mean (± SD), or percentage (number), stratified by smoking status.

	Smokers (n=25)	Non-smokers (n=25)	*p*
Male[1]	32 % (8)	52 % (13)	0.158
Elementary school (≤ 9 years)[1]	76 % (19)	36 % (9)	0.004
Age[2]	56.6 (2.07)	57.9 (1.86)	0.658
Body mass index[2]	22.9 (1.32)	23.62 (1.69)	0.742
Number of teeth[2]	23.5 (1.19)	25.8 (0.48)	0.076
Probing depth[2]	3.9 (0.12)	3.3 (0.10)	0.001
Clinical attachment level[2]	4.6 (0.19)	4.0 (0.11)	0.008
Bleeding index[2]	69.7 (4.00)	66.5 (3.06)	0.532
Plaque index[2]	51.6 (3.96)	56.3 (3.78)	0.415

[1] chi-square test
[2] two-sample independent t-test

Table 2. Collection site* characteristics at T0, T1, and T2, presented as mean (± SD) and percentage (number), stratified by smoking status.

	T0			T1			T2		
	Smokers	Non-smokers	*p*	Smokers	Non-smokers	*p*	Smokers	Non-smokers	*p*
Probing depth[1]	6.84 (0.27)	6.60 (0.22)	0.496	3.88 (0.28)^{A}	3.76 (0.19)^{A}	0.528	4.40 (0.27)^{C}	3.72 (0.26)^{C}	0.077
Clinical attachment level[1]	7.00 (0.30)	6.56 (0.27)	0.277	4.80 (0.36)^{A}	5.08 (0.32)^{A}	0.660	5.08 (0.32)^{C}	4.92 (0.27)^{C}	0.705
GCF volume (µl)[1]	0.46 (0.05)	0.58 (0.05)	0.077	0.37 (0.04)	0.34 (0.02)^{A}	0.565	0.43 (0.07)	0.42 (0.04)^{C}	0.952
Bleeding on probing, positive[2]	88% (22)	100% (25)	0.077	36% (9)^{A}	48% (12)^{A}	0.396	53% (13)^{C}	76% (19)^{C}	0.080
Plaque, positive[2]	84% (21)	96% (24)	0.164	36% (9)^{A}	36% (9)^{A}	1.000	60% (15)	72% (18)^{BC}	0.381
≥ 10^5 red complex species[2]	40% (10)	68% (17)	0.053	40% (10)	36% (9)^{A}	0.773	12% (3)^{B}	0% (0) ^{BC}	0.256
≥ 10^5 orange complex species[2]	92% (23)	96% (24)	0.552	76% (19)	84% (21)	0.480	60% (15)^{C}	60% (15)^{C}	1.000
≥ 10^5 *Aa* species[2]	24% (6)	52% (13)	0.041	52% (13)	44% (11)^{A}	0.571	24% (6)^{B}	32% (8)	0.529

*Collection site; the deepest periodontal pocket in each patient at T0

Aa; Aggregatibacter actinomycetemcomitans

Letters (A, B, and C) in superscript to the mean values denotes a significant difference (p<0.05) over time for that specific clinical and microbiological variable in smokers and in non-smokers. A: T0 vs T1; B: T1 vs T2; C: T0 vs T2.

[1] chi-square test

[2] two-sample independent t-test

Table 3a. Mean (± SEM) amount gingival crevicular fluid markers per site (pg/30 sec) in smokers and non-smokers at T0, T1, and T2.

	T0			T1			T2		
	Smokers	Non-smokers	*p*	Smokers	Non-smokers	*p*	Smokers	Non-smokers	*p*
IL-1β	116.06 (21.79)	242.74 (63.44)	0.365	128.93 (47.85)	59.48 (59.48)^{A}	0.194	104.28 (21.06)	67.8 (17.78)^{C}	0.113
TNF-α	12.38 (1.87)	19.39 (2.83)	0.037*	12.65 (1.95)	9.16 (0.94)^{A}	0.281	10.62 (0.84)	16.60 (2.81)^{B}	0.340
IL-6	5.34 (0.84)	10.28(2.37)	0.275	7.00(1.96)	5.42(1.09)	0.332	8.88(2.27)	9.41(1.89)	0.773
IL-7	1.49 (0.31)	1.70 (0.27)	0.653	1.12 (0.13)	0.96 (0.15)^{A}	0.276	1.47 (0.22)	2.42 (0.77)^{B}	0.619
IL-9	5.16 (1.11)	8.41 (1.02)	0.003*	4.05 (0.79)	3.88 (0.44)	0.678	3.59 (0.33)	7.62 (1.42)^{B}	0.011*
IL-17	235.73 (83.88)	331.66 (78.69)	0.060	249.44 (14.91)	161.53 (36.04)	0.949	123.28 (31.28)	215.70 (52.52)	0.111
G-CSF	74.71 (20.26)	137.07 (23.84)	0.008*	68.66 (14.91)	77.20 (16.36)	0.910	156.91 (49.27)	102.61 (15.03)	0.951
GM-CSF	134.69 (14.75)	173.02 (14.38)	0.033*	108.98(11.89)	91.39 (7.31)^{A}	0.161	124.95 (19.87)	119.75 (12.05)^{C}	0.967
IFN-γ	36.87 (7.49)	56.64 (7.93)	0.012*	28.46 (4.39)	30.47 (3.69)^{A}	0.652	24.22 (2.60)	62.60 (13.07)^{B}	0.006*
IL-1ra	3239.77 (725.91)	3037.24 (515.87)	0.978	2301.01(441.55)	2185.45 (504.33)	0.712	4502.04 (812.65)	2720.69 (551.70)	0.249
IL-4	0.70 (0.17)	1.02 (0.19)	0.136	0.62(0.10)	0.61(0.10)	0.557	0.53(0.07)	1.14 (0.23)	0.073
IL-10	6.26 (1.20)	7.47 (1.03)	0.183	4.17 (0.58)	4.13 (0.43) ^{A}	0.705	6.46 (1.08)	8.27 (1.20)^{B}	0.104
PDGF	6.44 (1.13)	10.87 (1.77)	0.077	4.99 (0.68)	5.41 (0.89)	0.625	4.84 (0.60)	11.97 (2.46)^{B}	0.016*
VEGF	76.40 (11.41)	117.73 (14.55)	0.013*	74.05 (11.81)	59.74 (6.43)^{A}	0.609	85.15 (13.64)	77.3 (9.14)	0.983
MIP-1α	3.42 (0.91)	5.53 (0.93)	0.014*	3.44 (0.72)	3.00 (0.41)	0.691	2.19 (0.33)	4.1 (0.77)	0.037*
MIP-1b	7.18 (1.61)	10.00 (1.44)	0.057	7.87(1.14)	7.96 (1.07)	0.860	7.7 (1.46)	10.86 (1.25)	0.021*
RANTES	25.74 (6.81)	47.05 (8.55)	0.037*	25.36 (6.09)	23.54 (4.00)	0.716	18.16 (3.26)	36.41 (6.49)	0.033*
IP-10	23.16 (7.51)	17.97 (2.22)	0.368	13.67 (2.40)	12.27 (2.12)^{A}	0.963	14.33 (2.67)	21.76 (3.61)^{B}	0.062
IL-8	113.44 (18.74)	93.61 (13.90)	0.340	98.63 (21.18)	85.55 (19.01)	0.633	220.68 (47.41)	107.23 (26.00)	0.034*
OPG	3.29 (0.17)	3.84 (0.20)	0.052	3.08 (0.18)	3.89 (3.48)	0.003*	3.66 (0.22)	3.94 (0.22)	0.371

Letters A and C in superscript to the mean values denotes a significant decrease ($p<0.05$) over time between the log mean values for that specific marker in smokers and in non-smokers. A: T0 vs T1; C: T0 vs T2. Letter B denotes a significant increase ($p<0.05$) over time. B: T1 vs T2.

Table 3b. Mean (± SEM) concentration gingival crevicular fluid markers per site (pg/mL) in smokers and non-smokers at T0, T1, and T2.

	T0			T1			T2		
	Smokers	Non-smokers	*p*	Smokers	Non-smokers	*p*	Smokers	Non-smokers	*p*
IL-1β	248.96 (29.08)	436.53 (128.04)	0.898	373.01 (142.30)	165.72 (128.04)	0.211	294.50 (67.44)	157.76 (35.87	0.092
TNF-α	28.11 (2.80)	32.69 (3.27)	0.350	34.92 (3.91)	27.36 (2.68)	0.288	29.80 (2.92)	41.45 (6.62)	0.278
IL-6	12.87 (1.78)	16.88 (3.07)	0.937	19.81 (5.08)	15.97 (2.41)	0.368	23.09 (5.61)	23.52 (4.00)	0.738
IL-7	3.67(0.68)	2.96 (0.40)	0.385	3.42 (0.43)	3.03 (0.51)	0.339	4.08 (0.67)	6.31 (2.29)	0.580
IL-9	10.69 (1.44)	14.50 (1.21)	0.019*	10.50 (1.15)	11.85 (1.35)	0.647	10.50 (1.15)	19.04 (3.71)	0.010*
IL-17	496.52 (141.28)	559.14 (110.94)	0.185	575.99 (144.20)	503.27 (118.80)	0.932	393.19 (112.77)	543.48 (118.30)	0.108
G-CSF	169.56 (42.01)	248.76 (51.57)	0.050	194.97 (27.28)	222.54 (35.75)	0.937	391.03 (104.83)^{C}	260.32 (35.99)	0.957
G-MCSF	300.50 (9.54)	300.04 (7.20)	0.909	296.02 (9.75)	263.82 (17.55)	0.132	293.21 (7.16)	298.04 (9.40)	0.841
IFN-γ	75.05 (7.57)	95.12 (9.14)	0.078	79.41 (10.12)	92.77 (10.88)	0.612	66.34 (6.83)	154.61 (34.45)	0.002*
IL-1ra	8203.28 (1659.82)	5615.66 (921.58)	0.310	7115.12 (1506.65)	6582.10 (1406.72)	0.751	12567.68 (2488.53)	7775.38 (1787.28)	0.258
IL-4	1.45 (0.19)	1.70 (0.22)	0.625	1.75 (0.23)	1.82 (0.29)	0.571	1.51 (0.20)	2.84 (0.54)	0.057
IL-10	15.09 (2.61)	13.38 (1.62)	0.739	12.71 (1.83)	12.21 (1.32)	0.682	17.23 (2.89)	21.35 (2.98)^{B}	0.084
PDGF	14.87 (2.57)	19.56 (3.55)	0.347	15.00 (2.13)	16.89 (2.98)	0.658	14.89 (2.21)	29.17 (5.88)^{B}	0.018*
VEGF	179.82 (18.35)	205.49 (19.60)	0.248	200.12 (20.52)	178.43 (18.18)	0.621	208.76 (21.31)	192.99 (12.89)	0.958
MIP-1α	6.94 (1.19)	9.33 (1.16)	0.086	8.95 (1.31)	9.03 (1.20)	0.661	6.44 (1.11)	9.89 (1.24)	0.030*
MIP-1b	16.24 (3.04)	17.74 (1.92)	0.387	22.40 (2.47)	23.55 (3.03)	0.832	19.40 (2.45)	27.45 (2.38)^{C}	0.009*
RANTES	53.46 (9.21)	80.21 (11.14)	0.147	63.02 (10.19)	71.54 (11.85)	0.735	51.48 (9.77)	87.90 (11.60)	0.022*
IP-10	58.18 (21.23)	31.22 (2.84)	0.997	37.41 (4.42)	37.45 (7.56)^{B}	0.986	35.54 (3.51)	55.14 (9.64)^{C}	0.028*
IL-8	272.68 (40.36)	180.11 (32.95)	0.057	285.25 (54.16)	224.93 (45.68)	0.650	517.44 (91.60)	254.58 (44.06)	0.017*
OPG	2.38 (0.23)	3.21 (0.22)	0.013*	1.99 (0.17)	2.79 (0.18)	0.002*	2.65 (0.23)	2.97 (0.24)	0.354

Letters A and C in superscript to the mean values denotes a significant decrease ($p<0.05$) over time between the log mean values for that specific marker in smokers and in non-smokers. A: T0 vs T1; C: T0 vs T2. Letter B denotes a significant increase ($p<0.05$) over time. B: T1 vs T2.

Table 4. Overall unadjusted and adjusted associations between gingival crevicular fluid markers (pg/30 sec) and cigarette smoking stratified by presence of plaque and numbers of red complex species.

	plaque negative sites		plaque positive sites		$< 10^5$ red complex species		$\geq 10^5$ red complex species	
	Coef (95 % CI)	*p*	Coef (95 % CI)	*p*	Coef (95 % CI)	*p*	Coef (95 % CI)	*p*
Unadjusted								
Pro-inflammatory markers	2.11 (-1.61,5.82)	0.257	-3.23 (-5.57,-0.90)	0.008*	-0.74 (-3.2,1.7)	0.552	-3.36 (-6.60,-0.13)	0.042*
Anti-inflammatory markers	0.29 (-0.80,1.39)	0.590	-0.69 (-1.39,0.06)	0.052	-0.02 (-0.74,0.69)	0.954	-1.19 (-2.11,-0.26)	0.012*
Chemokines	0.94 (-1.19,3.06)	0.377	-2.05 (-3.56,-0.55)	0.009*	-0.74 (-2.17,0.69)	0.306	-1.81 (-3.81,0.18)	0.074
Growthfactors	0.31 (-0.59,1.21)	0.487	-0.88 (-1.50,-0.26)	0.006*	-0.48 (-1.16,0.19)	0.158	-0.61 (-1.41,0.18)	0.125
Th1 / Th2 -ratio	-5.22 (-17.75,7.29)	0.402	-0.26(-0.93,0.42)	0.446	-3.79 (-10.81,3,22)	0.282	0.98 (-0.23,2.19)	0.108
OPG	-0.70 (-1.45,0.04)	0.062	-0.49 (-0.95,-0.03)	0.037*	-0.33 (-0.86,0.21)	0.227	-1.01 (-1.58,-0.45)	0.001*
Adjusted[§]								
Pro-inflammatory markers	12.04 (10.46,13.62)	<0.001*	-5.90 (-9.85,-1.96)	0.004*	-3.52 (-9.51,2.47)	0.236	-6.77 (-12.99,-0.55)	0.034*
Anti-inflammatory markers	0.50 (0.41,0.59)	<0.001*	-1.50 (-5.50,-0.81)	0.016*	-0.70 (-2.79,1.40)	0.498	-1.68 (-3.02,-0.34)	0.016*
Chemokines	7.26 (4.78,9.75)	0.001*	-3.15 (2.77,0.10)	0.010*	-1.94 (-5.21,1.33)	0.232	-3.00 (-6.98,0.99)	0.134
Growth factors	2.11 (1.63,2.58)	<0.001*	-1.32 (-2.26,-0.39)	0.007*	-0.98 (-2.32,0.34)	0.140	-1.41 (-2.97,0.14)	0.073
Th1 / Th2 -ratio	10.12 (-9.78,10.51)	<0.001*	-1.50 (-3.30,0.30)	0.101	-1.13 (-4.95,2.68)	0.544	0.71 (-2.69,1.28)	0.471
OPG	1.72 (1.05,2.38)	0.002*	-0.64 (-1.25,-0.03	0.042*	0.01 (-0.92,0.94)	0.982	-1.10 (-1.90,-0.28)	<0.011*

* significant *p*-value (<0.05); § regression analysis adjusted for age, gender, and education