J Clin Periodontol 2004; 31: 894–902 doi: 10.1111/j.1600-051X.2004.00585.x
Printed in Denmark. All rights reserved

Journal of Clinical Periodontology

Cytokine profile in gingival crevicular fluid of aggressive periodontitis: influence of smoking and stress

Joanna J. Kamma[1], Catherine Giannopoulou[2], Vassilis G. S. Vasdekis[3] and Andrea Mombelli[2]

[1]Private Practice, Athens, Greece; [2]Division of Physiopathology and Periodontology, School of Dentistry, Medical Faculty, University of Geneva, Switzerland; [3]University of Economics and Business, Athens, Greece

Kamma JJ, Giannopoulou C, Vasdekis VGS, Mombelli A: Cytokine profile in gingival crevicular fluid of aggressive periodontitis: influence of smoking and stress. J Clin Periodontol 2004; 31: 894–902. doi: 10.1111/j.1600-051X.2004.00585.x.

Abstract

Background: Cigarette smoking and stress are considered risk factors that have been associated with periodontal disease progression. Conflicting results have been reported concerning the direct influence of smoking on the subgingival microbiota of periodontitis patients. Cytokine production may also be influenced by smoking and stress leading to an imbalance that disturbs the host–parasite relationship.

Aim: The objective of the present study was to evaluate the influence of cigarette smoking on the gingival crevicular fluid (GCF) levels of interleukin (IL)-1β, IL-4, IL-6 and IL-8 in aggressive or early onset periodontitis (EOP) patients and in healthy controls (H), psychosocial stress being considered as modifying factor.

Material and Methods: Sixty-five EOP and 35 periodontally healthy individuals participated in this cross-sectional study. All the participants were interviewed about their smoking habits and their stressful social events. Clinical examination included the assessment of plaque index (PI), bleeding on probing (BOP), clinical attachment level (CAL) and probing pocket depth (PPD). GCF was collected using durapore strips, from four sites per patient, randomly selected in each quadrant. The total amounts of IL-1β, IL-4, IL-6 and IL-8 were measured in a total of 400 samples using commercially available enzyme-linked immunosorbent assays.

Results: All clinical parameters were significantly higher in the EOP group compared to the H group. There were no significant differences between EOP smokers and EOP non-smokers with regard to plaque accumulation, CAL and PPD of the sampling sites, whereas mean CAL and PPD of the diseased sites were greater in EOP smokers than in EOP non-smokers. In addition, EOP smokers seemed to have significantly less BOP and greater bone loss compared to EOP non-smokers. Significant interactions between ''EOP'' and ''smoking'' were present for total amounts of IL-1β and IL-4. IL-1β, IL-6 and IL-8 showed significant main effects with healthy smokers and healthy non-smokers, respectively. For IL-8, stress presented a statistically significant interaction with smoking status and EOP ($F = 4.742, p = 0.030$). More specifically EOP smokers were statistically affected by stress.

Conclusions: Smoking influences host-related factors including cytokine network. The relative importance of smoking and stress-related alterations and their precise mode of action in increasing the risk of aggressive periodontitis remains to be elucidated.

Key words: cytokines; early onset or aggressive periodontitis; gingival crevicular fluid; interleukin-1β; interleukin-4; interleukin-6; interleukin-8; smoking; stress

Accepted for publication 15 January 2004

The role of cigarette smoking in the pathogenesis of periodontal disease has been extensively studied and well documented by several investigators.

Cigarette smoking is a significant risk factor in the pathogenesis of periodontal disease, also associated with disease progression (Bergström & Preber 1994, Grossi et al. 1994).

Conflicting results have been reported concerning the influence of smoking on the subgingival microbiota of periodontitis patients (Stoltenberg et al. 1993, Zambon et al. 1996,

Boström et al. 1998a, 2001, Renvert et al. 1998, Kamma et al. 1999, Darby et al. 2000).

Regardless of the different microbial profiles that are identified in smokers and non-smokers in the majority of the investigations it is unclear whether the increased presence of certain micro-organisms is the cause or the consequence of a more severe disease condition.

It is known that bacterial products stimulate monocytes/macrophages and lymphocytes as well as resident cells (fibroblasts, endothelial cells) to secrete pro-inflammatory and immuno-regulatory cytokines. Smoking could have a negative impact on periodontal health by interfering with the immune system and modifying host response in the presence of plaque bacteria. (Barbour et al. 1997).

Interleukin (IL)-1β is a multifunctional pro-inflammatory mediator with a crucial role in the regulation of inflammatory reactions (Dinarello 1988). The observation that IL-1β can act on a large number of cells, like fibroblasts, chondrocytes, bone cells, neutrophils and lymphocytes, suggests that periodontal destruction and repair in periodontitis may in part be associated with this cytokine (Jandinski 1988).

IL-4 is a potent downregulator of macrophage function by inhibiting the secretion of IL-1β, tumor necrosis factor-α (TNF-α) and IL-6 (Te Velde et al. 1990). It also inhibits the secretion of prostaglandin (PG)E_2 by human monocytes which leads to bone resorption (Corcoran et al. 1992, Shapira et al. 1992). Furthermore, localized absence of IL-4 in diseased periodontal tissues has been associated with periodontal disease activity and progression (Shapira et al. 1992, Kabashima et al. 1996).

IL-6 is a multifunctional cytokine that contributes to the terminal differentiation of B-lymphocytes to plasma cells and stimulates secretion of immunoglobulin (Ig)A and IgG (Fujihashi et al. 1993). Smoking causes a depression of IgG and possibly IgA production in serum (Quinn et al. 1996). Of particular significance is the ability of IL-6 to induce bone resorption, both by itself and in conjunction with other bone-resorbing agents (Ishimi et al. 1990, Mundy 1991).

Finally, IL-8 is one of the chemokines, which has powerful chemotactic functions for polymorphonuclear leukocytes but also for lymphocytes and macrophages. Is a potent mediator of granulocyte accumulation at the sites of inflammation (Bickel 1993). Increased levels of IL-8 are found in the gingival crevicular fluid (GCF) of inflamed sites compared with ones (Baggiolini & Clark-Lewis, 1992, Tsai et al. 1995, Mathur et al. 1996).

Previous studies reported significantly increased levels of TNF-α, but not of IL-6 and IL-1β, in the GCF of current and former smokers with periodontal disease in comparison to non-smokers (Boström et al. 1998b, 1999, 2000).

In a recent study by Fredriksson et al. (2002), smoking was found to reduce the sensitivity of peripheral neutrophils to stimulation by IL-8 when comparing smoking to non-smoking patients.

This suggests that smoking may interfere with the inflammatory process by affecting the release of pro-inflammatory cytokines.

Stress has also been suggested to contribute to periodontal inflammation (Monteiro da Silva et al. 1996) by affecting both immune functions (Herbert & Cohen 1993) and susceptibility to infectious diseases (Cohen & Williamson 1991). The pathways mediating these interactions are still unexplored. Depressed immune responsiveness as a result of physical or mental stress has been postulated to be one of the several factors involved in the etiology of destructive periodontal disease (Ballieux 1991).

Increased levels of several cytokines such as IL-1, IL-2, IL-6, IL-8 and TNF-α have been observed in the GCF of patients with periodontal disease (Lee et al. 1995, Tsai et al. 1995, Wilson et al. 1996, Hirose et al. 1997, Giannopoulou et al. 2003). EOP patients showed a greater level of IL-1β, IL-6 and IL-8 in their GCF and lower level of IL-4 as compared to adult periodontitis patients and periodontally healthy individuals (Giannopoulou et al. 2003).

Therefore, the objective of the present study was to evaluate the influence of cigarette smoking on the GCF level of IL-1β, IL-4, IL-6 and IL-8 in early onset periodontitis (EOP) patients and periodontally healthy controls, psychosocial stress being considered as modifying factor.

Material and Methods

Subject population

Sixty-five aggressive periodontitis (EOP) patients and 35 periodontally healthy (H) individuals participated in this cross-sectional study. They were selected from a private practice limited to periodontics in Athens, Greece. All patients were systemically healthy, had not received antibiotics during the 6 months prior to entering the study and had no periodontal therapy during the previous year. Individuals who were pregnant or required pre-medication with a systemic antibiotic were excluded.

Aggressive periodontitis patients were selected according to clinical and radiographic criteria (less than 35 years old, loss of clinical attachment greater than 5 mm at two to three sites in more than 14 permanent teeth and radiographic evidence of bone loss).

In addition to aggressive periodontitis patients, 35 individuals without evidence of periodontitis (H) completed baseline measures. They were designated as healthy if their mean full-mouth clinical attachment loss values was less than 2 mm, and they had no radiographic evidence of alveolar bone loss.

The smoking history and the impact of life events (stress) were assessed on the patient's own report.

A subject was classified as smoker if he or she smoked regularly more than 10 cigarettes/day. Non-smokers were subjects who had never smoked. Former smokers, i.e. subjects who had previously been smokers but had stopped their habit, were excluded.

During an interview, the stressful social events were assessed using a questionnaire based on the modified and perceived stress scale (Linn 1986), and expressed as total perceived stress.

This scale measures total and average perceived stress in relation to major life events that happened during the previous year. While number of major life events gives a measure of objective stress, the total perceived stress score provides a measure of subjective stress within the person's environment over a given time. On the other hand, the average perceived stress score is presumed to reflect a person's usual way of perceiving stress. The instrument contained 30 life events applicable to adults. Subjects estimated on a 10-point scale (0 = none, 9 = extreme) the degree to which the event was perceived as stressful.

The demographic and behavioral data of each subject group are presented in Table 1.

Periodontal examination

The clinical and radiographic evaluations were performed by one periodontist (J. K.). The clinical examination included assessment of probing pocket depth (PPD), clinical attachment level (CAL) (Glavind & Löe 1967), plaque index (PI) (O'Leary et al. 1972) and bleeding upon probing (BOP) (Ainamo & Bay 1975) at six sites around each tooth, excluding third molars. Measurements were carried out to the nearest millimeter using a Goldman/Fox Williams periodontal probe. PPD was defined as the distance in millimeter from the most coronal margin of the free gingiva to the most apical penetration of the probe. CAL was defined as the distance in millimeter from the cemento-enamel junction to the most apical point of penetration of the probe. The number of teeth present in each patient was also recorded during the clinical examination.

Full-mouth standardized periapical radiographs were taken for all patients. Destruction of alveolar bone was assessed by the Schei method (Schei et al. 1959) and expressed as percentage bone loss. Measurements were made on the mesial and distal aspect of all teeth. The clinical parameters for each subject group are presented in Table 1.

GCF sampling

Clinical measurements were recorded and GCF sampling sites were pre-selected 1 week before sampling. GCF was collected in the four pre-selected sites, the deepest ones in each quadrant of each patient.

Briefly, teeth were air-dried and isolated with cotton rolls, supragingival plaque was removed and GCF was sampled with pre-cut durapore filter membranes 2 mm × 6 mm (pore size = 0.22 mm; Millipore Corp., Bedford, MA, USA). The first durapore strip was inserted 1 mm into the sulcus or pocket and left in place for 15 s. After removal of this first strip and waiting 3 min, a second durapore strip was inserted in the same site, in the same manner. The two strips were then placed into a microcentrifuge tube and immediately frozen at −70°C until the day of analysis. In case of visible contamination with blood, the strips were discarded and other sites fulfilling the same criteria were selected.

Clinical measurements were recorded and GCF sampling sites pre-selected 1 week before sampling. Clinical parameters were re-examined after GCF sampling and these values were used in the analysis.

Analysis of mediator production

The amounts of IL-1β, IL-4, IL-6 and IL-8 in the GCF were determined after centrifugal elution, by using commercially available enzyme-linked immunoadsorbent assays (ELISAS) (Ruwag Diagnostics Zurich, Switzerland), specific for each cytokine. The assays were sandwiched (ELISAs) and performed according to the manufacturer's instructions using human recombinant standards.

Results were calculated based on ELISA concentration values and reported as total cytokine amounts (in pg ± SD) per 30 s sample. Sites with cytokine levels below the limits of assay's detectability were scored as 0 pg.

Statistical analysis

An ANOVA model with two factors was fitted at a first stage in order to determine factors' effects. The two factors were ''EOP'' (with two levels: 1 if an individual was EOP and 0 if it was periodontally healthy) and ''smoking'' (1 for a smoker and 0 for a non-smoker). At a second stage, ''stress'' was added as a covariate with all possible interactions with ''EOP'' and ''smoking''. Due to data heterogeneity a weighted regression model was used (Montgomery et al. 2001).

Results

Patient biographical and clinical data

The demographic and behavioral data including gender, age, smoking habits and stress characteristics are summarized in Table 1. Interestingly, significantly more patients of the EOP group were found to be under more stressful conditions as compared to those in the H group. In addition, significantly more patients of the EOP-smokers group were under more stressful conditions as compared to the EOP-non-smokers group.

Table 1 also describes the periodontal status of each group including mean PPD, CAL of the four selected sites in each patient, as well as mean PPD and CAL of the diseased sites, namely sites exhibiting >5 mm PPD. Furthermore, Table 1 shows the percentage of sites with PI and BOP, as well as percentage of bone loss. As expected, all clinical parameters were significantly higher in the EOP group as compared to the H group. Concerning the EOP group, there were no significant differences between smokers and non-smokers with regard to PPD and CAL of the sampling sites and plaque accumulation, whereas mean PPD and CAL of the diseased sites were greater in EOP smokers as compared to EOP non-smokers. In addition, EOP smokers seemed to have significantly

Table 1. Demographical, behavioral and clinical characteristics of periodontally healthy and early onset periodontitis (EOP) or aggressive periodontitis patients

	Healthy		EOP	
	smokers	non-smokers	smokers	non-smokers
N	16	19	39	26
male/female	6/10	7/12	15/24	14/12
mean age	38 ± 8.8*	38 ± 11.8*	32.4 ± 2.9*	32.6 ± 2.8*
cigarettes	40 ± 25.9		30.5 ± 12.5	
stress	4.5 ± 12.8	3.2 ± 7.3	22.8 ± 21.4	10 ± 14.7
PPD (mm)/sampling sites	1.7 ± 0.2	2.2 ± 0.3	6.2 ± 1.7	6.5 ± 1.2
PPD (mm)/diseased sites (>5 mm)			6.7 ± 1.2†	5.9 ± 1.0
CAL (mm)/sampling sites	0	0	6.5 ± 1.9	6.6 ± 1.6
CAL (mm)/diseased sites (>5 mm)			7.2 ± 1.4†	6.7 ± 1.4
plaque	37.5 ± 0.4	36.8 ± 0.2	84.9 ± 0.1	89.4 ± 0.2
bleeding on probing (% of sites)	0	0	56.3 ± 0.0	78.9 ± 0.5†
mean bone loss (%)	0	0	46.9 ± 7.1	40.4 ± 7.6
teeth present (*N*)			25.9 ± 2.3	26.7 ± 2.6

*mean ± SD.

†ANOVA statistically significant differences.

PPD, probing pocket depth; CAL, clinical attachment level.

less BOP and greater bone loss as compared to EOP non-smokers.

GCF mediator level

Table 2 presents the mean total amounts of each cytokine in smokers and non-smokers for periodontally healthy (H) and for patients with EOP. When analyzing a factorial design, there are two classes of effects that we are interested in: the main effects and interactions. The differences between the means of the observations of the level of a factor (i.e. smoking, EOP, stress) are the main effects. If the main effects of one factor are not the same across the levels of another factor then these factors interact. Differences between smokers and non-smokers (main effects of smoking) were not the same for EOP and periodontally healthy individuals (interaction between EOP and smoking).

The results of the first stage of analysis showed that significant interactions between ''EOP'' and ''smoking'' were present for IL-1β and IL-4 expression ($F = 4.78$, $p = 0.029$ and $F = 6.67$, $p = 0.010$, respectively) (Table 3). These interactions are graphically depicted in Fig. 1. For IL-1β and for EOP individuals there was no significant difference between smokers and non-smokers (mean difference = 1.00, $p = 0.801$). For healthy individuals, however, there was a statistically significant difference in favor of smokers (mean difference = 10.12, $p < 0.001$). The difference between the two mean differences (δ) is statistically significant (-9.12, $p = 0.029$) indicating the presence of a statistically significant interaction of ''EOP'' and ''smoking'' status (Table 4). With regard to IL-4 in EOP individuals, smokers showed significantly increased levels of this cytokine as compared to non-smokers (1.19, $p = 0.002$), while in healthy individuals this difference was reversed (mean difference = -1.74, $p = 0.106$), (Table 4 and Fig. 2). Again the difference between the two mean differences (δ) is statistically significant (2.93, $p = 0.01$). Also, IL-6 in healthy individuals exhibited a statistically significant difference in favor of smokers (mean difference = 0.81, $p < 0.001$) (data not shown in table). Contrarily, IL-6 and IL-8, did not exhibit any significant interaction between smokers and EOP ($F = 0.051$, $p = 0.822$ for IL-6 and $F = 0.057$, $p = 0.811$ for IL-8). However, main effects were apparent (Table 3). Table 5 presents the means of IL-6 and IL-8 in EOP and healthy individuals, for smokers and non-smokers. IL-6 showed differences when comparing EOP and healthy individuals (mean difference = 4.23, $p < 0.001$) and between smokers and non-smokers (mean difference = 0.82, $p < 0.001$) (Fig. 3). Likewise, IL-8 showed differences between EOP and healthy individuals (mean difference = 48.34, $p < 0.001$) and between smokers and non-smokers (mean difference = -3.72, $p = 0.019$), the difference being in favor of non-smokers (Table 5, Fig. 4).

Table 2. Mean* GCF inflammatory mediator levels in periodontally healthy and early onset periodontitis (EOP) patients, smokers and non-smokers

	EOP		Healthy	
	smokers	non-smokers	smokers	non-smokers
IL-1β	62.37	61.37	17.97	7.85
IL-4	3.08	1.90	10.33	12.07
IL-6	6.04	5.04	1.70	0.89
IL-8	68.30	72.96	20.43	24.00

*Results are expressed as pg/30 s sample.
GCF, gingival crevicular fluid; IL, interleukin.

Table 3. Significance levels of main and interaction effects of the two variables between group (H, EOP) and smoking status

	IL-1β	IL-4	IL-6	IL-8
EOP × smoking	**0.029**	**0.010**	0.822	0.811
smoking	**0.008**	0.623	**0.003**	0.071
EOP	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Bold face denotes statistically significant interactions and main effects.
H, healthy; EOP, early onset periodontitis.

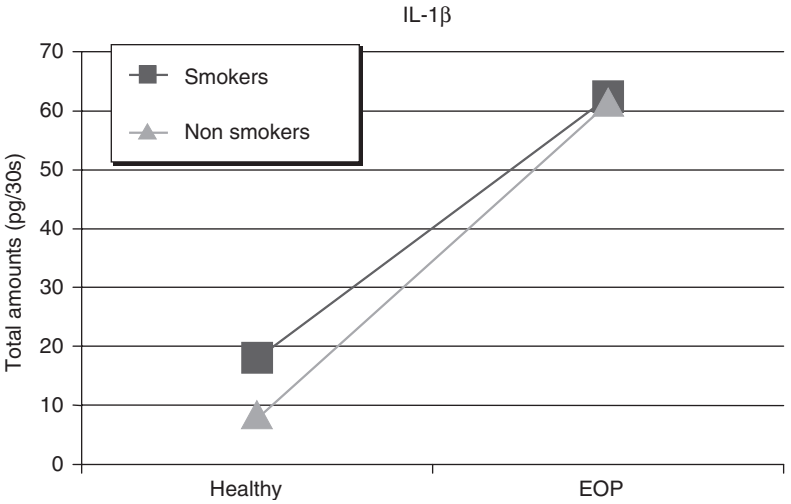

Fig. 1. Mean total amounts of interleukin (IL)-1β in early onset periodontitis (EOP) and healthy groups between smokers and non-smokers. Healthy exhibited greater differences in total amounts of IL-1β between smokers and non-smokers than EOP patients ($p < 0.001$).

At the second stage of analysis, stress entered as a covariate. Table 6 summarizes the statistically significant interactions of stress with EOP and/or smoking status. According to these results, the slopes of EOP and healthy individuals were different for IL-1β ($p = 0.007$). The same difference was noticed for IL-4 ($p = 0.006$) and IL-6 ($p = 0.001$). Likewise, significant differences in the slopes were observed between smokers and non-smokers in IL-4 ($p = 0.037$). IL-8 demonstrated a complex linear dependence with stress, the slopes being different for EOP, healthy, smokers and non-smokers.

Table 4. Differences* in total amounts of IL-1β, IL-4, IL-6 and IL-8 in EOP and healthy population between smokers and non-smokers

	IL-1β	IL-4
EOP smokers/non-smokers	1.00 ($p = 0.801$)	1.19 (**$p = 0.002$**)
H smokers/non–smokers	10.12 (**$p < 0.001$**)	− 1.74 ($p = 0.106$)
differences (δ)	− 9.12 (**$p = 0.029$**)†	2.93 (**$p = 0.01$**)†

*Weighted least-squares regression.
†Interaction effects (statistically significant differences of differences (EOP smokers/non-smokers) − (H smokers/non-smokers).
Bold face denotes statistically significant differences.
IL, interleukin; EOP, early onset periodontitis; H, healthy.

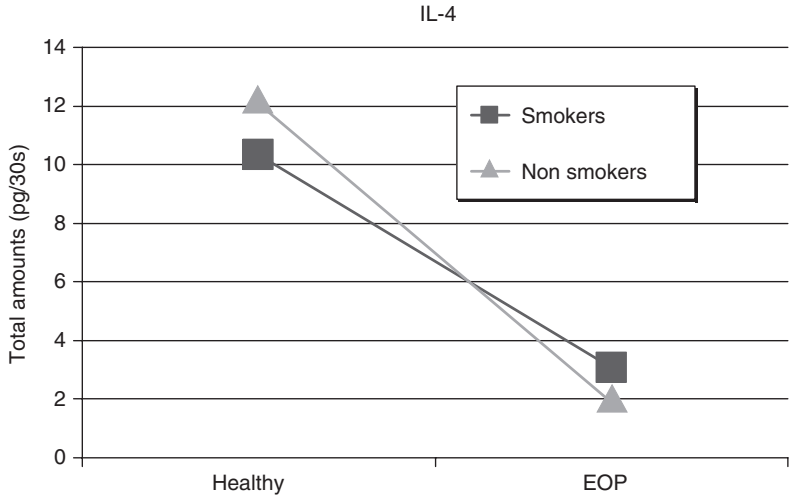

Fig. 2. Mean total amounts of interleukin (IL)-4 in early onset periodontitis (EOP) and healthy groups between smokers and non-smokers. EOP patients showed greater differences between smokers and non-smokers ($p < 0.001$). Healthy non-smokers had the greatest total amounts of IL-4.

Table 5. Estimated marginal mean* GCF inflammatory mediator levels in periodontally healthy and early onset periodontitis (EOP) patients, smokers and non-smokers

	Smokers	Non-smokers	δ	EOP	Healthy	δ
IL-6	3.83	3.01	0.82 **$p < 0.001$**	5.53	1.30	4.23 **$p < 0.001$**
IL-8	44.53	48.25	− 3.72 **$p = 0.019$**	70.56	22.22	48.34 **$p < 0.001$**

Weighted least-squares regression.
Bold face denotes statistically significant differences.
GCF, gingival crevicular fluid; IL, interleukin.

Specifically, for IL-1β, in healthy individuals, the slope of stress was of − 0.02 ($p = 0.736$) while in EOP patients this was of 0.31 (difference in slopes 0.33, $p = 0.007$) showing a positive correlation with stress. For IL-4, the slopes of stress in healthy individuals, smokers and non-smokers, were − 0.175 and − 0.134 ($p = 0.012$), respectively. The difference of the two slopes was statistically significant (− 0.0407, $p = 0.037$). EOP smokers and non-smokers had small slopes for stress (− 0.033 and 0.008, respectively). Stress showed no linear relationship with IL-6 in healthy individuals (− 0.0043, $p = 0.437$) and this relationship was positive and significant in EOP patients (0.074, $p = 0.001$). Finally, for IL-8 stress presented a statistically significant interaction with smoking status and EOP ($F = 4.742$, $p = 0.030$). The slope for EOP smokers was estimated as 0.307 ($p = 0.017$); for EOP non-smokers it was − 0.195 ($p = 0.368$), for healthy individuals who smoked it was 0.04 ($p = 0.659$) and finally, for healthy individuals who did not smoke, the slope was 0.23 ($p = 0.192$), showing that only EOP smokers were statistically affected by stress.

Discussion

The objective of this study was to evaluate the influence of smoking and stress on the clinical parameters and cytokine profile in a group of individuals exhibiting aggressive periodontitis.

Clinical parameters

The observation that PPD and CAL were greater in diseased sites in smokers as compared to non-smokers, confirms that smoking might be associated with increased disease severity. Furthermore, smokers exhibited greater bone loss than non-smokers. This outcome is in general agreement with observations presented by our group (Kamma et al. 1999) in an EOP population. Schenkein et al. (1995) observed that smokers with generalized EOP had significantly more teeth with at least 5 mm of clinical attachment loss than did non-smokers. Thus, the risk of smoking could greatly accelerate tooth loss in this group of relatively young individuals who are already at high risk for progressive periodontal attachment loss. Cigarette smoking was associated mostly with a greater increase in probing depth and attachment loss, as well as greater tooth loss at an earlier age (Chen et al. 2001). A strong relation between smoking and severe bone destruction in subjects with EOP was also found by Mullally et al. (1999). Payne et al. (2000) reported that postmenopausal female smokers were more likely to lose alveolar bone height and density as compared to non-smokers with a similar periodontitis, plaque and gingival bleeding experience.

Smoking has been shown to have similar negative impact in a group of 289 adult periodontitis patients studied by Haffajee & Socransky (2001). Current smokers had significantly more CAL, missing teeth, deeper pockets and fewer sites exhibiting bleeding on probing than past or never smokers. Recently, Bergström et al. (2000) in a 10-year prospective study of tobacco smoking and periodontal health, have shown that periodontal health is compromised in chronic smokers as evidenced by an increase of periodontally

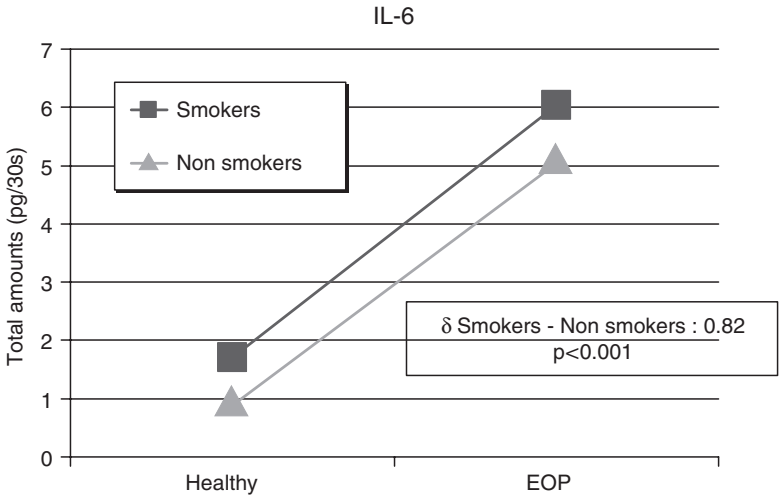

Fig. 3. Mean total amounts of interleukin (IL)-6 in early onset periodontitis (EOP) and healthy groups between smokers and non-smokers. Smokers exhibited greater total amounts of IL-6 than non-smokers ($p < 0.001$).

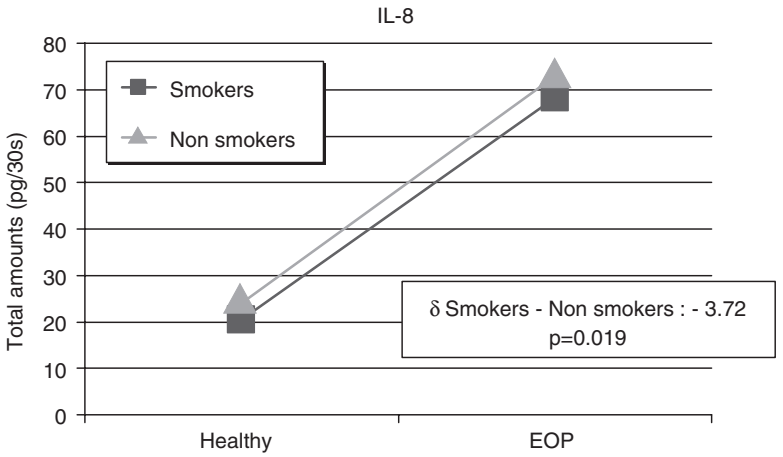

Fig. 4. Mean total amounts of interleukin (IL)-8 in early onset periodontitis (EOP) and healthy groups between smokers and non-smokers. Non-smokers had greater total amounts of IL-8 compared to smokers ($p = 0.019$).

Table 6. Statistically significant interactions of stress with EOP and smoking status

IL-1β	IL-4	IL-6	IL-8
EOP × stress $p = 0.007$	EOP × stress $p = 0.006$ smoking × stress $p = 0.037$	EOP × stress $p = 0.001$	EOP × smoking × stress $p = 0.030$

EOP, early onset periodontitis; IL, interleukin.

diseased sites concomitant with loss of bone height, as compared to non-smokers whose periodontal health condition remained unaltered throughout the 10-year period of investigation.

With respect to plaque accumulation, we found no significant difference between EOP subjects who smoke and EOP subjects who did not smoke. However, smokers had fewer bleeding sites.

Nicotine has been shown to have a vasoconstrictive effect on gingival blood vessels thereby reducing gingival blood flow (Clarke et al. 1981). However, another study has yielded contradictory results (Baab & Oberg 1987).

According to Bergström et al. (1988), the reduced inflammatory response is considered to be caused by a lower increase in the number of gingival blood vessels in smokers as compared to non-smokers.

Using the experimental gingivitis model, Bergström & Preber (1986) showed an increase in inflammation during the experiment which was less pronounced in smokers than in non-smokers.

Using the same model, Lie et al. (1998) revealed that smokers have a lower bleeding tendency after 14 days of experimentally induced gingivitis than non-smokers. These two studies suggest that in smokers the inflammatory response measured clinically during experimental gingivitis seems to be ''delayed''.

Biochemical parameters

In the present study, the total amounts of IL-1β, IL-4, IL-6 and IL-8 were analyzed in the GCF of periodontally healthy (H) and early onset or aggressive periodontitis patients (EOP) smokers and non-smokers.

Because of the inability of measuring the extremely small quantities of GCF available from healthy sites, the levels of the biochemical compounds have been reported as total amounts per 30 s sample, as an alternative to concentrations. This is in accordance with the findings of several authors (Eley & Cox, 1992, Smith et al. 1992, Nakashima et al. 1996), suggesting that total amounts rather than concentrations of GCF components should be used when estimating periodontal disease activity.

IL-1β, IL-6 and IL-8 showed significant main effects with EOP. This finding is consistent with the observations of our previous study showing that elevated total amounts of IL-1β, IL-6 and IL-8 were associated with sites of periodontal destruction. Indeed, these three markers increased significantly in sites belonging to the aggressive periodontitis and EOP patients, compared to those belonging to the H and gingivitis groups (Giannopoulou et al. 2003).

Cytokine profiles are of considerable value when studying periodontal tissue destruction. The penetration of bacteria and/or bacterial products into the tissues results in recruitment and activation of

the monocyte/T lymphocyte axis. This leads in turn to the enhanced monocytic release of TNF-α, IL-1β and IL-6, associated with periodontal tissue destruction. IL-8 secreted by monocytic cells but also from keratinocytes, endothelial cells and fibroblasts, induces matrix metalloproteinase (MMP)-8 release by neutrophils. This MMP is a potent collagenase and plays a critical role in degrading connective tissue at the site of inflammation during formation of periodontal pocket.

The microflora of EOP individuals, and more specifically the one of smokers, consists of an elevated number of periodontal pathogens, as well as species pertaining to exogenous flora, and causes more severe and widespread periodontal destruction (Kamma et al. 1999). In summary, in diseased sites an imbalance in the cytokine network is locally induced by the bacterial challenge, thus contributing to the development of elevated B cell responses in the inflamed gingival tissue. The fact that all cytokines were detected in periodontally healthy sites of both smokers and non-smokers, is attributable to the presence of a small number of macrophages and mononuclear cells in the gingival tissues and/or to the neutrophils of GCF. Finally, the wide range in total amounts obtained for all four cytokines, is mostly related to the diversity of cell types which can produce these mediators.

IL-1β showed a clear association with smoking, the difference being statistically significant in favor of healthy smokers. In contrast Boström et al. (2000) who analyzed GCF levels of IL-1β and its receptor antagonist IL-1ra with respect to smoking in patients with moderate-to-severe periodontal disease, showed no association between GCF levels of these molecules with smoking. In our previous study as well, no correlation was demonstrated between smokers with different forms of periodontitis. When nicotine was applied in vitro, on peripheral blood monocytes and lymphocytes and on gingival mononuclear cells of patients with periodontitis, no effect on IL-1β secretion was observed, suggesting that nicotine cannot activate more cells, possibly due to maximal previous stimulation in the periodontitis lesion (Payne et al. 1996, Bernzweig et al. 1998). Therefore, smoking affects the expression of IL-1β in healthy individuals only.

In the present study, IL-6 and IL-8 showed significant relationships with smoking. IL-6 showed an evident association with smoking, the difference being statistically significant in favor of healthy smokers.

In individuals who smoke, an imbalance in cytokine production seems to occur. Boström et al. (1999) observed no significant differences regarding the GCF IL-6 level between current smokers, former smokers and non-smokers, in patients with moderate-to-severe forms of periodontal disease. In contrast, elevated concentrations of IL-6 were observed in the plasma of smokers (Tappia et al. 1995), as well as in the alveolar cells of healthy donors stimulated by tobacco glycoprotein (TGP), a phenol-rich glycoprotein, present in tobacco leaves and cigarette smoke condensate (Francus et al. 1992).

IL-8 demonstrated a negative association with smoking, the difference being significant in favor of non-smokers. In a recent study by Fredriksson et al. (2002), smoking was found to reduce the sensitivity of peripheral neutrophils to stimulation of IL-8 when comparing smoking with non-smoking patients, and Kuschner et al. (1996) reported a dose-dependent effect of smoking on levels of host cytokines such as IL-1, IL-6, IL-8 and monocyte chemotactic protein-1 levels.

The results from studies of nicotine effects on cell cytokines and cytokine levels in smokers appear to be contradictory. Careful clinical and laboratory studies are needed considering that smoking effects are more complex than just increasing the in vitro nicotine concentration. The concentrations of nicotine and those of other chemicals or noxious stimuli related to smoking, should be included into such studies. These investigations may have importance given the weight currently given to the theories that cytokines' overproduction may be detrimental to the host response predisposing such a way an individual to periodontitis (Kornman & di Giovine 1998).

Stress in this study showed interaction with EOP in GCF IL-1β, IL-6 and IL-8 levels. Stress is suggested as an important disruptive factor in the homeostatic regulation between oral bacteria and the host's immune system (Genco 1992, Seymour et al. 1993, Ainamo & Ainamo 1996). We may thus expect stress experiences to have both direct (through biochemical mediators) and indirect effects (through altered compliance and health behavior). Recent research has provided evidence that products from the nervous and neuroendocrine systems, released by emotional stress, may influence immune activities by immune cells, via alterations in the production of cytokines (Blalock 1994), thus affecting the T helper (Th)1/Th2 balance (for review, see Breivik et al. 1996). For example, stress from academic examination significantly increased serum IL-1β, IL-6 and IL-10 and decreased IFN-γ production (Paik et al. 2000). IL-6 serum concentrations were increased in patients with posttraumatic stress disorder (Maes et al. 1999) as well as after speaking and exercise tasks (Goebel et al. 2000). Students with high-stress perception during the stressful condition had a significantly higher production of TNF-α and IL-6 as compared to students with a low-stress perception (Maes et al. 1998). IL-6 has often been called ''stress-inducible cytokine''. IL-1β is the only cytokine studied in GCF in association to stress and this is by Deinzer et al. (1999, 2000) who measured GCF IL-1β in a group of medical students who participated in a major medical examination. The authors observed significantly higher amounts of GCF cytokine levels as compared to a group of students not participating in the exam. These studies, together with the present one, suggest that there is an interaction between endocrine and immune systems in response to a physiological stress.

The present study demonstrated a significant interaction between smoking, stress and increased levels of IL-8 in EOP patients. IL-8 can be induced by a variety of stimuli including cytokines, like IL-1β, bacterial products or viral products. In periodontal disease, specific Gram-negative bacteria and their products are inducers of pro-inflammatory cytokine secretion (Birkedal-Hansen 1993). One of the best examples for cytokine network mechanisms is provided by IL-8 and IL-1β, where IL-1β controls the local levels of IL-8 (Wilson et al. 1996). In addition, Deinzer et al. (1999) suggested that stress might affect periodontal health by increasing levels of IL-1β locally, especially when oral hygiene is neglected.

Summing up, IL-1β, IL-4, IL-6, IL-8 showed a clear association with EOP. This association was dependent on smoking for IL-1β and IL-4. IL-1β and

IL-6 showed a significant association with smoking, in favor of healthy smokers while IL-8 showed significant association with healthy non-smokers. Stress showed significant relationship with IL-1β, IL-6, IL-8 and EOP. Also, stress demonstrated a significant interaction with smoking and increased levels of IL-8 in EOP patients.

In conclusion, the present study suggests that smokers and the stressed may have more disease because both smoking and stress influence host-related factors including cytokine network, thereby modifying the microbial flora to be more pathogenic.

However, the relative importance of smoking and stress-related alterations and their precise mode of action in increasing the risk of aggressive periodontitis remains to be elucidated.

Acknowledgments

The authors gratefully acknowledge the Swiss Society of Odontostomatology (SSO) for kindly supporting this study.

References

Ainamo, J. & Ainamo, A. (1996) Risk assessment of recurrence of disease during supportive periodontal care. Epidemiological considerations. *Journal of Clinical Periodontology* **23**, 232–239.

Ainamo, J. & Bay, I. (1975) Problems and proposals for recording gingivitis and plaque. *International Dental Journal* **25**, 229–235.

Baab, D. A. & Oberg, P. A. (1987) The effect of cigarette smoking on gingival blood flow in humans. *Journal of Clinical Periodontology* **14**, 418–424.

Baggiolini, M. & Clark-Lewis, I. (1992) Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS* **307**, 97–101.

Ballieux, R. E. (1991) Impact of mental stress on the immune response. *Journal of Clinical Periodontology* **18**, 27–430.

Barbour, S. E., Nakashima, K., Zhang, J. B., Tangada, S., Hahn, C. I., Schenkein, H. & Tew, J. G. (1997) Tobacco and smoking environmental factors that modify the host response (immune system) and have an impact on periodontal health. *Critical Reviews in Oral Biology & Medicine* **8**, 437–460.

Bergström, J., Eliasson, S. & Dock, J. (2000) A 10-year prospective study of tobacco smoking and periodontal health. *Journal of Periodontology* **71**, 1338–1347.

Bergström, J., Persson, L. & Preber, H. (1988) Influence of cigarette smoking on vascular reaction during experimental gingivitis. *Scandinavian Journal of Dental Research* **96**, 34–39.

Bergström, J. & Preber, H. (1986) The influence of cigarette smoking on the development of experimental gingivitis. *Journal of Periodontal Research* **21**, 668–676.

Bergström, J. & Preber, H. (1994) Tobacco use as a risk factor. *Journal of Periodontology* **65**, 545–550.

Bernzweig, E., Payne, J. B., Reinhardt, R. A., Dyer, J. K. & Patil, K. D. (1998) Nicotine and smokeless tobacco effects on gingival and peripheral blood mononuclear cells. *Journal of Clinical Periodontology* **25**, 246–252.

Bickel, M. (1993) The role of IL-8 in inflammation and mechanisms of regulation. *Journal of Periodontology* **64**, 456–460.

Birkedal-Hansen, H. (1993) Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research* **28**, 500–510.

Blalock, J. E. (1994) The syntax of immune-endocrine communication. *Immunology Today* **15**, 504–511.

Boström, L., Bergström, J., Dahlén, G. & Linder, L. E. (2001) Smoking and subgingival microflora in periodontal disease. *Journal of Clinical Periodontology* **28**, 212–219.

Boström, L., Linder, L. E. & Bergström, J. (1998a) Influence of smoking on the outcome of periodontal surgery. A 5-year follow-up. *Journal of Clinical Periodontology* **25**, 194–201.

Boström, L., Linder, L. E. & Bergström, J. (1998b) Clinical expression of smoking-associated periodontal disease. *Journal of Clinical Periodontology* **25**, 767–773.

Boström, L., Linder, L. E. & Bergström, J. (1999) Smoking and crevicular fluid levels of IL-6 and TNF-alpha in periodontal disease. *Journal of Clinical Periodontology* **26**, 352–357.

Boström, L., Linder, L. E. & Bergström, J. (2000) Smoking and GCF levels of IL-1beta and IL-1ra in periodontal disease. *Journal of Clinical Periodontology* **27**, 250–255.

Breivik, T., Thrane, P. S., Murison, R. & Gjermo, P. (1996) Emotional stress effects on immunity gingivitis and periodontitis. *European Journal of Oral Sciences* **104**, 327–334.

Chen, X., Wolff, L., Aeppli, D., Guo, Z., Luan, W., Baelum, V. & Fejeskov, O. (2001) Cigarette smoking, salivary/gingival crevicular fluid cotinine and periodontal status. A 10-year longitudinal study. *Journal of Clinical Periodontology* **28**, 331–339.

Clarke, N. G., Shephard, B. C. & Hirsch, R. S. (1981) The effects of intra-arterial epinephrine and nicotine on gingival circulation. *Oral Surgery Oral Medicine Oral Pathology* **52**, 577–582.

Cohen, S. & Williamson, G. M. (1991) Stress and infectious disease in humans. *Psychological Bulletin* **109**, 5–24.

Corcoran, M. L., Stetler-Stevenson, W. G., Brown, P. D. & Wahl, L. M. (1992) IL-4 inhibition of PGE_2 synthesis block interstitial collagenase and 92Kd type IV collagenase/gelatinase production of human monocytes. *Journal of Biological Chemistry* **267**, 515–519.

Darby, I. B., Hodge, P. J., Riggio, M. P. & Kinane, D. F. (2000) Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction. *Journal of Clinical Periodontology* **27**, 417–424.

Deinzer, R., Förster, P., Fuck, L., Herforth, A., Stiller-Winkler, R. & Idel, H. (1999) Increase of crevicular interleukin 1β under academic stress at experimental gingivitis sites and at sites of perfect oral hygiene. *Journal of Clinical Periodontology* **26**, 1–8.

Deinzer, R., Kottmann, W., Forster, P., Herforth, A., Stiller-Winkler, R. & Idel, H. (2000) After-effects of stress on crevicular interleukin-1 beta. *Journal of Clinical Periodontology* **27**, 74–77.

Dinarello, C. A. (1988) Biology of interleukin 1. *FASEB* **2**, 108–115.

Eley, B. M. & Cox, S. W. (1992) Cathepsin B/L-, elastase-, tryptase-, trypsin- and dipeptidyl peptidase IV activities in gingival crevicular fluid: correlation with clinical parameters in untreated chronic periodontitis patients. *Journal of Periodontal Research* **27**, 62–69.

Francus, T., Romano, P. M., Manzo, G., Fonacier, L., Arango, N. & Szabo, P. (1992) IL-1, IL-6 and PDGF mRNA expression in alveolar cells following stimulation with a tobacco-derived antigen. *Cell Immunology* **145**, 156–174.

Fredriksson, M., Bergström, J. & Åsman, B. (2002) IL-8 and TNF-α from peripheral neutrophils and acute -phase proteins in periodontitis. Effect of cigarette smoking: a pilot study. *Journal of Clinical Periodontology* **29**, 123–128.

Fujihashi, K., Kona, Y., Beagley, K. W., Yamamoto, M., McGhee, J. R., Mestecky, J. & Kiyono, H. (1993) Cytokines and periodontal disease: immunopathological role of interleukins for B cell responses in chronic inflamed gingival tissues. *Journal of Periodontology* **64**, 400–406.

Genco, R. (1992) Host responses in periodontal diseases: current concepts. *Journal of Periodontology* **63**, 338–355.

Giannopoulou, C., Kamma, J. & Mombelli, A. (2003) Effect of inflammation, smoking and stress on gingival crevicular fluid cytokine level. *Journal of Clinical Periodontology* **30**, 145–153.

Glavind, L. & Löe, H. (1967) Errors in the clinical assessment of periodontal destruction. *Journal of Periodontal Research* **2**, 180–184.

Goebel, M. U., Mills, P. J., Irwin, M. R. & Ziegler, M. G. (2000) Interleukin-6 and tumor necrosis factor-alpha production after acute psychological stress, exercise, and infused isoproternol: differential effects and pathways. *Psychosomatic Medicine* **62**, 591–598.

Grossi, S. G., Zambon, J. L., Hu, A. W., Koch, G., Dunford, R. G., Machtei, E. E., Norderyd, J. J. & Genco, R. J. (1994) Assessment of risk for periodontal disease. I. Risk indicators for

attachment loss. *Journal of Periodontology* **65**, 260–267.

Haffajee, A. D. & Socransky, S. S. (2001) Relationship of cigarette smoking to the subgingival microbiota. *Journal of Clinical Periodontology* **28**, 377–388.

Herbert, T. B. & Cohen, S. (1993) Stress and immunity in humans: a meta-analytic review. *Psychosomatic Medicine* **55**, 364–379.

Hirose, K., Isogai, E., Miura, H. & Ueda, I. (1997) Levels of *Porphyromonas gingivalis* fimbriae and inflammatory cytokines in gingival crevicular fluid from adult human subjects. *Microbiology and Immunology* **41**, 21–26.

Ishimi, Y., Miyaura, C., Jin, C. H., Akatsu, T., Abe, E., Nakamura, Y., Yamaguchi, A., Yoshiki, S., Matsuda, T. & Hirano, T. (1990) IL-6 is produced by osteoblasts and induces bone resorption. *Journal of Immunology* **145**, 3297–3303.

Jandinski, J. J. (1988) Osteoclast activating is now interleukin-1 beta: historical perspective and biological implications. *Journal of Oral Pathology* **17**, 145–152.

Kabashima, H., Nagata, K., Hashiguchi, I., Toriya, Y., Iijima, T., Maki, K. & Maeda, K. (1996) Interleukin-1 receptor antagonist and interleukin-4 in gingival crevicular fluid of patients with inflammatory periodontal disease. *Journal of Oral Pathology Medicine* **25**, 449–455.

Kamma, J. J., Nakou, M. & Baehni, P. C. (1999) Clinical and microbiological characteristics of smokers with early onset periodontitis. *Journal of Periodontal Research* **34**, 25–33.

Kornman, K. S. & di Giovine, F. S. (1998) Genetic variation in cytokine expression: a risk factor for severity of adult periodontitis. *Annals of Periodontology* **3**, 327–338.

Kuschner, W. G., D'Alessandro, A., Wong, H. & Blanc, P. D. (1996) Dose-dependent cigarette smoking - related inflammatory response in healthy adults. *European Respiratory Journal* **9**, 1989–1994.

Lee, H. J., Kang, I. K., Chung, C. P. & Choi, S. M. (1995) The subgingival microflora and gingival crevicular fluid cytokines in refractory periodontitis. *Journal of Clinical Periodontology* **22**, 885–890.

Lie, M. A., Timmerman, M. F., van der Velden, U. & van der Weijden, G. A. (1998) Evaluation of 2 methods to assess gingival bleeding in smokers and non-smokers in natural and experimental gingivitis. *Journal of Clinical Periodontology* **25**, 695–700.

Linn, M. (1986) Modifiers and perceived stress scale. *Journal of Consulting Clinical Psychology* **54**, 507–513.

Maes, M., Lin, A. H., Delmeire, L., Van Gastel, A., Kenis, G., De Jongh, R. & Bosmans, E. (1999) Elevated serum interleukin-6 (IL-6) and IL-6 receptor concentrations in posttraumatic stress disorder following accidental man-made traumatic events. *Biological Psychiatry* **45**, 833–839.

Maes, M., Song, C., Lin, A., De Jongh, R., Van Gastel, A., Kenis, G., Bosmans, E., de Meester, I., Benoy, I., Neels, H., Demedts, P., Janca, A., Scharpe, S. & Smith, R. S. (1998) The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and Th1-like response in stress-induced anxiety. *Cytokine* **10**, 313–318.

Mathur, A., Michalowicz, B., Castillo, M. & Aeppli, D. (1996) Interleukin-1 alpha, interleukin-8 and interferon-alpha levels in gingival crevicular fluid. *Journal of Periodontal Research* **31**, 489–495.

Monteiro da Silva, A. M., Oakley, D. A., Newman, H. N., Nohl, F. S. & Nohl, H. M. (1996) Psychosocial factors and adult and rapidly progressive periodontitis. *Journal of Clinical Periodontology* **23**, 789–794.

Montgomery, D. C., Peck, E. A. & Vining, G. G. (2001) *Introduction to Linear Regression Analysis*, 3rd edition. New York: John Wiley & Sons.

Mullally, B. H., Breen, B. & Linden, G. J. (1999) Smoking and patterns of bone loss in early-onset periodontitis. *Journal of Periodontology* **70**, 394–401.

Mundy, G. (1991) Inflammatory mediators and the destruction of bone. *Journal of Periodontal Research* **26**, 213–217.

Nakashima, K., Giannopoulou, C., Andersen, E., Roehrich, N., Brochut, P., Dubrez, B. & Cimasoni, G. (1996) A longitudinal study of various crevicular fluid components as markers of periodontal disease activity. *Journal of Clinical Periodontology* **23**, 832–838.

O'Leary, T. J., Drake, R. B. & Naylor, J. E. (1972) The plaque control record. *Journal of Periodontology* **43**, 38.

Paik, I. H., Toh, K. Y., Lee, C., Kim, J. J. & Lee, S. J. (2000) Psychological stress may induce increased humoral and decreased cellular immunity. *Behavioral Medicine* **26**, 139–141.

Payne, J. B., Johnson, G. K., Reinhardt, R. A., Dyer, J. K., Maze, C. A. & Dunning, D. G. (1996) Nicotine effects on PGE2 and IL-1 beta release by LPS-treated human monocytes. *Journal of Periodontal Research* **31**, 99–104.

Payne, J. B., Reinhardt, R. A., Nummikoski, P. V., Dunning, D. G. & Patil, K. D. (2000) The association of cigarette smoking with alveolar bone loss in postmenopausal females. *Journal of Clinical Periodontology* **27**, 658–664.

Quinn, S. M., Zhang, J. B., Gunsolley, J. C., Schenkein, J. C., Schenkein, H. A. & Tew, J. G. (1996) Influence of smoking and race on immunoglobulin G2 subclass concentration in early onset periodontitis patients. *Infection and Immunity* **64**, 2500–2505.

Renvert, S., Dahlen, G. & Wikstrom, M. (1998) The clinical and microbiological effects of non-surgical periodontal therapy in smokers and non-smokers. *Journal of Clinical Periodontology* **25**, 153–157.

Schei, O., Waerhaug, J., Lovdal, A. & Arno, A. (1959) Alveolar bone loss as related to oral hygiene and age. *Journal of Periodontology* **30**, 7–16.

Schenkein, H. A., Gunsolley, J. C., Koerge, T. E., Schenkein, J. G. & Tew, J. G. (1995) Smoking and its effects on early onset periodontitis. *Journal of the American Dental Association* **126**, 1107–1113.

Seymour, G. J., Gemmell, E., Reinhardt, R., Eastcott, J. & Taubman, M. A. (1993) Immunopathogenesis of chronic periodontal disease: cellular and molecular mechanisms. *Journal of Periodontal Research* **28**, 478–486.

Shapira, L., van Dyke, T. E. & Hart, T. C. (1992) A localized absence of interleukin-4 triggers periodontal disease activity: a novel hypothesis. *Medical Hypotheses* **39**, 319–322.

Smith, Q. T., Au, G. S., Freese, P. L., Osborn, J. B. & Stoltenberg, J. L. (1992) Five parameters of gingival crevicular fluid from eight surfaces in periodontal health and disease. *Journal of Periodontal Research* **27**, 466–475.

Stoltenberg, J. L., Osborne, J. B., Philstrom, B. L., Hertzberg, M. C., Aeppli, D. M., Wolf, L. F. & Fischer, G. E. (1993) Association between cigarette smoking, bacterial pathogens and periodontal status. *Journal of Periodontology* **64**, 1225–1230.

Tappia, P. S., Troughton, K. L., Langley-Evans, S. C. & Grimble, R. F. (1995) Cigarette smoking influences cytokine production and antioxidant defences. *Clinical Sciences* **88**, 485–489.

Te Velde, a. A., Huijbens, R. J., Heije, K., de Vries, J. E. & Figdor, C. G. (1990) Interleukin 4 (IL-4) inhibits secretion of IL-beta, tumor necrosis factor alpha and IL-6 by human monocytes. *Blood* **76**, 1392–1397.

Tsai, C. C., Ho, Y. P. & Chen, C. C. (1995) Levels of interleukin-1β and interleukin-8 in gingival crevicular fluids in adult periodontitis. *Journal of Periodontology* **66**, 852–859.

Wilson, M., Reddi, K. & Henderson, B. (1996) Cytokine-inducing components of periodontopathogenic bacteria. *Journal of Periodontal Research* **31**, 393–407.

Zambon, J. J., Grossi, S. G., Machtei, E. E., Ho, A. W., Dunford, R. & Genco, R. J. (1996) Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. *Journal of Periodontology* **67** (suppl.), 1050–1054.

Address:
Joanna J. Kamma
6-8 Freattidos St.
GR-185 37 Piraeus
Greece
Fax: +30 210 4525 935
E-mail: j.kamma@periodontology.gr