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Association between passive smoking and salivary markers related to periodontitis

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Abstract

Objectives: The mechanism of passive smoking in terms of development of periodontitis has not been investigated. This study examined the effect of passive smoking on salivary markers related to periodontitis.

Methods: Periodontal status was evaluated on the basis of probing pocket depth and clinical attachment level in 273 workers. Salivary marker levels were determined by enzyme assay including enzyme-linked immunosorbent assay. Six periodontal pathogens in saliva were assessed using real-time PCR methodology. Non-, passive and active smokers were defined as subjects exhibiting salivary cotinine levels of 0 (53 subjects), 1–7 (118) and $\geqslant$8 ng/ml (102).

Results: Levels of salivary markers, including IL-1β, lactoferrin, albumin and aspartate aminotransferase (AST), were elevated significantly in passive smokers relative to non-smokers. Additionally, these marker levels, with the exception of IL-1β, decreased significantly in active smokers in comparison with passive smokers. However, no meaningful differences in percentages of periodontal pathogens were observed between non- and passive smokers. Multiple linear regression analyses were performed for each marker utilizing age, gender, cotinine level and periodontal status as independent variables. IL-1β, albumin and AST were independently associated with cotinine level.

Conclusion: Passive smoke exposure leads to elevation of IL-1β, albumin and AST levels in saliva.

Key words: cotinine; epidemiology; passive smoking; periodontitis; salivary biomarkers

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Periodontal diseases are infectious diseases caused by a small group of predominantly anaerobic Gram-negative bacteria. However, a susceptible host is essential and host factors are determinative with respect to occurrence of periodontitis (Madianos et al. 2005). The local host response to these bacteria includes recruitment of leucocytes and subsequent release of inflammatory mediator and pro-inflammatory cytokines. Cytokines such as interleukin (IL)-1β and tumour necrosis factor-α induce and enhance the production of prostaglandin E_2 (PGE_2) and matrix metalloproteinases (MMPs). These molecules mediate destruction of the extracellular matrix of gingiva and periodontal ligament as well as resorption of alveolar bone (Madianos et al. 2005).

Numerous studies suggest that smoking leads to epidemiologically and biologically enhanced risk of periodontal disease (Johnson & Hill 2004). Various factors contribute to the deleterious periodontal effects of smoking, including alteration of both microbial and host response factors. Although some investigators noted no difference in the prevalence of subgingival bacteria associated with periodontitis (Lie et al. 1998, Boström et al. 2001, Mager et al. 2003), several recent reports documented higher prevalence or counts of certain organisms in smokers (Haffajee & Socransky 2001, van Winkelhoff et al. 2001). On the other hand, smoking also affects the human immune system and the cellular and humoral inflammatory system; moreover, smoking may exert effects throughout the cytokine network (Kinane & Chestnutt 2000, Palmer et al. 2005). However, the importance of these smoking-related alterations and the corresponding mechanisms in terms of increased risk of periodontitis remain unclear.

Recently, Arbes et al. (2001) reported that the adjusted odds of periodontal disease were 1.6 times greater for persons exposed to passive smoke than for persons not exposed based on evaluation of self-reported environmental tobacco smoke exposure. We previously established that passive smoking classified in terms of

salivary cotinine level is independently associated with periodontitis (Yamamoto et al. 2005). Multiple logistic regression analysis revealed significantly higher periodontitis odds ratios in passive smokers relative to non-smokers following adjustment for other lifestyle factors such as age, sex, alcohol consumption and body mass index (BMI); the odds ratio was 2.87. These results suggest the presence of a harmful effect in connection with passive smoking with respect to periodontal diseases. However, the changes in the host–microbe interactions involved in periodontal destruction in passive smokers are poorly understood.

Saliva is easily collected and it contains locally and systemically derived markers of periodontal disease (Kaufman & Lamster 2000); consequently, whole saliva was employed in the current investigation for the measurement of various biomarkers. The objective of the present study was to evaluate the influence of passive smoking on periodontal pathogens and various biomarkers possibly related to development of periodontitis including inflammatory mediator and pro-inflammatory cytokines in saliva.

Subjects and Methods

Study population

Three hundred and sixty Japanese factory workers employed at a manufacturing company were available for evaluation; in 2003, 273 (75.8%) of these individuals (236 males and 37 females, aged 18–62 years) were surveyed via an oral examination and a self-administered questionnaire as a part of the annual health check-up. Oral status was not assessed in 61 subjects due to reasons corresponding to their work; additionally, 26 participants refused to provide saliva. No subjects were using prescription medications for diabetes, hyperlipidaemia or hypertension. Furthermore, participants were not exposed to sources of nicotine other than cigarettes, including smokeless tobacco, nicotine gum and nicotine patches. Informed consent was obtained from all subjects. This study population was introduced previously (Yamamoto et al. 2005). Based on results from receiver-operating characteristic (ROC) plots for cotinine-level classification derived from self-reported smoking status, we determined that 8 ng/ml salivary cotinine was the most suitable cut-off value for distinguishing active smokers from non- or passive smokers. However, an association between self-reported exposure to passive smoking and salivary cotinine concentration was not observed in ROC analysis. In order to justify substitution of biochemical measures of smoking behaviour for self-reported cigarette smoking to quantify risk, correlation with disease outcomes must be demonstrated (Perez-Stable et al. 1995). No meaningful difference was detected in periodontal status between non- and passive smokers classified by self-reporting; in contrast, passive smokers (1–7 ng/ml) defined by salivary cotinine displayed significantly more severe periodontal status in comparison with non-smokers (0 ng/ml). Consequently, non-, passive and active smokers were defined as those subjects exhibiting cotinine levels of 0, 1–7 and $\geqslant$8 ng/ml, respectively (Yamamoto et al. 2005); 53, 118 and 102 subjects were classified as non-smokers, passive smokers and active smokers, respectively (Table 1). Permission for this investigation was obtained from the Ethical Committee for Clinical Research of Osaka University Graduate School of Dentistry.

Collection of saliva

Participants were asked to chew a piece of paraffin gum for 30 s at the annual health check-up in order to stimulate salivary flow. Subsequently, saliva samples were obtained by expectoration; subjects were asked to spit approximately 2.0 ml of saliva into a test tube. Saliva samples were collected between 09:00 and 12:00 hours. Samples, which were stored at -80°C until use, were delivered to the laboratory for analyses.

Assessment of salivary biomarkers

Before assay, frozen salivary samples were thawed completely and centrifuged at 1500 $\times$ *g* for 15 min. Cotinine levels were measured by a competitive enzyme-linked immunosorbent assay (ELISA) as described previously (Yamamoto et al. 2005). ELISA plates (Nunc A/S, Roskilde, Denmark) were coated (0.1 ml/well) with a solution of rabbit polyclonal anti-goat immunoglobulin G (IgG) (10 μg/ml) (Dako Cytomation A/S, Glostrup, Denmark) in tris-buffer, pH 8.4, and incubated overnight at 4°C. The plates were blocked with 0.2 ml of 10 mM phosphate buffer, pH 7.5, containing 0.1% bovine serum albumin (BSA) (phosphate-BSA buffer); subsequently, plates were incubated for 1 h at room temperature and stored at 4°C. A standard inhibition curve was generated by serial dilution (1:2) of a solution consisting of cotinine (160 ng/ml) in phosphate-BSA buffer to obtain seven dilutions of known concentration. Each dilution was tested in duplicate via addition of 50 μl of cotinine solution, 50 μl of (1/10,000) goat polyclonal anti-cotinine reagent (Affiniti Research Product Ltd., Exeter, UK) and 50 μl of cotinine conjugated with horseradish peroxidase, which was derived from carboxyl-cotinine (Aldrich Chem Co., Milw., WI, USA) and horseradish peroxidase (Sigma Co., St. Louis, MO, USA) as described previously by Grabarek & Gergely (1990). Each unknown sample was also tested in duplicate with 50 μl of saliva at 1:2 dilution and 50 μl of anti-cotinine reagent and horseradish-conjugated cotinine reagent. Following a 1-h incubation at 25°C, plates were washed three times with 0.3 ml of distilled water. Substrate solution (100 μl) containing tetramethylbenzidine (TMB) was then added and plates were incubated for 30 min at 25°C in the dark. Color development was terminated upon the introduction of 100 μl (1 M) of phosphoric acid. The optical density of each well was determined with a microplate reader at 450 nm. The minimum limitation of the measurement for salivary cotinine was 1 ng/ml in this study. The coefficients of variation of the assay were 5.8% within batch and 9.6% between batches.

Table 1. Study population by sex, age and salivary continine levels

	N	Sex		Age (years)			Salivary continine levels		
		male	female	mean		SE	mean		SE
Non-smokers (0 ng/ml)*	53	36	17	39.2	±	12.8	0	±	0
Passive smokers (1–7 ng/ml)	118	103	15	40.6	±	11.1	3	±	0
Active smokers ($\geqslant$8 ng/ml)	102	97	5	41.4	±	10.8	143	±	9
Total	273	236	37	40.6	±	0.7	55	±	5

*Salivary cotinine level.

Standards for IL-1β (0–400 pg/ml), PGE_2 (0–1000 pg/ml), MMP-8 (0–4 ng/ml), MMP-9 (0–128 ng/ml), lactoferrin (0–1000 ng/ml), secretory immunoglobulin (S-Ig)A (0–2000 ng/ml) and albumin (0–1000 ng/ml) were prepared. PGE_2 was determined with a competitive ELISA (Cayman Chemical Co., Ann Arbor, MI, USA). IL-1β (human IL-1β ELISA Kit, Pierce Biotechnology, Rockfold, IL, USA), MMP-8 (MMP-8 Human Biotrak ELISA System, GE Healthcare Bio-Sciences, Piscataway, NJ, USA; this system recognized precursor and active forms of MMP-8) and MMP-9 (MMP-9 Human Biotrak ELISA System, Amersham Bioscience Corp.; this system recognized pro-MMP-9 and 83 kDa MMP-9) were determined via conventional sandwich ELISA. Analyses were performed according to the manufacturers' recommended protocols. Lactoferrin, S-IgA and albumin were determined via a conventional ELISA using polyclonal antibody (Dakopatts, Glostrup, Denmark) and alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany). Aspartate aminotransferase (AST) was measured via enzymatic methods with an automatic analyzer (Auto Analyzer AU-5242, Olympus, Tokyo, Japan) and Cica Liquid AST (Kanto Kagaku, Tokyo, Japan).

Assessment of periodontal pathogens

For determination of numbers of six periodontal pathogens and total cells present in saliva, the quantitative real-time polymerase chain reaction (PCR) method was used with a LightCycler™ system (Roche Diagnostic, Mannheim, Germany) (Kuboniwa et al. 2004). Percentage of each pathogen in total cells served as a quantitative parameter. Salivary periodontal pathogens examined in this study included *Porphyromonas gingivalis*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, *Treponema denticola*, *Prevotella intermedia* and *Prevotella nigrescens*. Species-specific probe and primer sets were designed from the variable regions of the 16S rRNA gene sequences (Kuboniwa et al. 2004); additionally, a universal probe and primer set was utilized (Nadkarni et al. 2002). Fluorescence intensity was monitored at the annealing temperature in single acquisition mode. The dye signals generated during a run were measured in fluorimeter channel 2 (*F*2, 640 ± 30 nm) and channel 1 (*F*1, 530 ± 30 nm); furthermore, results were indicated as the *F*2/*F*1 ratio, which was considered adequate for a TaqMan probe conjugated with FAM and TAMRA. Fluorescent data were analysed with LightCycler Data Analysis software version 3.5 (Roche Diagnostic). Thirty-eight subjects refused the measurement of periodontal pathogens in saliva, whereas permission was obtained from 235 subjects; subsequently, detection of periodontal pathogens was implemented.

Assessment of periodontal status

Periodontal condition, which was measured as probing pocket depth (PPD) and clinical attachment level (CAL) in millimetres, was recorded by three examiners using an automated probe equipped with a 0.5-mm ball tip (Vivacare TPS Probe™, Schaan, Liechtenstein) involving a constant force (20 g). Probing was performed at six sites per tooth for all teeth (excluding the third molar); moreover, the deepest reading was recorded for each. In two selected quadrants – one maxillary and one mandibular – CAL was calculated based on the probed distances (in millimetres) from the free gingival margin to the cemento-enamel junctions and the base of the sulcus; the greatest CAL was recorded for each tooth. Calibrated examiners conducted the periodontal examinations. The mean κ values among the examiners were 0.71 and 0.77 for assessment of PPD and CAL, respectively, when PPD or CAL of 3.5 mm served as the cut-off point.

Statistical analysis

Data were analysed with a statistical package (Stat View, SAS Institute, Cary, NC, USA). The Kruskal–Wallis and Mann–Whitney *U* tests were utilized to evaluate the differences in salivary biomarkers or periodontal pathogens among non-, passive and active smokers. Multiple linear regression analyses were performed for each biomarker utilizing age, sex, cotinine level, number of teeth with PPD $\geqslant$ 3.5 and number of teeth with CAL $\geqslant$ 3.5 mm as independent variables in non- and passive smokers. All reported *p*-values are two-tailed; moreover, those *p*-values less than 0.05 were considered statistically significant.

Results

The characteristics of the study population with regard to smoking are presented in Table 1. The total number of participants in this study was 273 (236 males and 37 females). The mean ages were quite similar in non-, active and passive smokers, approximately 40 years of age. In addition, the mean salivary cotinine levels were 0 ng/ml for non-smokers, 3 ng/ml for passive smokers and 143 ng/ml for active smokers (Table 1). Periodontal status was characterized based on the number of teeth exhibiting PPD $\geqslant$ 3.5 mm and CAL $\geqslant$ 3.5 mm. The mean (± SE) numbers of teeth with PPD $\geqslant$ 3.5 mm and CAL $\geqslant$ 3.5 mm were 4.8 (± 0.3) and 1.6 (± 0.1), respectively. The number of teeth displaying PPD $\geqslant$ 3.5 mm varied from a low of 0 to a high of 25, whereas the number of teeth with CAL $\geqslant$ 3.5 mm varied from a low of 0 to a high of 11.

Table 2 displays the mean values of the salivary biomarkers by smoking status. Levels of salivary markers, including IL-1β, lactoferrin, albumin and AST, were elevated significantly in passive smokers in comparison with non-smokers. These marker levels, with the exception of IL-1β, decreased significantly in active smokers relative to passive smokers.

The mean percentages of periodontal pathogens by smoking status are summarized in Table 3. Percentages of *P. gingivalis*, *P. intermedia* and *P. nigrescens* in passive smokers were slightly elevated in comparison with non-smokers; however, the differences were not statistically significant. However, percentage of *P. gingivalis* in active smokers was significantly higher than that in non- and passive smokers.

Multiple linear regression analysis was conducted using salivary cotinine level, the number of teeth with PPD $\geqslant$ 3.5 mm or CAL $\geqslant$ 3.5 mm, age and sex as independent variables; IL-1β, lactoferrin, albumin and AST served as dependent variables in non- and passive smokers (Table 4). IL-1β, albumin and AST exhibited significant standardized regression coefficients with salivary cotinine levels, but not with the number of teeth displaying CAL $\geqslant$ 3.5 mm. However, AST was independently associated with the number of teeth characterized by PPD $\geqslant$ 3.5 mm.

Discussion

The previous investigation, which involved the same subject population as the current study (Yamamoto et al. 2005), revealed that the mean numbers of teeth with

Table 2. Mean values of salivary biomarkers by smoking status

	N	Salivary biomarkers															
		Interleukin-1β (pg/ml)		Prostaglandin E_2 (pg/ml)		Matrix metalloproteinase-8 (ng/ml)		Matrix metalloproteinase-9 (ng/ml)		Lactoferrin (μg/ml)		Secretory immunoglobulin A (μg/ml)		Albumin (μg/ml)		Aspartate aminotransferase (IU/1)	
		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Non-smokers (0 ng/ml)†	53	164.3	31.8	91.5	19.3	45.7	5.4	278.8	36.4	6.6	0.9	79.4	4.5	62.4	7.5	50.3	4.7
Passive smokers (1–7 ng/ml)	118	199.0	17.4	96.6	13.3	43.3	2.8	263.8	12.3	11.8	1.4	94.1	6.0	90.6	7.0	71.2	4.5
Active smokers (⩾8 ng/ml)	102	167.0	18.6	90.5	21.7	41.1	3.4	228.1	15.2	6.9	0.6	89.0	6.0	58.5	8.0	48.3	3.3
p-value (Kruskal–Wallis test)			<0.05		<0.02		NS		<0.02		<0.0001		NS		<0.0001		<0.0001

* $p<0.05$ (Mann–Whitney *U*-test).
** $p<0.01$ (Mann–Whitney *U*-test).
*** $p<0.001$ (Mann–Whitney *U*-test).
**** $p<0.0001$ (Mann–Whitney *U*-test).
† Salivary cotinine level.
NS, not significant.

CAL ⩾ 3.5 mm were 0.9 (± 0.3) for non-smokers, 1.6 (± 0.2) for passive smokers and 1.9 (± 0.2) for active smokers. Active and passive smokers displayed significantly greater numbers of teeth characterized by CAL ⩾ 3.5 mm than non-smokers ($p<0.05$). In addition, the mean numbers of teeth exhibiting PPD ⩾ 3.5 mm were 2.3 (± 0.3) for non-smokers, 4.5 (± 0.5) for passive smokers and 6.2 (± 0.6) for active smokers. Active smokers demonstrated significantly greater numbers of teeth with PPD ⩾ 3.5 mm in comparison with non- or passive smokers ($p<0.05$).

The present study revealed that levels of salivary biomarkers, including IL-1β, lactoferrin, albumin and AST, were elevated significantly in passive smokers in comparison with non-smokers. These marker levels, with the exception of IL-1β, decreased significantly in active smokers relative to passive smokers. Furthermore, IL-1β, albumin and AST exhibited independent association with salivary cotinine levels. However, no meaningful differences were observed with respect to proportions of periodontal pathogens in passive smokers in comparison with non-smokers.

The local host response to periodontitis has been examined generally via biochemical analysis of gingival crevicular fluid (GCF). However, measurement of biochemical compounds in GCF from all sites of the mouth, especially healthy sites, is difficult due to extremely small quantities of GCF. Saliva can be collected more readily, in larger amounts and with less subject discomfort in comparison with GCF. In addition, whole saliva contains locally produced microbial and host response mediators as well as systemic markers; consequently, salivary components including host inflammatory mediators and enzymes may be useful biomarkers of periodontal disease (Kaufman & Lamster 2000, Ozmeric 2004). Biomarker concentrations in whole saliva served as the parameters in the present study. Saliva flow rate has been shown to affect saliva biomarker concentrations markedly in periodontitis subjects (Brock et al. 2004). In order to neutralize the influence of salivary flow rate to as great an extent as possible, biomarker concentrations were adjusted with total protein or inorganic phosphorus; however, these parameters did not provide satisfactory differentiation with respect to smoking status (data not shown).

IL-1β demonstrated a clear association with passive smoking. Elevated cytokine levels, i.e., IL-1β, resulted in

Table 3. Mean proportions of periodontal pathogens by smoking status

	N	Periodontal pathogens											
		Porphyromonas gingivalis (%)		*Tannerella forsythia* (%)		*Actinobacillus actinomycetemcomitans* (%)		*Treponema denticola* (%)		*Prevotella intermedia* (%)		*Prevotella nigrescens* (%)	
		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Non-smokers (0 ng/ml)†	45	0.09	0.04	0.01	0.00	0.00	0.00	0.01	0.00	0.30	0.11	0.30	0.06
Passive smokers (1–7 ng/ml)	103	0.12	0.04	0.01	0.00	0.00	0.00	0.01	0.00	0.44	0.10	0.33	0.07
Active smokers (⩾8 ng/ml)	87	0.29	0.09	0.01	0.00	0.00	0.00	0.02	0.01	0.49	0.12	0.24	0.06
p-value (Kruskal–Wallis test)			<0.03		NS		NS		NS		NS		NS

*$p < 0.05$ (Mann–Whitney *U*-test).
†Salivary cotinine level.
NS, not significant.

Table 4. Association of salivary biomarkers with cotinine levels

	Salivary biomarkers							
	Interleukin-1β (pg/ml)		Lactoferrin (μg/ml)		Albumin (μg/ml)		Aspartate aminotransferase (IU/1)	
	β	*p*-value	β	*p*-value	β	*p*-value	β	*p*-value
Age (years)	0.170	<0.05	0.095	NS	0.025	NS	0.147	NS
Sex*	−0.141	NS	−0.019	NS	0.002	NS.	0.078	NS
Salivary cotinine levels (ng/ml)	0.211	<0.01	0.137	NS	0.154	<0.05	0.209	<0.01
Number of teeth with CAL⩾3.5 mm	−0.073	NS	0.060	NS	−0.083	NS	−0.090	NS
Number of teeth with PPD⩾3.5 mm	0.134	NS	−0.037	NS	0.114	NS	0.204	<0.05

*0; female 1; male
CAL, clinical attachment level; PPD, probing pocket depth; β; standardized regression coefficient; NS; not significant.

production of PGE_2 and MMPs (Madianos et al. 2005); however, changes in PGE_2, MMP-8 and MMP-9 saliva levels were not observed in passive smokers. Recent reports suggest that smoking influences host cytokine levels. Boström et al. (1998) noted that smokers displayed significantly higher quantities of GCF TNF-α than did non-smokers among periodontal patients. IL-1β also significantly increased in terms of periodontally healthy sites in smokers (Kamma et al. 2004). Kuschner et al. (1996) documented a dose-dependent effect of smoking on IL-1, IL-6, IL-8 and monocyte chemotactic protein levels. In contrast to the results of these studies, no difference in GCF IL-1β was evident between non-smokers and smokers (Boström et al. 2000). In addition, decreased GCF IL-1β was detected in deep bleeding periodontitis sites in smokers (Rawlinson et al. 2003). It is difficult to compare these findings with those of other investigations due to variations in the population, experimental methodology and mathematical treatment of the data. In particular, IL-1β in saliva was measured in the current study. Although cytokines have been identified in whole saliva (Leigh et al. 1998), the present observations appear to be the first regarding the influence of passive smoking on IL-1β in saliva.

Levels of albumin, which is an antioxidant in saliva, were not influenced by smoking status (Zappacosta et al. 1999; Zuabi et al. 1999). However, an association between periodontal disease and lower antioxidant capacity was detected (Sculley & Langley-Evans 2003). Significantly higher levels of albumin were observed in passive smokers in the current study. It is hypothesized that salivary albumin may counteract the harmful action of free radical and reactive oxygen species derived from passive smoke or inflammatory cells in order to protect periodontal tissue and/or oral mucosa.

Zappacosta et al. (2002) described inhibition of salivary AST activity following smoking of a single cigarette. However, the chronic effect of smoking on salivary AST remains unclear. On the other hand, AST levels in saliva were correlated with periodontal diseases evaluated with the community periodontal index, suggesting that periodontal destruction such as periodontal pockets, gingival bleeding and suppuration may be related to higher AST levels in saliva (Cesco et al. 2003). Passive smokers displayed significantly higher CAL in comparison with non-smokers (Yamamoto et al. 2005); as a result, elevated AST levels in passive smokers may be associated with periodontal destruction. Nakamura et al. (2000) have suggested that AST activity may be positively correlated with levels of IL-1β, PGE_2 and collagenase. These findings may lend support to the elevated levels of AST and IL-1β in passive smokers observed in the present investigation.

Recently, reports regarding a higher prevalence of certain organisms in smokers have appeared in the literature (Haffajee & Socransky 2001, van Winkelhoff et al. 2001). In the current investigation, somewhat greater differences between passive smokers and non-smokers in terms of percentages of *P. gingivalis* and *P. intermedia* were also detected; however, these differences were not statistically significant. It is unlikely that passive smoke reaches subgingival plaque directly; therefore, passive smoking may not affect these bacteria. Indeed, a number of studies have indicated that smoking has little effect on the subgingival microflora (Lie et al. 1998; Boström et al. 2001; Mager et al. 2003).

Levels of IL-1β, lactoferrin, albumin and AST in saliva increased in passive smokers; thus, passive smoke exposure may stimulate inflammatory responses of periodontal tissue. These responses may be intended to eliminate the microbial challenge in periodontal pockets. Actually, passive smokers did not exhibit increased proportions of periodontal pathogens in spite of periodontal destruction. On the other hand, active smoking led to decreased levels of some inflammatory markers including PGE_2, MMP-9, lactoferrin, albumin and AST as well as to an increase in the proportion of *P. gingivalis* in saliva relative to passive smoking. In general, gingival inflammatory responses are reduced in active smokers (Palmer et al. 2005). Several investigations have demonstrated impairment of neutrophil function in GCF of smokers (Persson et al. 2001, Söder et al. 2002, Guntsch et al. 2006). Neutrophil function in active smokers may affect lower levels of PGE_2 and lactoferrin observed in this study. Our findings suggest that active smoke exposure may impair an inflammatory response in the development of periodontitis. In addition, suppression of inflammatory responses in active smokers may result in an increase in some periodontal pathogens.

However, this study was characterized by several limitations, the most important of which was the cross-sectional design. Data pertaining to periodontal disease and levels of cotinine and other biomarkers in saliva were collected simultaneously. Moreover, biomarkers and periodontal pathogens were obtained from whole saliva samples. Despite these limitations, the current investigation possessed considerable strength in terms of the result that passive smoke exposure affects biomarkers related to periodontitis. Longitudinal studies involving larger populations are necessary in order to provide stronger evidence with respect to the biological mechanisms of passive smoking in the development of periodontitis.

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References

Arbes, S. J. Jr., Agustsdottir, H. & Slade, G. D. (2001) Environmental tobacco smoke and periodontal disease in the United States. *American Journal of Public Health* **91**, 253–257.

Boström, L., Bergström, J., Dahlen, 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. (1998) Clinical expression of TNF-α in smoking associated periodontal disease. *Journal of Clinical Periodontology* **25**, 767–773.

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.

Brock, R. G., Butterworth, J. C., Matthews, B. J. & Chapple, C. L. (2004) Local and systemic total antioxidant capacity in periodontitis and health. *Journal of Clinical Periodontology* **31**, 515–521.

Cesco, Rde T., Ito, I. Y. & de Albuquerque, R. F. Jr. (2003) Levels of aspartate aminotransferase (AST) in saliva of patients with differ-

ent periodontal conditions. *Journal of Clinical Periodontology* **30**, 752–755.
Grabarek, Z. & Gergely, J. (1990) Zero-length crosslinking procedure with the use of active esters. *Analytical Biochemistry* **185**, 131–135.
Guntsch, A., Erler, M., Preshaw, P. M., Sigusch, B. W., Klinger, G & Glockmann, E. (2006) Effect of smoking on crevicular polymorphonuclear neutrophil function in periodontally healthy subjects. *Journal of Periodontal Research* **41**, 184–188.
Haffajee, A. D. & Socransky, S. S. (2001) Relationship of cigarette smoking to the subgingival microbiota. *Journal of Clinical Periodontology* **28**, 377–388.
Johnson, G. K. & Hill, M. (2004) Cigarette smoking and the periodontal patient. *Journal of Periodontology* **75**, 196–209.
Kamma, J. J., Giannopoulou, C., Vasdekis, V. G. & Mombelli, A. (2004) Cytokine profile in gingival crevicular fluid of aggressive periodontitis, influence of smoking and stress. *Journal of Clinical Periodontology* **31**, 894–902.
Kaufman, E. & Lamster, I. B. (2000) Analysis of saliva for periodontal diagnosis – a review. *Journal of Clinical Periodontology* **27**, 453–465.
Kinane, D. F. & Chestnutt, I. G. (2000) Smoking and periodontal disease. *Critical Review in Oral Biology and Medicine* **11**, 356–365.
Kuboniwa, M., Amano, A., Kimura, K. R., Sekine, S., Kato, S., Yamamoto, Y., Okahashi, N., Iida, T. & Shizukuishi, S. (2004) Quantitative detection of periodontal pathogens using real-time polymerase chain reaction with taqman probes. *Oral Microbiology and Immunology* **19**, 168–176.
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.
Leigh, J. E., Steele, C., Wormley, F. L. Jr., Luo, W., Clark, R. A., Gallaher, W. & Fidel, P. L. Jr. (1998) Th1/Th2 cytokine expression in saliva of HIV-positive and HIV-negative individuals, a pilot study in HIV-positive individuals with oropharyngeal candidiasis. *Journal of Acquired Immune Deficiency Syndromes and Human Retroviology* **19**, 373–380.
Lie, T., Bruun, G. & Boe, O. E. (1998) Effects of topical metronidazole and tetracycline in treatment of adult periodontitis. *Journal of Periodontology* **69**, 819–827.
Madianos, P. N., Bobetsis, Y. A. & Kinane, D. F. (2005) Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. *Journal of Clinical Periodontology* **32** (Suppl. 6), 57–71.
Mager, D. Çk., Haffajee, A. D. & Socransky, S. S. (2003) Effects of periodontitis and smoking on the microbiota of oral mucous membranes and saliva in systemically healthy subjects. *Journal of Clinical Periodontology* **30**, 1031–1037.
Nadkarni, M. A., Martin, F. E., Jacques, N. A. & Hunter, N. (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primer set. *Microbiology* **148**, 257–266.
Nakamura, T., Kido, J., Kido, R., Ohishi, K., Yamauchi, N., Kataoka, M. & Nagata, T. (2000) The association of calprotectin level in gingival crevicular fluid with gingival index and the activities of collagenase and aspartate aminotransferase in adult periodontitis patients. *Journal of Periodontology* **71**, 361–367.
Ozmeric, N. (2004) Advances in periodontal disease markers. *Clinica Chimica Acta; International Journal of Clinical Chemistry* **343**, 1–16.
Palmer, R. M., Wilson, R. F., Hasan, A. S. & Scott, D. A. (2005) Mechanisms of action of environmental factors – tobacco smoking. *Journal of Clinical Periodontology* **32** (Suppl. 6), 180–195.
Perez-Stable, E. J., Benowitz, N. L. & Marin, G. (1995) Is serum cotinine a better measure of cigarette smoking than self-report? *Preventive Medicine* **24**, 171–179.
Persson, L., Bergström, J., Ito, H. & Gustafsson, A. (2001) Tobacco smoking and neutrophil activity in patients with periodontal disease. *Journal of Periodontology* **72**, 90–95.
Rawlinson, A., Grummitt, J. M., Walsh, T. F. & Ian Douglas, C. W. (2003) Interleukin 1 and receptor antagonist levels in gingival crevicular fluid in heavy smokers *versus* non-smokers. *Journal of Clinical Periodontology* **30**, 42–48.
Sculley, D. V. & Langley-Evans, S. C. (2003) Periodontal disease is associated with lower antioxidant capacity in whole saliva and evidence of increased protein oxidation. *Clinical Science (London)* **105**, 167–172.
Söder, B., Jin, J. L. & Wickholm, S. (2002) Granulocyte elastase, matrix metalloproteinase-8 and prostaglandin E2 in gingival crevicular fluid in mached clinical sites in smokers and non-smokers with persistent periodontitis. *Journal of Clinical Periodontology* **29**, 384–391.
van Winkelhoff, A. J., Bosch-Tijhof, C. J., Winkel, E. G. & van der Reijden, W. A. (2001) Smoking affects the subgingival microflora in periodontitis. *Journal of Periodontology* **72**, 666–671.
Yamamoto, Y., Nishida, N., Tanaka, M., Hayashi, N., Matsuse, R., Nakayama, K., Morimoto, K. & Shizukuishi, S. (2005) Association between active and passive smoking evaluated by salivary cotinine and periodontitis. *Journal of Clinical Periodontology* **32**, 1041–1046.
Zappacosta, B., Persichilli, S., De Sole, P., Mordente, A. & Giardina, B. (1999) Effect of smoking one cigarette on antioxidant metabolites in the saliva of healthy smokers. *Archives of Oral Biology* **44**, 485–488.
Zappacosta, B., Persichilli, S., Mordente, A., Minucci, A., Lazzaro, D., Meucci, E. & Giardina, B. (2002) Inhibition of salivary enzymes by cigarette smoke and the protective role of glutathione. *Human and Experimental Toxicology* **21**, 7–11.
Zuabi, O., Machtei, E. E., Ben-Aryeh, H., Ardekian, L., Peled, M. & Laufer, D. (1999) The effect of smoking and periodontal treatment on salivary composition in patients with established periodontitis. *Journal of Periodontology* **70**, 1240–1246.

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Clinical Relevance

Recently, several investigations demonstrated the presence of a harmful effect in connection with passive smoking with respect to periodontal diseases. However, changes in the host–microbe interactions involved in periodontal destruction in passive smokers are poorly understood. In this study, no meaningful differences in the population of periodontal pathogens were detected. However, passive smoke exposure leads to elevation of IL-1β, albumin, lactoferrin and AST levels in saliva. These findings should provide important information regarding the pathologic mechanisms via which passive smoking affects the inflammatory responses in the development of periodontitis.