ORAL DISEASES

Oral Diseases (2012) 18, 410–416 doi:10.1111/j.1601-0825.2011.01891.x

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ORIGINAL ARTICLE

Smoking influences salivary histamine levels in periodontal disease

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OBJECTIVES: Histamine, a potent vasoactive amine, is increased in saliva of periodontitis patients. The present study aimed to further investigate the diagnostic potential of histamine for periodontal disease and assessed smoking, a major risk factor of periodontitis, as a possible influencing factor.

METHODS: Salivary and serum samples of 106 participants (60 periodontitis patients, 46 controls) were collected. Salivary histamine was determined by a commercially available ELISA kit, and serum C-reactive protein was measured by a routine laboratory test. Cigarettes per day and packyears were assessed as smoking exposure parameters.

RESULTS: Statistically significantly increased levels of salivary histamine and serum C-reactive protein were detected between the patient and control group ($P = 0.022$ and $P = 0.001$). Salivary histamine levels were significantly higher in smoking compared with non-smoking patients ($P < 0.001$), and salivary histamine as well as serum C-reactive protein correlated significantly positively with smoking exposure parameters ($P < 0.05$).

CONCLUSIONS: Smoking, an established and common risk factor of periodontitis, was assessed as a possible influencing factor for salivary histamine. Most interestingly, salivary histamine differed highly significantly between smoking and non-smoking periodontitis patients. Our results suggest a possible involvement of histamine in tobacco-exacerbated periodontal disease, but do not suggest salivary histamine as a reliable diagnostic marker for periodontitis.

Oral Diseases (2012) **18**, 410–416

Keywords: saliva; periodontal disease; smoking; periodontal diagnostics

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Received 27 July 2011; revised 4 December 2011; accepted 8 December 2011

Introduction

Periodontitis is a chronic infectious inflammatory disease affecting the tooth-supporting structures. It is initiated by a bacterial biofilm accumulating on the surfaces of the teeth, leading to an excessive inflammatory response, which is influenced by several risk factors, such as stress and/or host-specific factors or habits, such as smoking. Uncontrolled inflammation causes loss of connective tissue, alveolar bone, and finally, the tooth itself (Brook, 2003; Hugoson *et al*, 2008; Holtfreter *et al*, 2010). The pathologic alterations induced by this disease are mostly irreversible.

Histamine is a potent vasoactive amine that participates in allergic and inflammatory processes. It can be formed, stored, and released after activation of mast cells by allergic triggers, bacterial antigens, or cytokines (Theoharides and Cochrane, 2004). In the oral cavity, histamine can also be produced by histidine decarboxylase, which is expressed in neutrophils, macrophages, and gingival fibroblasts and is up-regulated by bacterial and viral products (Kahlson and Rosengren, 1968; Endo, 2001). The contribution of histamine to periodontal disease has been investigated in clinical, animal, and *in vitro* studies (Hyyppa, 1981; Van Dyke *et al*, 2005; Hasturk *et al*, 2006; Venza *et al*, 2006; Minami *et al*, 2007). Histamine enhances interleukin-8 and prostaglandin E_2 production, as well as the expression of cyclooxygenase and Toll-like receptors 2 and 4, and augments the inflammatory response in gingival fibroblasts *in vitro* (Minami *et al*, 2007; Gutierrez-Venegas *et al*, 2011). Topical application of the histamine H_2 receptor antagonist cimetidine in a rabbit model appears to arrest periodontal inflammation induced by *Porphyromonas gingivalis* (Hasturk *et al*, 2006). A cimetidine oral rinse solution in humans improves the antibacterial function of gingival crevicular neutrophils (Van Dyke *et al*, 2005).

Previous studies have described increased salivary histamine levels in periodontitis patients (Hyyppa, 1984; Venza *et al*, 2006). Venza *et al*. (2006) suggested that salivary histamine could be used as a predictive index for periodontitis, because elevated levels of salivary

histamine preceded the onset of clinical signs of periodontal disease and correlated with the severity of periodontitis. However, in that study of Venza and colleagues, only non-smoking individuals were included. Yet, *in vitro* and *in vivo* studies have reported an influence of cigarette smoke on histamine release by mast cells (Walter and Walter, 1982; Thomas *et al*, 1992). Moreover, smoking, which is an established and common risk factor for periodontal disease (Tonetti, 1998; Tomar and Asma, 2000), influences serum and salivary parameters in periodontitis patients (Kibayashi *et al*, 2007; Nishida *et al*, 2008; Heikkinen *et al*, 2010; Ozcaka *et al*, 2011a,b). Thus, one can assume that smoking might influence salivary histamine levels, and this should be considered in the assessment of this parameter as a diagnostic marker for periodontitis. Therefore, the main aim of the present study was to further investigate salivary histamine as a parameter for periodontitis, and smoking was assessed as a possible influencing factor.

Further, periodontitis is a well-known local inflammatory disease, which possibly induces systemic reactions. C-reactive protein (CRP) is a well-established parameter indicating various inflammatory diseases (Rosa Neto *et al*, 2009). Particularly, in periodontal disease, elevated CRP levels are reported (Buhlin *et al*, 2009; Andrukhov *et al*, 2010; Duarte *et al*, 2010; Nakajima *et al*, 2010; Shimada *et al*, 2010), and previous studies suggested to additionally determine serum CRP to prove the systemic inflammatory burden in periodontitis (Rosania *et al*, 2009; Rai *et al*, 2011). Therefore, in the present study, serum CRP was additionally investigated to assess a possible association of the local inflammatory marker histamine with systemic inflammatory reaction.

Methods

Patient recruitment and clinical periodontal examination
The protocol for the present cross-sectional study was approved by the ethics committee of the Medical University of Vienna (EK 623/2007). Written informed consent was obtained from all participants. This study included 106 participants (mean age ± S.D., 37.56 ± 9.05; 53 males, 53 females; 60 periodontitis patients, 46 periodontally healthy individuals), and clinical history of all participants was recorded (personal data and medical history). Exclusion criteria were defined as follows: periodontal treatment or antibiotic therapy within the preceding 3 months, presence of any systemic disease or diseases of the salivary glands, usage of a dental prosthesis, and acute infection. Smoking history was assessed according to the following parameters: cigarettes smoked per day (cig/day) and packyears (PY – number of cigarettes smoked per day, multiplied by the number of years of smoking, divided by 20). Panoramic radiographs were taken for each participant for the determination of alveolar bone loss. The 'periodontitis patients' group included individuals with severe (loss of supporting bone ≥1/3 of the root length) and generalized (≥30% affected sites) periodontal disease (Armitage, 1999), with at least five sites with a probing depth (PD) ≥5 mm. The control group consisted of individuals without a history of periodontal disease and attachment loss, as well as with probing PD ≤3 mm and papilla bleeding index (PBI) simplified $< 20\%$ to exclude the presence of gingivitis. PD, clinical attachment level (CAL), and bleeding on probing (BoP) were measured on 6 sites per tooth in periodontitis patients. BoP is expressed in percent (sites positive for bleeding multiplied by 100 divided by the number of measured sites).

Sample size calculation
Based on preliminary results, two sample size calculations (80% power, alpha value 0.05) were performed prior to patient recruitment. Our main interest was to investigate a possible influence of smoking on salivary histamine. The first sample size calculation led to the conclusion that 40 participants/group (smoking *vs* non-smoking) should be included. The second sample size calculation determined that at least 22 periodontally diseased subjects/group (smoking *vs* non-smoking) would be essential. Therefore, in the present study population, 41 smokers and 65 non-smokers, and, within the group of periodontitis patients, 26 smoking and 34 non-smoking individuals were included.

Saliva and serum analysis
Sample collection was carried out between 7:30 and 10:30 A.M. Participants were asked to refrain from eating, drinking (except water), smoking, chewing gum, brushing their teeth, and using mouth rinsing solutions beginning at midnight before sampling occurred, to exclude any possible influences. Stimulated salivary samples were collected by the use of the saliva extraction solution (4 ml, citrate buffer pH 4.2) of the saliva collection system® (Greiner Bio-One, Kremsmünster, Austria) according to the manufacturer's instructions. Patients were instructed to rinse out the oral cavity with the solution for 2 min. The stimulated whole saliva mixed with the extraction solution is collected in a beaker. The yellow food dye in the extraction solution serves as internal standard for photometric saliva volume determination in the collected samples by means of a Saliva Quantification Kit at 450 nm (Greiner Bio-One, Kremsmünster, Austria). Venous blood was drawn from the antecubital vein into serum gel tubes (Vacuette®; Greiner Bio-One) and sera were isolated by centrifugation (10 min at 2220 ***g*** at 4°C). All samples were stored at −40°C until analysis. Salivary histamine levels were determined with an ELISA kit (Labor Diagnostika Nord, Nordhorn, Germany) according to the manufacturer's instructions. The standard range was 0.3–30 ng/ml with a sensitivity of 0.1 ng/ml. Serum levels of CRP were measured on a Chemistry Immuno-System Olympus AU640. A highly sensitive CRP assay with a lower detection limit of 0.08 mg/l was applied. Linear measurements are possible from 0.08 to 80 mg/l.

Statistical analysis
Data were checked for normal distribution by the Kolmogorov–Smirnov test. If the data sets were

normally distributed, an independent samples *t*-test was performed; otherwise, the Mann–Whitney test was applied. Possible correlations between salivary histamine levels and clinical and smoking parameters were assessed by the Spearman correlation coefficient. Statistical analyses were performed with SPSS Version 17.0 (SPSS, Chicago, IL, USA), and *P*-values ≤0.05 were considered statistically significant.

Results

The mean clinical parameters of control and test groups are presented in Table 1. All parameters were highly significantly increased in the test group compared with the control group ($P < 0.001$). Mean age was higher in the periodontitis group, but salivary histamine levels did not correlate with age in the study population (Spearman correlation, $\rho = 0.149$, $P = 0.127$; data not shown). No significant difference in salivary histamine levels was detectable between male and female participants (control group, $P = 0.130$; whole study population, $P = 0.056$; data not shown). Serum CRP and salivary histamine levels of the periodontitis patients and the control group are shown in Figures 1 and 2. Periodontitis patients exhibited up to two times higher levels of CRP ($P = 0.001$) and salivary histamine ($P = 0.022$) compared with the control group. However, no statistically significant correlation of these parameters with clinical parameters (PD, CAL, number of teeth with PD ≥5 mm, and BoP) in periodontitis patients was observed (all *P*-values >0.09, data not shown).

Table 1 Clinical periodontal parameters of periodontally diseased and healthy probands (independent t-test)

	Control	*Perio*	P-*value*
Probing depth (PD) (mm)	1.65 ± 0.04	3.56 ± 0.12	**<0.001**
Clinical attachment level (mm)	1.65 ± 0.04	4.06 ± 0.17	**<0.001**
nr. of teeth (PD ≥ 5 mm)	0.00 ± 0.00	15.48 ± 0.89	**<0.001**
Bleeding on probing (%)	4.43 ± 0.74	35.77 ± 3.88	**<0.001**

Data are expressed as mean ± S.E.M.
Bold values indicate statistical significance ($P < 0.05$).

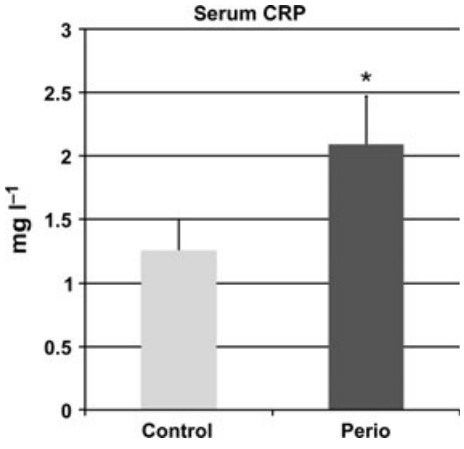

Figure 1 Serum C-reactive protein (CRP) levels (mg/L) in the periodontitis patients and the control group. Data are expressed as mean ± S.E.M. * serum CRP levels were significantly higher in the periodontitis group than in the control group ($P = 0.001$)

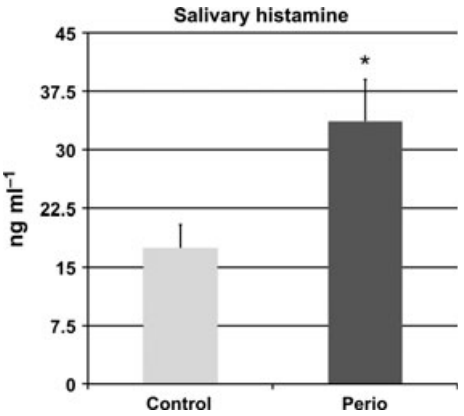

Figure 2 Salivary histamine levels (ng/mL) in the periodontitis patients and the control group. Data are expressed as mean ± S.E.M. * salivary histamine levels were significantly higher in the periodontitis group than in the control group ($P = 0.022$)

Salivary histamine levels and serum CRP levels in periodontitis patients and healthy controls, depending on their smoking status, are presented in Figure 3. Within the control group, there was no significant difference of salivary histamine levels between smokers and non-smokers (Figure 3a; $P = 0.126$). However, in the periodontitis patients, salivary histamine levels were three times higher in smoking individuals compared with non-smokers (Figure 3a; $P < 0.001$). No significant difference was observed for serum CRP levels between smokers and non-smokers (Figure 3b; control group, $P = 0.888$; periodontitis group, $P = 0.146$). Salivary histamine levels of smoking individuals correlated significantly positively with the parameters cig/day ($P = 0.006$; Figure 4a) and packyears (PY) ($P = 0.05$; Figure 4b). Serum CRP levels and clinical periodontal parameters significantly correlated positive with smoking exposure parameters (cig/day, PY) (Table 2). Clinical periodontal parameters of periodontitis patients, depending on smoking status, are given in Table 3. Smoking patients presented a tendency toward elevated PD, CAL, number of teeth with PD ≥5 mm, and lowered BoP compared with non-smoking patients, but these differences were not statistically significant. Notably, smoking parameters (cig/day, PY) of periodontitis patients were significantly higher than those of smoking control participants (Table 4).

Discussion

Saliva has become increasingly interesting for the diagnosis and monitoring of diseases, because saliva collection is non-invasive and thus is well accepted by patients (Giannobile *et al*, 2009; Zhang *et al*, 2009; Buduneli and Kinane, 2011). Recently, saliva was investigated for diagnosing oral diseases, particularly caries (Larmas, 1992), oral cancer (Li *et al*, 2004), salivary gland diseases (Hu *et al*, 2009), and periodontitis (Zhang *et al*, 2009; Buduneli and Kinane, 2011).

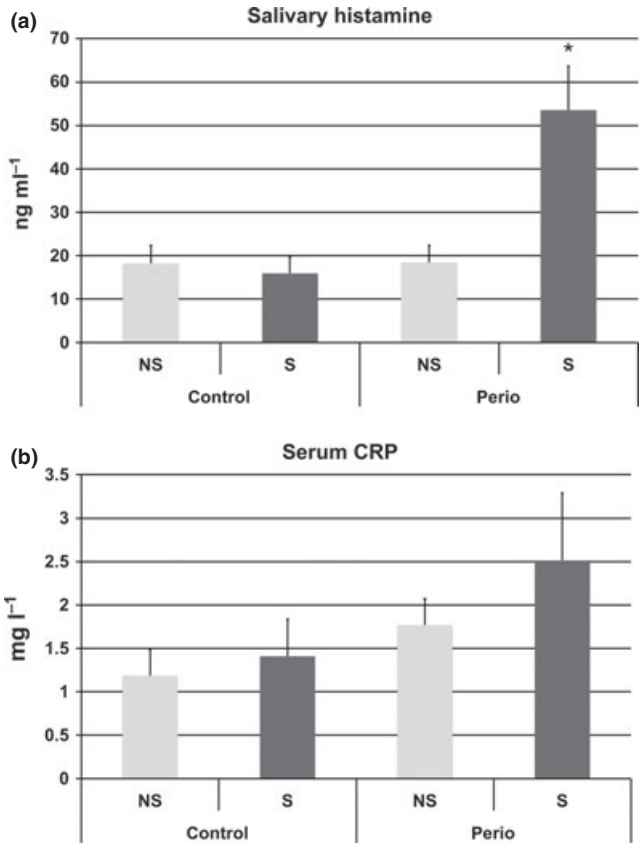

Figure 3 (**a** and **b**) Salivary histamine levels (**a**) and serum C-reactive protein (CRP) levels (**b**) in the periodontitis patients and the healthy controls depending on smoking status. Data are expressed as mean ± S.E.M. * salivary histamine levels were significantly higher in the smoking periodontitis patients than in the non-smoking periodontitis patients ($P < 0.001$); non-smokers (NS), smokers (S)

Nevertheless, no laboratory tests have so far been developed for diagnosing, monitoring, or evaluating periodontal disease, and measuring clinical parameters remains the most reliable method (Buduneli and Kinane, 2011). Histamine has been discussed as a potential diagnostic or even predictive index for periodontitis (Venza *et al*, 2006). As much as possible, diagnostic factors should be independent of confounding factors, such as smoking. Therefore, the aim of the present study was to investigate the influence of smoking on salivary histamine levels and further assess its practicability as a diagnostic parameter for periodontitis.

Our results revealed significantly increased salivary histamine levels of periodontitis patients compared with those of the control group, which is in line with previous reports (Hyyppa, 1984; Venza *et al*, 2006). However, among the non-smoking participants, a significant difference in salivary histamine levels between the patients and the control group could not be confirmed. To date, there have been few studies quantifying salivary histamine by different collection and measurement methods, which impedes comparison (Hyyppa, 1984; Venza *et al*, 2006). In our study, stimulated whole saliva was collected by means of a system, which provides a rather reliable and accurate quantification of the recov-

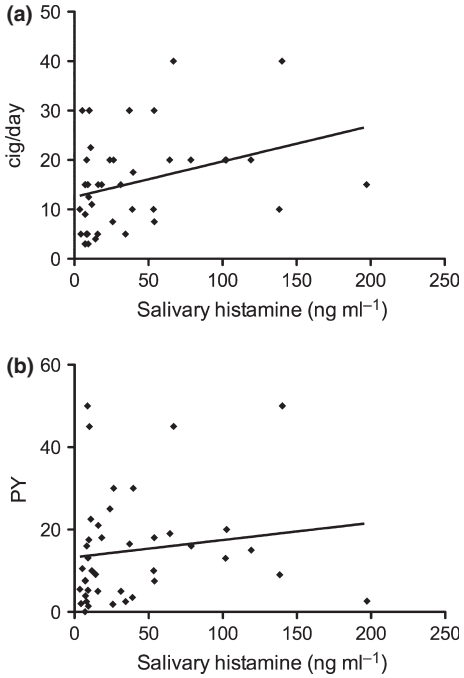

Figure 4 (**a** and **b**) Correlation of salivary histamine levels of smoking individuals with smoking parameters - cig/day (a – $\rho = 0.419$, $P = 0.006$), packyears (PY) (b – $\rho = 0.308$, $P = 0.05$) – (Spearman correlation coefficient)

Table 2 Correlation of clinical periodontal parameters and serum C-reactive protein (CRP) levels of smoking individuals with smoking parameters (Spearman correlation coefficient)

	Cig/day		*Packyears*	
	ρ	P-*value*	ρ	P-*value*
Probing depth (PD)	0.495	**0.002**	0.670	**< 0.001**
Clinical attachment level	0.515	**0.001**	0.660	**< 0.001**
nr. of teeth (PD ≥ 5 mm)	0.400	**0.010**	0.644	**< 0.001**
Bleeding on probing	0.105	0.543	0.391	**0.018**
Serum CRP	0.392	**0.011**	0.391	**0.011**

Bold values indicate statistical significance ($P < 0.05$).

Table 3 Clinical periodontal parameters of smoking and non-smoking periodontitis patients

	Smoker	*Non-smoker*	P-*value*
Probing depth (PD) (mm)	3.81 ± 0.18	3.38 ± 0.16	0.073
Clinical attachment level (mm)	4.34 ± 0.28	3.84 ± 0.22	0.156
nr. of teeth (PD ≥ 5 mm)	16.46 ± 1.29	14.74 ± 1.23	0.292
Bleeding on probing (%)	32.78 ± 5.61	38.05 ± 5.37	0.488

Data are expressed as mean ± S.E.M.

Table 4 Smoking parameters of periodontally healthy and diseased smoking individuals

	Control	*Perio*	P-*value*
cig/day	10.23 ± 1.77	18.35 ± 1.94	**0.004**
Packyears	5.19 ± 0.87	20.59 ± 2.70	**< 0.001**

Data are expressed as mean ± S.E.M.
Bold values indicate statistical significance ($P < 0.05$).

ered saliva volume compared to conventional methods (Raggam *et al*, 2008; Nunes *et al*, 2011). The salivary histamine levels of the control group presented a mean value of 17.5 ng/ml. This value seems near to that reported in the study by Kejr *et al*. (2010), which assessed salivary histamine values with two different methods (ELISA technique and high-performance liquid chromatography). They presented values between 0.31 and 12.4 ng/ml. Venza *et al*. (2006) collected unstimulated saliva from the sublingual region and detected substantially higher values of salivary histamine (mean value of the control group: 6.2 μg/ml). Under stimulation, saliva is produced mainly by the parotid gland (Llena-Puy, 2006). Wolff *et al*. (2008) reported significantly decreased stimulated and unstimulated flow rates of submandibular/sublingual glands, but not of parotid glands, in individuals taking antihistamine medication. This suggests that histamine production might be connected to submandibular and sublingual glands. Saliva collected primarily from these glands could contain higher amounts of histamine, whereas collecting whole stimulated saliva would dilute the released histamine.

Significantly increased serum CRP levels in periodontitis patients implied systemic effects of the local periodontal infection (Buhlin *et al*, 2009; Andrukhov *et al*, 2010; Duarte *et al*, 2010; Nakajima *et al*, 2010; Shimada *et al*, 2010). However, in the present study, no correlation was found between salivary histamine levels and serum CRP level, number of teeth with PD ≥5 mm, or BoP. In addition, no significant effect of age or gender on salivary histamine levels was detected. Previous studies also report no effect of age, but a possible higher allergen-induced histamine release of mast cells in male subjects (Atkins *et al*, 1993; Petersen *et al*, 1996; Saarinen *et al*, 2000). In our study, male subjects presented slightly increased values, but not statistically significant.

Smoking is a well-known risk factor for periodontal disease (Tonetti, 1998; Tomar and Asma, 2000). Similar to our results, clinical parameters indicating local inflammation, such as bleeding indices, are frequently decreased comparing to non-smokers, in spite of increased pocket depths (Preber and Bergstrom, 1985; Heikkinen *et al*, 2010). Although controversially discussed, smoking might cause localized vascular dysfunction and reduce gingival blood flow and crevicular fluid, which could be responsible for the decreased overt inflammation in smoking periodontitis patients (Meekin *et al*, 2000; Mavropoulos *et al*, 2003, 2007; Morozumi *et al*, 2004). Moreover, active and passive smoking influences inflammatory parameters and bone metabolism in periodontitis patients (Nishida *et al*, 2008; Gurlek *et al*, 2009; Heikkinen *et al*, 2010; Ozcaka *et al*, 2011a,b). The concentrations of several systemic and local parameters have been specifically compared between smoking and non-smoking periodontitis patients (Ozcaka *et al*, 2011a,b). Those investigators observed other inflammatory parameters, such as myeloperoxidase, neutrophil elastase, matrix metalloproteinases and its inhibitors, or osteocalcin, between periodontally diseased smokers and non-smokers. As with histamine, there are differences between smokers and non-smokers in the periodontitis patients, but not always in the control group. Interestingly, decreased salivary osteocalcin levels were discussed to imply increased periodontal tissue destruction among smokers (Gurlek *et al*, 2009; Ozcaka *et al*, 2011b). Altogether, these studies underline the important role of smoking by assessing potential markers for periodontitis.

Previous clinical observations suggest an influence of smoking on histamine production. Exemplary, in smokers, an increased histamine concentration has been detected in the bronchoalveolar lavage fluid (Walter and Walter, 1982; Kalenderian *et al*, 1988; Thomas *et al*, 1992). Concerning the mechanisms, direct and indirect influence of cigarette smoke on histamine release by mast cells has been discussed. It is supposed to be directly released by mast cells after nicotine exposure. Histamine release by mast cells upon cigarette smoke stimulation has been observed *in vitro* and *in vivo* (Walter and Walter, 1982; Thomas *et al*, 1992). Alternatively, nicotine increases the interleukin-1 production of macrophages *in vitro*, which in turn is able to release histamine by human basophils and mast cells (Subramanian and Bray, 1987; Kalenderian *et al*, 1988). In periodontitis patients, previous studies did not consider the effect of smoking on salivary histamine levels (Hyyppa, 1981; Venza *et al*, 2006). Our results demonstrated that smoking significantly influences salivary histamine levels in periodontitis patients. However, in the present study, cig/day and PY indicating current and previous smoking exposure differed significantly between periodontally healthy and diseased participants. This might be a possible explanation for elevated salivary histamine levels solely in periodontally diseased smoking subjects. Another one is the availability and responsiveness of the mast cells. Mast cell density is increased in chronic periodontitis and gingivitis lesions (Batista *et al*, 2005). Consequently, a higher number of mast cells in combination with increased smoking exposure may trigger increased salivary histamine levels, which were present only in the smoking periodontitis patients in this study population. Future studies on the effects of smoking might additionally determine salivary cotinine, which is another suggested parameter to assess smoking load beside cig/day and PY (Blackford *et al*, 2006). A linear relationship between salivary cotinine and cig/day is reported up to 20 cig/day (Blackford *et al*, 2006). However, in the present study, 54% of the periodontally diseased smokers were loaded over 20 cig/day, which is beyond the exact determination range of salivary cotinine.

Elevated histamine release under smoking load could be involved in the pathogenesis of periodontal destruction. Histamine influences the behavior of many cells of the periodontium, such as gingival fibroblasts, periodontal ligament cells, osteoclasts, or osteoblasts (Niisato *et al*, 1996; Ikawa *et al*, 2007; Minami *et al*, 2007; Biosse-Duplan *et al*, 2009; Gutierrez-Venegas *et al*, 2011). Histamine causes increased cytokine and prostaglandin production and expression of Toll-like

receptors 2 and 4 in human gingival fibroblasts (Minami *et al*, 2007; Gutierrez-Venegas *et al*, 2011), as well as increased prostaglandin E_2 production in human periodontal ligament cells (Niisato *et al*, 1996). Prostaglandin E_2 plays an especially important role in the pathogenesis of periodontal disease and is a strong osteolytic factor (Nakashima *et al*, 1994; Van Dyke and Serhan, 2003). Moreover, histamine directly influences the process of osteoclastogenesis as well. Particularly, histamine increases the number osteoclasts and elevates the expression of RANKL by osteoblasts resulting in bone resorption (Ikawa *et al*, 2007; Biosse-Duplan *et al*, 2009). As recently reviewed, histamine is also involved in immune response and modulation (O'Mahony *et al*, 2011). An appropriate amount of histamine seems to be necessary for an appropriate immune reaction in infection diseases. However, strongly elevated histamine levels up-regulate the immune reaction, resulting in further destruction of the soft and hard periodontal tissue.

In the present clinical cross-sectional study, histamine was examined as a new marker for diagnosing periodontal disease and smoking was assessed as a possible influencing factor. Salivary histamine levels differed significantly between smoking and non-smoking periodontally diseased patients. Our results suggest a possible involvement of histamine in tobacco-exacerbated periodontal disease, but do not suggest salivary histamine as a reliable diagnostic marker for periodontitis.

Acknowledgements

The authors thank Mrs Rutschek Hedwig and Mrs. Nguyen Phuong Quynh (both in the Department of Periodontology, Bernhard Gottlieb School of Dentistry, Medical University of Vienna, Austria) and Dr Ildiko Wichart for their help in performing the experiments. Greiner Bio-One supported this study by financing materials (reagents and saliva collection system) (Medical University of Vienna, §26, project number AP00345OFF). All authors have no conflict of interest to declare, have read and approved the manuscript, and have agreed to its submission to this journal.

Author contributions

Kristina Bertl: concept/design, patient recruitment, acquisition of data, data analysis, data interpretation, drafting of the manuscript, approval of the article. Hady Haririan: patient recruitment, acquisition of data, critical revision of the manuscript, approval of the article. Markus Laky: patient recruitment, acquisition of data, critical revision of the manuscript, approval of the article. Michael Matejka: concept/design, critical revision of the manuscript, approval of the article. Oleh Andrukhov: data analysis, data interpretation, critical revision of the manuscript, approval of the article. Xiaohui Rausch-Fan: concept/design, data analysis, data interpretation, critical revision of the manuscript, approval of the article.

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