ORAL DISEASES

Oral Diseases (2010) 16, 365–374. doi:10.1111/j.1601-0825.2009.01648.x

http://www.blackwellmunksgaard.com

ORIGINAL ARTICLE

Neuropeptides in saliva of subjects with burning mouth syndrome: a pilot study

V Borelli[1], A Marchioli[1], R Di Taranto[2], M Romano[1], S Chiandussi[2], R Di Lenarda[2], M Biasotto[2], G Zabucchi[1]

[1]*Department of Life Sciences, University of Trieste, Trieste, Italy;* [2]*Department of Biomedicine, Unit of Dental Sciences and Biomaterials, University of Trieste, Trieste, Italy*

OBJECTIVE: A neuropathic basis has been suggested for burning mouth syndrome (BMS) and an altered concentration of neuropeptides has been reported in lingual oral mucosa and saliva in this disease.

The aims of this study were to compare the levels of nerve growth factor (NGF), substance P (SP) and degranulation products from mast cells and neutrophils in the saliva of BMS subjects with those of control subjects.

MATERIAL AND METHODS: Salivary flow rate, protein concentration, NGF peptide and mRNA, SP, mast cells tryptase, neutrophil myeloperoxidase and calprotectin were analyzed in saliva of 20 BMS subjects and of 20 age- and gender-matched healthy subjects.

RESULTS AND CONCLUSIONS: NGF peptide and tryptase activity were shown to be significantly and persistently higher in saliva of BMS subjects, with respect to control values. Conversely the salivary levels of SP were shown to be significantly lower, while neutrophil markers didn't show any change. We conclude that the neuropathic origin of the disease is confirmed at salivary level. Furthermore, the higher tryptase activity indicates a possible involvement of mast cells. The salivary neuropeptide concentration in BMS subjects, together with mast cell derived compounds, could be useful biomarkers for diagnosis and monitoring of this disease.

Oral Diseases (2010) **16**, 365–374

Keywords: burning mouth syndrome; nerve growth factor; neutrophils; mast cells; tryptase; substance P; neuropeptides

Introduction

Burning mouth syndrome (BMS) is a chronic disease characterized by burning of the oral mucosa associated with sensation of dry-mouth and/or taste alterations (Grushka *et al*, 2002), in the absence of visible pathologic lesions or abnormal laboratory tests.

BMS is a relatively common condition. The estimated prevalence of BMS reported in recent studies ranges between 0.7 and 4.6% of the general population (Maltsman-Tseikhin *et al*, 2007). Such variability reflects the lack of accurate diagnostic criteria for BMS, with studies often including all patients with oral burning symptoms. Many local and/or systemic alterations could lead to a burning type of symptomatology localized in the oral cavity (local or systemic BMS), but this does not constitute a sufficient requisite to indicate a diagnosis of true BMS (idiopathic or essential BMS) (Sardella and Carrassi, 2001). The overall prevalence of BMS is higher among women, especially after menopause, with a male: female ratio of 1:33 (Scala *et al*, 2003).

Pain is a moderate to severe burning sensation, affecting mainly the lateral borders and the tip of the tongue and may involve also the lips and the buccal mucosa (Cerchiari *et al*, 2006). Regardless of the nature of pain, once the oral burning starts, it often persists for many years (Maina *et al*, 2005). The cause of BMS is currently unknown. The etiology is presumed to be multifactorial involving the interaction between biological (neurophysiological mechanisms) and psychological factors (Zakrzewska, 1995). There are increasing numbers of studies to suggest that this condition is not due to psychological factors alone, but may be a form of neuropathic pain that results in psychological effects (Zakrzewska, 2009). In the last decade, clinical, psychophysical, and, more recently, electrophysiological studies suggested that primary neuropathic dysfunction might be involved in the pathogenesis of BMS. (Svensson *et al*, 1993; Jääskeläinen *et al*, 1997; Gao *et al*, 2000; Heckmann *et al*, 2001; Forssell *et al*, 2002).

A neuropathic basis for BMS has been supported by observations that it is frequently accompanied by changes in taste, altered pain or sensory perceptions (Formaker and Frank, 2000). Recently Eliav *et al*, showed that BMS patients had a dysfunction of their

Correspondence: Violetta Borelli, Department of Life Sciences, University of Trieste, via Valerio 28, 34127 Trieste, Italy. Tel: +39 40 5584028, Fax: +39 40 5584023, E-mail: borelliv@units.it
Received 27 February 2009; revised 5 October 2009; accepted 28 October 2009

chorda tympani (Eliav *et al*, 2007). Superficial biopsy of the tongue demonstrated diffuse degeneration of epithelial and sub-papillary nerve fibers in the anterior two-thirds of the tongue as a sign of a small-fiber sensory trigeminal nerve neuropathy (Lauria *et al*, 2005). Decreased levels of salivary concentration of the neuropeptide calcitonin gene-related peptide (CGRP) in BMS subjects recently reported by Zidverc-Trajkovic *et al* (2009), could be additional proof of the trigeminal nerve degeneration in this disorder.

Intriguingly, neuropeptides are providing increasingly impressive database for their roles in the nociceptive processing which leads to neuropathic pain and/or hyperalgesia (Payan *et al*, 1984; Rothwell and Hopkins, 1995; Thacker *et al*, 2007). In a recent study, Yilmaz *et al* demonstrated that BMS associates with increased level of nerve growth factor (NGF) in nerve fibers, as a result of the partial denervation observed in BMS tissues (Yilmaz *et al*, 2007).

NGF, the prototypical member of neurotrophin family, is crucial for survival of nociceptive neurons during development and has been shown to play an important role in nociceptive function in adults (Thoenen, 1991; Indo *et al*, 1996; Shu and Mendell, 1999). In addition to neuronal sensitization, NGF directly interacts with some immune cell types. Mast cells (MC) are considered important components in the action of neuropeptides (Kulka *et al*, 2008). There is strong evidence for functional interactions between MC and nerves in human oral mucosa (Walsh, 2003). MC might play an important role in BMS tissue since, upon stimulation by neuropeptides, they release a number of mediators involved in inflammation (Metcalfe *et al*, 1997; Galli *et al*, 2005; Kulka *et al*, 2008) and neuropathic pain (e.g. tryptase) (Kawabata *et al*, 2001; Vergnolle *et al*, 2001) as well as NGF itself (Leon *et al*, 1994). Furthermore NGF and NGF-induced MC activation can lead to neutrophil accumulation which may be critical for the sensitizing actions of NGF (Amann *et al*, 1996; Bennett *et al*, 1998), but may also have an anti-inflammatory and anti-nociceptive role via calprotectin, capable of inhibiting inflammatory pain in mice (Giorgi *et al*, 1998).

Neurogenic inflammation should be regarded as a protective mechanism which form the first line of defense and protect tissue integrity. However prolonged noxious stimulation may result in an overt inflammatory response (Scardina *et al*, 2007a). The main mediator of neurogenic inflammation, beside CGRP, is substance P (SP) (Scardina *et al*, 2007b) an undecapeptide belonging to the tachykinin family, which, like NGF, is involved in the process of nociception (Sharma *et al*, 1990; Malmberg and Yaksh, 1992) and has a variety of proinflammatory functions (Levine *et al*, 1987; Fischer *et al*, 1998).

Altogether these data suggest the following scenario: a still unknown triggering event induces nerve fibers damage and a compensatory NGF production and release; this neurotrophin, together with other neuropeptides released by nerve fibers (e.g. SP) (Payan *et al*, 1984; Saria *et al*, 1986), could induce hypersensitivity by acting on mouth peripheral nerves and amplify its own action by stimulating resident MC and recruiting neutrophils, which in turn could maintain the neurogenic inflammation.

With the aim of testing this pathway and finding BMS diagnostic markers, we compared the salivary amount of NGF (at peptide and mRNA level), of SP and of degranulation products released from MCs (tryptase) and neutrophils (myeloperoxidase and calprotectin) of subjects with BMS with age- and gender-matched controls.

Materials and methods

BMS and control subjects

We observed a total of 20 non-smoking subjects, 17 females and 3 men (age range 41–89 years, mean 69.0 years), without oral lesions possibly responsible for the burning, and 20 healthy non-smoking subjects (16 females and 4 men, age range 41–77 years, mean 68.3 years) who were not taking any medications for at least 1 month. None of the BMS subjects had been treated before. The control group matched the study group in number, gender, age and none of them complained of oral burning.

Prior to establishment of BMS diagnosis each subject underwent an intra-oral examination to exclude parafunctional activity (such as tongue thrusting) and signs of mucosal diseases. A swab was carried out to exclude oral candidal or bacterial infection. A blood test (glucose and haematinies) was performed to exclude diabetes and anemia and none of the subjects had any systemic disease which may contribute to the symptoms of burning.

The Community Periodontal Index (CPI) was recorded and only participants without periodontal disease were included in this study.

Essential BMS was diagnosed according to the International Headache Classification criteria. A questionnaire was proposed to BMS subjects to reveal related symptoms as: pain, extent of burning sensation (burning sensation involving only tongue, extending from tongue to other mouth sites or spreading in the whole oral cavity), presence of subjective feeling of dry mouth and/or presence of taste alteration. Pain intensity was assessed using a visual analogue scale (VAS).

After a complete description of the study, written informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

The recruited subjects were instructed not to eat or drink for 1 h before the collection of saliva. Saliva was collected, between 8 and 12 AM, by simple spitting method (Navazesh, 1993) in sitting position. The saliva was collected for 5 min into calibrated containers to measure the salivary flow rate (ml min^{-1}).

To minimise the influence of peptidase activity and bacterial growth, saliva samples were immediately cooled on ice, centrifuged at 1000 *g* in a precooled centrifuge and the supernatants were stored at −80°C until use.

The sample collection was repeated for 10 BMS and control subjects after 6 months and during this period

the subjects did not receive any treatment and did not show any change in their condition.

Reagents
Bradford reagent, dapsone (4,4′-diaminodiphenylsulfone), bovine-serum-albumine (BSA), tetramethylbenzidine (TMB), hydrogen peroxide, H_2SO_4 were purchased from Sigma Aldrich (St Louis, MO, USA).

ELISA assays
NGF and Substance P. NGF (Promega Corporation, Madison, WI, USA) and Human SP (USCN Life Sciences & Technology Co. Ltd, Wuhan, China) immunoassay kits were used to determine the concentration of NGF and SP in the whole-saliva samples both from BMS and control subjects. The assays were performed according to the manufacturer's instructions and the results referred to a calibration curve expressed in pg ml^{-1}. Samples were assayed in triplicate.

Calprotectin. Salivary calprotectin levels were measured by enzyme-linked-immunosorbent assay using a commercial kit (Calprest, Eurospital, Italy). Samples were assayed in triplicate. The assays were performed according to the manufacturer's instructions, and the results referred to a calibration curve expressed in μg ml^{-1}.

Biochemical and enzymatic assays
Salivary protein content. The salivary protein content was quantitated with the Bradford method (Bradford, 1976) using BSA as standard and expressed in mg ml^{-1}.

Salivary tryptase. Mast cell degranulation colorimetric assay kit from Millipore Corporation (Billerica, MA, USA) was used to determine the concentration of tryptase in the whole-saliva samples both from patients and controls. The assay is based on spectrophotometric detection of the chromophore *p*-nitroaniline (pNA) after cleavage from the labeled substrate tosyl-gly-pro-lys-pNA. The free pNA can then be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. The assays were performed according to the manufacturer's instructions. Samples were assayed in triplicate and salivary tryptase was expressed in μg ml^{-1}.

Salivary myeloperoxidase. Neutrophil myeloperoxidase (MPO) is responsible for a large portion of peroxidase-catalyzed reactions in saliva (Thomas *et al*, 1994). To distinguish MPO from salivary peroxidase [lactoperoxidase (LPO) which is secreted by the salivary glands] in whole saliva, we used dapsone, a LPO specific inhibitor, which has two primary aromatic amine moieties and allows identification and quantification of MPO in human saliva (Thomas *et al*, 1994; Sakamoto *et al*, 2008). Salivary MPO activity was calculated from the rate of H_2O_2-dependent oxidation of TMB (Menegazzi *et al*, 1992).

Briefly the activity of salivary MPO was determined at room temperature in 96-well microtiter plate using a reaction mixture containing 1 mM (final concentration) TMB added as substrate (from a 25 mM stock solution in dimethylsulphoxide), 0.02% cetyltrimethylammonium bromide (CTAB) and 1 mM dapsone. The reaction was started by adding 0.30 mM hydrogen peroxide (final concentration), and stopped after 2 min by adding 0.4 N H_2SO_4 (final concentration). The reaction product was quantified spectrophotometrically at 413 nm. The concentration of MPO in saliva was calculated by referring to a calibration curve (Karhuvaara *et al*, 1990) obtained with pure human MPO (Rz 0.8) prepared from human neutrophils as previously described (Zabucchi *et al*, 1988) and was expressed in μg ml^{-1}.

Reverse transcription-polymerase chain reaction analysis
RNA was extracted from whole saliva, using the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed with 200 U of M-MLV Reverse Transcriptase (Invitrogen, Merelbeke, Belgium) with poly-(dT) primer.

PCRs were carried out with the primers 5′-TGC-ATAGCGTAATGTCCATG-3′ and 5′-AAGTCCAG ATCCTGAGTGTC-3′ in order to amplify a 325-bp fragment of the human NGF-β mRNA; and the primers 5′-CTTCCTGGGCATGGAGTCC-3′ and 5′-CGCTC AGGAGGAGCAATGAT-3′ in order to amplify a 214-bp fragment of the human housekeeping β-actin gene.

The conditions used for the PCRs were the following: 94°C for 3 min for the initial denaturation, 94°C for 20 s, 58°C for 20 s, 72°C for 45 s for 30 cycles (β-actin) or 40 cycles (NGF), and 72°C for 7 min for the final extension. The PCRs were optimised to be in the exponential phase of amplification. The results are representative of three independent RT-experiments. The PCR products were separated on a 2% agarose gel and then stained by ethidium bromide. The relative amount NFG was quantified by optical densitometry with ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2004).

Statistical analysis
Statistical analysis was performed by Graph Pad Prism (Graph Pad Software Inc., La Jolla, CA, USA). The data are presented as arithmetic mean ± s.e.m. The Kolmogorov–Smirnov test was applied to assess the normality of the studied data. Independent samples *t*-test was used to compare data between the two groups.

The effectiveness of pairing between the first and the second evaluation within the same group was assessed by calculating the Pearson correlation coefficient, *r*, and the corresponding *P*-value. Paired *t*-test was used to analyze, in the same group, the mean difference between the first and the second evaluation.

Spearman's Rho correlation coefficient was used to analyze the relationship of salivary NGF, SP and tryptase levels with pain scores (VAS) and the duration of the disorder.

As far as reverse transcription-polymerase chain reaction analysis is concerned statistical analysis and graphics were generated with EZAnalyze and Merlin

Excel Add-ins and the significance of differences was determined by Student's *t*-test.

In all instances the level of significance for statistical analysis was set at 5% ($P < 0.05$).

Results

Our samples consisted of 20 patients diagnosed with essential BMS. Mean age of the studied BMS subjects was 69.0 years, 16 subjects were older than 60 years. Only patients who had complained of BMS for more than 6 months were included. Fourteen patients suffered from BMS disorder longer than 1 year (range 6–48 months). Subjective feeling of dry mouth was reported by 13 patients (65%) and taste disturbances by seven patients (35%). The distribution of BMS complaints which were confined to the tongue had four patients (20%), while 13 patients (65%) complained of burning sensation extending from the tongue to the hard palate, lips or alveolar ridges. Burning sensation in the whole oral cavity was reported by three patients (15%). Table 1 shows that mean values of salivary flow and protein concentration in BMS subjects were 0.43 ± 0.07 ml min^{-1} and 1.88 ± 0.12 mg ml^{-1} respectively, which were not significantly different than those found in healthy subjects (0.64 ± 0.09 ml min^{-1} and 1.87 ± 0.11 mg ml^{-1} respectively).

Table 1 and Figure 1 show NGF, SP levels and enzymatic markers from neutrophils (MPO and calprotectin) and MC (tryptase) measured in whole saliva together with the relative statistical analysis.

Salivary NGF concentration was significantly higher, about twofold, in BMS subjects as compared to the control group. Conversely the salivary SP concentration was significantly lower on the average, in BMS subjects as compared to control group. The specific mast cells marker, tryptase in BMS samples was significantly higher with respect to the control group. As far as neutrophil markers are regarded no significant difference in salivary level of MPO and calprotectin was found between BMS subjects and the control group (Table 1).

Tables 2 and 3 show that none of the evaluated salivary components changed significantly between two evaluations spaced out 6 months in 10 subjects from both the BMS and the control group. A complete statistical analysis for the mean values and the effectiveness of pairing between the first and the second evaluation is reported in Table 3. Notably the average value of salivary NGF and tryptase concentration remained significantly higher in BMS subjects as compared to the control group either at the first or the second determination.

Salivary NGF, SP and tryptase levels were compared within the group of BMS subjects according to different demographic features (Table 4). No significant difference was found in NGF, tryptase and SP levels of BMS subjects according to gender and age. Salivary NGF, SP

Table 1 Salivary analysis in BMS and control subjects. Salivary flow rate, protein concentration, NGF and SP content, neutrophils markers (MPO and calprotectin) and mast cells marker (tryptase) in the saliva of patients with BMS and healthy individuals

Parameter	*BMS*	*Controls*	*P-value*
Flow rate			
(ml min^{-1})	0.43 ± 0.07	0.64 ± 0.09	0.0657NS
Lower 95% CI of mean	0.29	0.46	
Upper 95% CI of mean	0.56	0.82	
Protein			
(mg ml^{-1})	1.88 ± 0.12	1.87 ± 0.11	0.9547NS
Lower 95% CI of mean	1.63	1.63	
Upper 95% CI of mean	2.12	2.11	
NGF			
(pg ml^{-1})	697.8 ± 72.5	342.6 ± 55.8	0.0010S
Lower 95% CI of mean	546.1	225.2	
Upper 95% CI of mean	849.6	458.8	
Substance P*			
(pg ml^{-1})	37.24 ± 4.68	57.72 ± 8.52	0.0437S
Lower 95% CI of mean	27.28	39.55	
Upper 95% CI of mean	47.21	75.89	
Tryptase			
(μg ml^{-1})	0.182 ± 0.024	0.090 ± 0.017	0.0008S
Lower 95% CI of mean	0.131	0.055	
Upper 95% CI of mean	0.233	0.125	
MPO			
(μg ml^{-1})	2.95 ± 0.27	2.48 ± 0.24	0.1949NS
Lower 95% CI of mean	0.24	0.20	
Upper 95% CI of mean	0.35	0.30	
Calprotectin			
(μg ml^{-1})	1.01 ± 0.04	0.91 ± 0.04	0.1228NS
Lower 95% CI of mean	0.91	0.83	
Upper 95% CI of mean	1.10	1.00	

Values are the mean ± s.e.m. obtained from 20 subjects of each group. The results present 95% CI of mean and statistical significance determined by the Student's unpaired *t*-test analysis to compare data between the two groups. The level of significance for analysis was set at 5% ($P < 0.05$). S = significant; NS = not significant. *SP values are obtained from 16 subjects of each group.

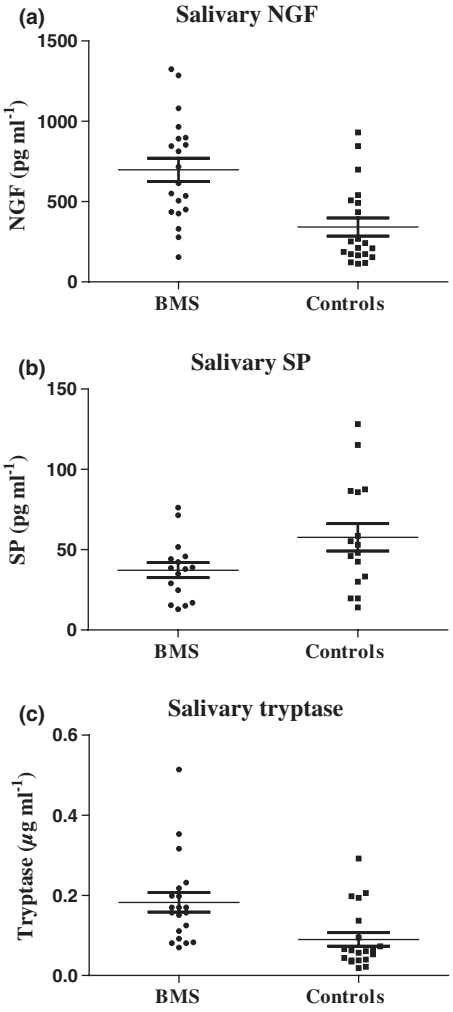

Figure 1 Salivary NGF, SP and tryptase levels in BMS and Control group. Salivary NGF (**a**), SP (**b**) and tryptase (**c**) levels in BMS (●) and Control group (■). The results present single values and mean ± s.e.m. obtained from 20 subjects of each group. SP values are obtained from 16 subjects of each group

and tryptase levels were also compared within the BMS group according to different BMS features (Table 5). No significant difference was found when subjective feeling of dry mouth and distribution of complaints were considered. NGF peptide levels only appeared to be significantly lower in BMS subjects with taste disturbances.

Furthermore, correlation analysis showed that in BMS subjects salivary NGF, SP and tryptase levels were independent of pain scores and the duration of the disorder without any association (Table 6).

With the aim of confirming salivary NGF peptide data, obtained by ELISA, we performed a semiquantitative RT-PCR examination of NGF mRNA in whole saliva of BMS and control subjects (Figure 2a). The box plot (Figure 2b) shows the integrated optical density of NGF RT-PCR bands normalized to β-actin (ratio NGF/actin). The results are representative of three independent RT-PCR experiments. No significant differences in the expression levels of NGF mRNA were found between BMS and control subjects ($P = 0.780$).

Discussion

The examined group of BMS patients consisted of 20 subjects. Eighty-five percent of them were women and majority of patients were seniors. The demographical features detected in our patients' group are in accordance with results of other authors who estimated that BMS is most frequently reported by post-menopausal women (Scala *et al*, 2003).

Patients with BMS frequently have other complaints, and amongst these symptoms, xerostomia and loss or altered sense of taste are most frequently mentioned (Scala *et al*, 2003). A majority of our BMS subjects complained of subjective feeling of dry mouth (65%) and more than one-third of them reported abnormalities in taste perception. All our patients had burning sensation of the tongue, and 80% of them complained that these sensations extended to other mouth sites or the whole oral cavity. Their mean salivary flow rate and salivary protein concentration were not different when compared with those of healthy subjects as previously reported (Hershkovich and Nagler, 2004; Granot and Nagler, 2005; de Moura *et al*, 2007).

BMS has been included among the chronic orofacial pain disorders (Woda and Pinchon, 2000; Scala *et al*, 2003) or the 'dynias' (Wesselmann and Reich, 1996) and the symptoms are the result of an undetermined neuropathic process (Scala *et al*, 2003). Loss of nerve fibers has been reported in the lingual mucosa affected by BMS suggesting that in some patients BMS may represent a peripheral sensory small-fibers neuropathy (Lauria *et al*, 2005). Recently Yilmaz *et al* demonstrated

Table 2 Salivary analysis in BMS and control subjects in two evaluation. Salivary flow rate, protein concentration, NGF content, neutrophils markers (MPO and calprotectin) and mast cells marker (tryptase) in the saliva of patients with BMS and healthy individuals

	First evaluation		*Second evaluation*	
Parameter	*BMS*	*Controls*	*BMS*	*Controls*
Flow rate (ml min^{-1})	0.56 ± 0.17	0.58 ± 0.07	0.50 ± 0.12	0.63 ± 0.08
Protein (mg ml^{-1})	1.77 ± 0.12	1.74 ± 0.14	1.95 ± 0.13	1.97 ± 0.15
NGF (pg ml^{-1})	761.3 ± 199.4	353.3 ± 80.3	688.8 ± 185.1	318.0 ± 82.8
MPO (μg ml^{-1})	2.90 ± 0.80	2.66 ± 0.28	4.16 ± 0.86	2.94 ± 0.46
Calprotectin (μg ml^{-1})	1.02 ± 0.09	1.11 ± 0.04	1.01 ± 0.14	0.91 ± 0.20
Tryptase (μg ml^{-1})	0.212 ± 0.049	0.078 ± 0.014	0.154 ± 0.025	0.074 ± 0.015

Values are the mean ± s.e.m. obtained from 10 subjects of each group in two evaluation spaced out 6 months.

	First vs second evaluation		*BMS vs* controls	
Parameter	*BMS*	*Controls*	*First evaluation*	*Second evaluation*
Flow rate (ml min^{-1})				
Means difference	NS	NS	NS	NS
	$P = 0.2971$	$P = 0.3916$	$P = 0.9231$	$P = 0.3675$
Effective pairing	S	S		
	$P < 0.0001$	$P = 0.0064$		
	$r = 0.9760$	$r = 0.7820$		
Protein (mg ml^{-1})				
Means difference	NS	NS	NS	NS
	$P = 0.0614$	$P = 0.0621$	$P = 0.8837$	$P = 0.9157$
Effective pairing	S	S		
	$P = 0.0096$	$P = 0.0329$		
	$r = 0.7919$	$r = 0.6355$		
NGF (pg ml^{-1})				
Means difference	NS	NS	S	S
	$P = 0.6388$	$P = 0.5135$	$P = 0.0392$	$P = 0.0444$
Effective pairing	S	S		
	$P = 0.0250$	$P = 0.0082$		
	$r = 0.7067$	$r = 0.8032$		
MPO (μg ml^{-1})				
Means difference	NS	NS	NS	NS
	$P = 0.5701$	$P = 0.4089$	$P = 0.7588$	$P = 0.2085$
Effective pairing	S	S		
	$P = 0.0448$	$P = 0.0129$		
	$r = 0.6081$	$r = 0.9222$		
Calprotectin (μg ml^{-1})				
Means difference	NS	NS	NS	NS
	$P = 0.1250$	$P = 0.2548$	$P = 0.3677$	$P = 0.6702$
Effective pairing	S	S		
	$P = 0.0417$	$P = 0.0083$		
	$r = 0.9487$	$r = 0.8929$		
Tryptase (μg ml^{-1})				
Means difference	NS	NS	S	S
	$P = 0.1016$	$P = 0.8281$	$P = 0.0070$	$P = 0.0072$
Effective pairing	S	S		
	$P = 0.0056$	$P = 0.0033$		
	$r = 0.8682$	$r = 0.8571$		

Table 3 Statistical analysis of data reported in Table 2. Results from statistical analysis of data reported in Table 2. The results are expressed as mean ± s.e.m. Statistical analysis was performed using the Student's *t*-test. The effectiveness of pairing between the first and the second evaluation in the same group (First *vs* Second evaluation column) was assessed by calculating the Pearson correlation coefficient, *r*, and the corresponding *P* value. Paired *t*-test was used to compare data between the two evaluation in the same group and *P* values are reported as far as the significance about the means is concerned. Unpaired *t*-test was used to compare data between the two groups (BMS *vs* Controls column) and *P* values are reported. The level of significance for analysis was set at 5% ($P < 0.05$). S = significant, NS = not significant

Table 4 Salivary parameters in BMS subjects compared according to different demographical features. Salivary NGF, tryptase and SP levels in BMS subjects compared according to different demographic features

Demographic feature	*NGF levels (pg ml^{-1})*	*Tryptase levels (μg ml^{-1})*	*SP* levels (pg ml^{-1})*
Gender: female *vs* males	733.7 ± 80.70 $N = 17$ *vs*	0.183 ± 0.028 $N = 17$ *vs*	39.38 ± 5.43 $N = 13$ *vs*
	494.4 ± 115.3 $N = 3$	0.177 ± 0.020 $N = 3$	28.00 ± 7.50 $N = 3$
	$P = 0.2487$ NS	$P = 0.7504$ NS	$P = 0.3600$ NS
Age: younger than 60years *vs* older than 60 years	459.3 ± 147.6 N = 4 *vs*	0.203 ± 0.048 $N = 4$ *vs*	23.40 ± 7.26 $N = 3$ *vs*
	757.5 ± 77.77 N = 16	0.177 ± 0.028 $N = 16$	40.44 ± 5.20 $N = 13$
	$P = 0.1008$ NS	$P = 0.3940$ NS	$P = 0.1618$ NS

Values are the mean ± s.e.m. obtained from 20 subjects of the study group. The results present mean ± s.e.m., and statistical significance determined by the Student's unpaired *t*-test. The level of significance for analysis was set at 5% ($P < 0.05$). NS = not significant. *SP values are obtained from 16 subjects of each group.

an over-expression of NGF by the surviving fibers of nerve terminal and in the basal epidermal cells (Yilmaz *et al*, 2007). Although the cause of decrease in intra-epithelial fibers remains unknown, these authors hypothesized that this partial denervation may induce a compensative production of NGF by non-nerve associated cells, such as the basal epidermal layer, as shown in rodent skin (Mearow *et al*, 1993), which in turn may lead to a persistent hypersensitivity (Yilmaz *et al*, 2007).

In addition to affect neuronal sensitization, NGF can directly interact with some immune cell types: MC and neutrophils.

Mast cells degranulate at the site of nerve lesion (Olsson, 1967; Zuo *et al*, 2003) and can discharge their secretory granule content, including histamine, proteinases (Metcalfe *et al*, 1997; Galli *et al*, 2005) and NGF itself (Leon *et al*, 1994), in response to neuropeptides stimulation (Horigome *et al*, 1993; Gentner *et al*, 1996; Kulka *et al*, 2008).

Table 5 Salivary parameters in BMS subjects compared according to different BMS features. Salivary NGF, tryptase and SP levels in BMS subjects compared according different BMS features

BMS feature	*NGF levels (pg ml^{-1})*	*Tryptase levels (μg ml^{-1})*	*SP* levels (pg ml^{-1})*
Subjective feeling of dry mouth: reported *vs* not reported	804.0 ± 215.7 $N = 13$ *vs* 741.7 ± 169.8 $N = 7$ $P = 0.7513$ NS	0.204 ± 0.035 N = 13 *vs* 0.142 ± 0.016 $N = 7$ $P = 0.2294$ NS	36.88 ± 6.03 $N = 10$ *vs* 37.85 ± 8.10 $N = 6$ $P = 0.9241$ NS
Taste disturbances: reported *vs* not reported	452.7 ± 89.52 $N = 7$ *vs* 959.6 ± 211.6 $N = 13$ $P = 0.0476$ S	0.1414 ± 0.024 N = 7 vs 0.204 ± 0.033 N = 13 P = 0.3407 NS	34.24 ± 5.39 N = 7 vs 9.58 ± 7.37 N = 9 P = 0.5892 NS
Distribution of BMS: tongue *vs* extension to other mouth sites	466.2 ± 150.4 $N = 4$ *vs* 861.1 ± 179.3 $N = 16$ $P = 0.3030$ NS	0.117 ± 0.019 $N = 4$ *vs* 0.199 ± 0.028 $N = 16$ $P = 0.1845$ NS	41.07 ± 16.92 $N = 3$ *vs* 36.36 ± 4.72 $N = 13$ $P = 0.7086$ NS

Values are the mean ± s.e.m. obtained from 20 subjects of the study group each group. The results present mean ± s.e.m., and statistical significance determined by the Student's unpaired *t*-test. The level of significance for analysis was set at 5% ($P < 0.05$). S = significant; NS = not significant. *SP values are obtained from 16 subjects of each group.

Table 6 Salivary parameters in BMS subjects compared according to pain scores and duration of the disorder. Salivary NGF, SP and tryptase levels relationship with pain scores (VAS) and duration of the disorder. Spearman's Rho correlation and *P* are reported. Probability value of $P < 0.05$ was accepted as statistically significant

BMS feature	*NGF levels (pg ml^{-1})*	*Tryptase levels (μg ml^{-1})*	*SP* levels (pg ml^{-1})*
Pain (VAS)	$r = -0.04538$ P value = 0.8493	$r = 0.1720$ P value = 0.4684	$r = 0.04802$ P value = 0.8598
Duration of the disorder (months)	$r = -0.02762$ P value = 0.9080	$r = 0.09779$ P value = 0.8609	$r = 0.1308$ P value = 0.6293

*SP values are obtained from 16 subjects of each group.

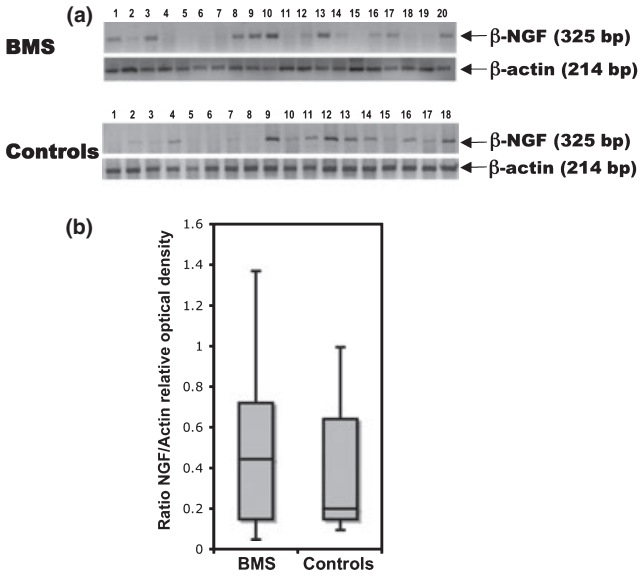

Figure 2 Semiquantitative RT-PCR analysis to assess the NGF mRNA levels in saliva samples. The expression of NGF in saliva samples from BMS patients and control subjects was analyzed by RT-PCR. Amplified PCR products obtained with mRNA after reverse transcription were size-fractionated on a 2.0% agarose gel (**a**). Upper gel, BMS patients. Lower gel Control subjects. The box plot (**b**) shows the integrated optical density of NGF RT-PCR bands normalized to ß-actin (ratio NGF/actin). The results are representative of three independent RT-PCR experiments. No significant differences in the expression levels of NGF mRNA were found between BMS patients and control subjects ($P = 0.780$)

NGF may also produce peripheral sensitization via activation of the 5-lipoxgyenase pathway, which can lead to local neutrophil accumulation (Amann *et al*, 1996; Bennett *et al*, 1998). Depletion of neutrophils in animals prevents NGF-induced thermal hyperalgesia, indicating that neutrophil accumulation may be critical for sensitizing to the NGF actions (Bennett *et al*, 1998).

Neutrophils are an essential part of the innate immune system and can contribute to inflammatory hyperalgesia (Levine *et al*, 1985; White *et al*, 1990; Bennett *et al*, 1998; Witko-Sarsat *et al*, 2000). While neutrophils are not found in normal nerves, a significant numbers of them are recruited at the site of peripheral nerve injury (Perry *et al*, 1987; Clatworthy *et al*, 1995; Perkins and Tracey, 2000; Zuo *et al*, 2003).

Finally, it is worth noting that neutrophils may also have an anti-nociceptive role. Calprotectin (MRP8-14) is a Ca^{2+}-binding heterodimeric protein that forms a significant portion of the cytoplasmic and granular proteins in neutrophils (Hessian *et al*, 1993) and can suppress inflammatory pain in mice (Giorgi *et al*, 1998).

Neurogenic components have been implicated in oral inflammatory diseases (Scardina *et al*, 2007a). It also has been hypothesized that inflammation, which is not clinically apparent, might lead to burning symptoms which would then result in altered cytokine profile (Boras *et al*, 2006; Simcić *et al*, 2006; Pekiner *et al*, 2009).

Measurement of neuropeptides (NGF, SP and CGRP) in human saliva could provide a valuable tool for the study of patients with chronic painful disorders (tension headache, cluster headache, migraine, chronic back pain) (Fischer *et al*, 1998). Despite of this, only one study has been carried out for evaluating the salivary level of mediators involved in peripheral neuropathic pain (CGRP) (Zidverc-Trajkovic *et al*, 2009), and up to now, neither the evaluation of the salivary level of

neuropeptides involved in neurogenic inflammation (SP and NGF) nor the functional involvement of inflammatory cells (MC and neutrophils) has been addressed.

In this study a persistent significantly higher salivary concentration of NGF in BMS subjects was recorded. We cannot exclude that the increased amount of NGF in BMS patients could derive from salivary glands, but we think this possibility very unlikely since salivary flow rate and protein concentrations were found unchanged. It is more likely that NGF mainly derives from the basal epidermal cells where its level appeared increased in BMS (Yilmaz *et al*, 2007).

Human oral keratinocytes express NGF mRNA (Hayashi *et al*, 2007) and 3000 species of mRNA are contained in saliva of healthy subjects (Li *et al*, 2004) which could be exploited as biomarkers for oral cancer (St John *et al*, 2004). With this concepts in mind, we examined NGF also at the mRNA level in saliva of both BMS and control subjects, but were unable to find any difference. Salivary RNA appears to enter the oral cavity through many different routes, including circulation, and show a differential degree of stability (Park *et al*, 2006). We think it possible that these features of salivary mRNA could explain our failure to correlate the increased amount of salivary NGF with an increased expression of its coding gene.

Our findings report absolute values for NGF concentrations which are lower than those reported previously (Ruhl *et al*, 2004; Nam *et al*, 2007). The reason for this discrepancy is unknown, but differences in the subject age, collection time and procedure of evaluation might explain it.

In this study we evaluated also the salivary level of another neuropeptide, SP, present in considerable quantities in nerves within the oral cavity (Wakisaka, 1990) and closely related to CGRP (Tamatani *et al*, 1989), the only neuropeptide evaluated in saliva of BMS subjects up to now.

In certain painful conditions, particularly tension headache, significantly higher concentrations of salivary SP have been found (Marukawa *et al*, 1996).

As far as salivary levels of SP are concerned, a significantly lower concentration in patients with BMS was recorded. Since SP and CGRP often co-exist in nerve fibers (Gibson *et al*, 1984; Lundberg *et al*, 1986) and frequently are released simultaneously (Payan *et al*, 1984; Saria *et al*, 1986), our findings are in agreement with the data reported in BMS subjects by Zidverc-Trajkovic *et al* (2009).

The spatial association of nerves and mast cells facilitates the effects of neuropeptides that are secretagogues for mast cells (Kulka *et al*, 2008). Accordingly the levels of tryptase, a specific granule stored trypsin-like serine proteinase, released upon degranulation, which serves as a useful indicator of local mast cells activity, was analyzed. A significantly persistent higher concentration of tryptase in saliva of patients was observed, indicating a mast cell involvement in BMS. We suggest that the activation of MC degranulation could be induced by NGF, and that MC could be a further source of this neurotrophin. Finally tryptase could be involved also in the initiation of neuropathic pain since this enzyme can activates the protease-activated receptor-2 (PAR-2) of primary sensory neurons, which can trigger inflammatory hyperalgesia and nociceptive behavior in rats (Kawabata *et al*, 2001; Vergnolle *et al*, 2001).

Since NGF and NGF-dependent mast cell activation can trigger the recruitment of neutrophils, the salivary levels of two neutrophil markers, MPO and calprotectin (MRP8-14), were evaluated. We did not find any change in both MPO and calprotectin salivary concentration in BMS patients with respect to those of healthy controls. Altogether, these data seem to exclude a consistent, if any, neutrophil involvement in BMS.

We analyzed also the possible relationship between demographic elements and clinical BMS features with the salivary parameters. Gender, distribution of burning sensations, duration of the disorder, pain and subjective feelings of dry mouth did not show any significant correlation with the NGF, SP and tryptase levels of our BMS subjects. Subjects with taste disturbances had significantly lower levels of salivary NGF. There have been several studies regarding the modulation of gustatory responses by neuropeptides (Simon *et al*, 2003) and in BMS subjects dysfunction of *chorda tympani,* has been reported (Eliav *et al*, 2007). NGF could be involved in this pathway since it is expressed by the *chorda tympany* fungiform papilla associated neurons (Farbman *et al*, 2004) and has shown to enhance functional recovery, after injury to the *chorda tympani* and lingual nerves (Smith *et al*, 2004).

In conclusion the data reported in this paper support the neurogenic origin of BMS which is strongly suggested by the altered neuropeptide salivary profile observed in BMS subjects. We cannot exclude a role of neuropeptides in other chronic or neuropathic pain conditions. However as far as salivary SP levels in BMS are concerned we observed a decrement of this neuropeptide, while in other painful conditions (migraine, tension headache) a significant increment is reported (Marukawa *et al*, 1996), indicating that this neuropeptide could discriminate between BMS and other chronic painful conditions. We put forward the hypothesis that considering a more complete neuropeptide panel, together with MC activation markers, should be considered useful for early diagnosis and monitoring of BMS.

Acknowledgements

We would like to thank Dr Fulvio Marcori from Eurospital, Trieste (Italy) and Dr Erika Russi (Department of Biomedicine, Unit of Dental Sciences and Biomaterials, University of Trieste, Trieste, Italy) for their technical assistance.

References

Amann R, Schuligoi R, Lanz I, Peskar BA (1996). Effect of a 5-lipoxygenase inhibitor on nerve growth factor-induced thermal hyperalgesia in the rat. *Eur J Pharmacol* **306:** 89–91.

Bennett G, Al-Rashed S, Hoult JR, Brain SD (1998). Nerve growth factor induced hyperalgesia in the rat hind paw is dependent on circulating neutrophils. *Pain* **77:** 315–322.

Boras VV, Brailo V, Lukac J, Kordić D, Blazić-Potocki Z (2006). Salivary interleukin-6 and tumor necrosis factor-alpha in patients with burning mouth syndrome. *Oral Dis* **12:** 353–355.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–54.

Cerchiari DP, de Moricz RD, Sanjar FA *et al* (2006). Burning mouth syndrome: etiology. *Braz J Otorhinolaryngol* **72:** 419–423.

Clatworthy AL, Illich PA, Castro GA, Walters ET (1995). Role of peri-axonal inflammation in the development of thermal hyperalgesia and guarding behavior in a rat model of neuropathic pain. *Neurosci Lett* **184:** 5–8.

Eliav E, Kamran B, Schaham R *et al* (2007). Evidence of chorda tympani dysfunction in patients with burning mouth syndrome. *J Am Dent Assoc* **138:** 628–633.

Farbman AI, Guagliardo N, Sollars SI, Hill DL (2004). Each sensory nerve arising from the geniculate ganglion expresses a unique fingerprint of neurotrophin and neurotrophin receptor genes. *J Neurosci Res* **78:** 659–667.

Fischer HP, Eich W, Russell IJ (1998). A possible role for saliva as a diagnostic fluid in patients with chronic pain. *Semin Arthritis Rheum* **27:** 348–359.

Formaker BK, Frank ME (2000). Taste function in patients with oral burning. *Chem Senses* **25:** 575–581.

Forssell H, Jääskeläinen S, Tenovuo O, Hinkka S (2002). Sensory dysfunction in burning mouth syndrome. *Pain* **99:** 41–47.

Galli SJ, Nakae S, Tsai M (2005). Mast cells in the development of adaptive immune responses. *Nat Immunol* **6:** 135–142.

Gao S, Wang Y, Wang Z (2000). Assessment of trigeminal somatosensory evoked potentials in burning mouth syndrome. *Chin J Dent Res* **3:** 40–46.

Gentner MR, Savage NW, Walsh LJ (1996). Modulation of dental pulp adhesion molecule expression in vitro. *Aust Endodont Newsl* **22:** 32–34.

Gibson SJ, Polak JM, Bloom SR *et al* (1984). Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. *J Neurosci* **4:** 3101–3111.

Giorgi R, Pagano RL, Dias MA *et al* (1998). Antinociceptive effect of the calciumbinding protein MRP-14 and the role played by neutrophils on the control of inflammatory pain. *J Leukocyte Biol* **64:** 214–220.

Granot M, Nagler RM (2005). Association between regional idiopathic neuropathy and salivary involvement as the possible mechanism for oral sensory complaints. *J Pain* **6:** 581–587.

Grushka M, Epstein JB, Gorsky M (2002). Burning mouth syndrome. *Am Fam Physician* **65:** 615–620.

Hayashi K, Storesund T, Schreurs O *et al* (2007). Nerve growth factor beta/pro-nerve growth factor and their receptors in normal human oral mucosa. *Eur J Oral Sci Oct* **115:** 44–354.

Heckmann SM, Heckmann JG, HiIz MJ *et al* (2001). Oral-mucosal blood flow in patients with burning mouth syndrome. *Pain* **90:** 281–286.

Hershkovich O, Nagler RM (2004). Biochemical analysis of saliva and taste acuity evaluation in patients with burning mouth syndrome, xerostomia and/or gustatory disturbances. *Arch Oral Biol* **49:** 515–522.

Hessian PA, Edgeworth J, Hogg N (1993). MRP-8 and MRP-14, two abundant Ca^{2+}-binding proteins of neutrophils and monocytes. *J Leukoc Biol* **53:** 197–204.

Horigome K, Pryor JC, Bullock ED, Johnson EM (1993). Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. *J Biol Chem* **268:** 14881–14887.

Indo Y, Tsuruta M, Hayashida Y *et al* (1996). Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. *Nat Genet* **13:** 485–488.

Jääskeläinen SK, Forssell H, Tenovuo O (1997). Abnormalities of the blink reflex in burning mouth syndrome. *Pain* **3:** 455–460.

Karhuvaara L, Tenovuo J, Sievers G (1990). Crevicular fluid myeloperoxidase-an indicator of acute gingival inflammation. *Proc Finn Dent Soc* **86:** 3–8.

Kawabata A, Kawao N, Kuroda R *et al* (2001). Peripheral PAR-2 triggers thermal hyperalgesia and nociceptive responses in rats. *NeuroReport* **12:** 715–719.

Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP (2008). Neuropeptides activate human mast cell degranulation and chemokine production. *Immunology* **123:** 398–410.

Lauria G, Majorana A, Borgna M *et al* (2005). Trigeminal small-fiber sensory neuropathy causes burning mouth syndrome. *Pain* **115:** 332–337.

Leon A, Buriani A, Dal Toso R *et al* (1994). Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci U S A* **91:** 3739–3743.

Levine JD, Gooding J, Donatoni P, Borden L, Goetzl EJ (1985). The role of the polymorphonuclear leukocyte in hyperalgesia. *J Neurosci* **5:** 3025–3029.

Levine JD, Goetzl EJ, Basbaum AI (1987). Contribution of the nervous system to the pathophysiology of rheumatoid arthritis and other polyarthritides. *Rheum Dis Clin North Am* **13:** 369–383.

Li Y, Zhou X, St John MA, Wong DT (2004). RNA profiling of cell-free saliva using microarray technology. *J Dent Res* **83:** 199–203.

Lundberg JM, Franco-Cereceda A, Hua X, Hokfelt T, Fischer MG (1986). Coexistence of substance P and calcitonin gene-related peptide in sensory nerves in relation to cardiovascular and bronchoconstriction effects of capsaicin. *Eur J Pharmacol* **108:** 315–319.

Maina G, Albert U, Gandolfo S, Vitalucci A, Bogetto F (2005). Personality disorders in patients with burning mouth syndrome. *J Pers Disord* **19:** 84–93.

Malmberg AB, Yaksh TL (1992). Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. *Science* **257:** 1276–1279.

Maltsman-Tseikhin A, Moricca P, Niv D (2007). Burning mouth syndrome: will better understanding yield better management? *Pain Pract* **7:** 151–162.

Marukawa H, Shimomura T, Takahashi K (1996). Salivary substance P, 5-hydroxytryptamine, and gamma-aminobutyric acid levels in migraine and tension-type headache. *Headache* **36:** 100–104.

Mearow KM, Kril Y, Diamond J (1993). Increased NGF mRNA expression in denervated rat skin. *Neuroreport* **4:** 351–354.

Menegazzi R, Zabucchi G, Knowles A, Cramer R, Patriarca P (1992). A new, one-step assay on whole cell suspensions for peroxidase secretion by human neutrophils and eosinophils. *J Leukoc Biol* **52:** 619–624.

Metcalfe DD, Baram D, Mekori YA (1997). Mast cells. *Physiol Rev* **77:** 1033–1079.

de Moura SA, de Sousa JM, Lima DF *et al* (2007). Burning mouth syndrome (BMS): sialometric and sialochemical analysis and salivary protein profile. *Gerodontology* **24:** 173–176.

Nam JW, Chung JW, Kho HS, Chung SC, Kim YK (2007). Nerve growth factor concentration in human saliva. *Oral Dis* **13:** 187–192.

Navazesh M (1993). Methods for collecting saliva. *Ann N Y Acad Sci* **694:** 72–77.

Olsson Y (1967). Degranulation of mast cells in peripheral nerve injuries. *Acta Neurol Scand* **43:** 365–374.

Park NJ, Li Y, Yu T, Brinkman BM, Wong DT (2006). Characterization of RNA in saliva. *Clin Chem* **52:** 988–994.

Payan DG, Levine JD, Goetzl J (1984). Modulation of immunity and hypersensitivity by sensory neuropeptides. *J Immunol* **132:** 1601–1605.

Pekiner FN, Gümrü B, Demirel GY, Ozbayrak S (2009). Burning mouth syndrome and saliva: detection of salivary trace elements and cytokines. *J Oral Pathol Med* **38:** 269–275.

Perkins NM, Tracey DJ (2000). Hyperalgesia due to nerve injury: role of neutrophils. *Neuroscience* **101:** 745–757.

Perry VH, Brown MC, Gordon S (1987). The macrophage response to central and peripheral nerve injury. A possible role for macrophages in regeneration. *J Exp Med* **165:** 1218–1223.

Rothwell NJ, Hopkins SJ (1995). Cytokines and the nervous system II: actions and mechanisms of action. *Trends Neurosci* **18:** 130–136.

Ruhl S, Hamberger S, Betz R *et al* (2004). Salivary proteins and cytokines in drug-induced gingival overgrowth. *J Dent Res* **83:** 322–326.

Sakamoto W, Fujii Y, Kanehira T, Asano K, Izumi H (2008). A novel assay system for myeloperoxidase activity in whole saliva. *Clin Biochem* **41:** 584–590.

Sardella A, Carrassi A. (2001). BMS: S for syndrome or S for symptom? A reappraisal of the burning mouth syndrome. *Minerva Stomatol* **50:** 241–246.

Saria A, Gamse R, Petermann J *et al* (1986). Simultaneous release of several tachykinins and calcitonin gene-related peptide from rat spinal cord slices. *Neurosci Lett* **63:** 310–314.

Scala A, Checchi L, Montevecchi M, Marini I, Giamberardino MA. (2003). Update on burning mouth syndrome: overview and patient management. *Crit Rev Oral Biol Med* **14:** 275–291.

Scardina GA, Ruggeri A, Messina P. (2007a). Neurologic inflammation and oral disease. An aetiopathogenic hypothesis. *Recenti Prog Med* **98:** 653–655.

Scardina GA, Pisano T, Messina P. (2007b). Burning mouth syndrome. *Recenti Prog Med* **98:** 120–128.

Sharma HS, Nyberg F, Olsson Y, Dey PK (1990). Alteration of substance P after trauma to the spinal cord: an experimental study in the rat. *Neuroscience* **38:** 205–212.

Shu XQ, Mendell LM (1999). Neurotrophins and hyperalgesia. *Proc Natl Acad Sci U S A* **96:** 7693–7696.

Simcić D, Pezelj-Ribarić S, Grzić R, Horvat J, Brumini G, Muhvić-Urek M (2006). Detection of salivary interleukin 2 and interleukin 6 in patients with burning mouth syndrome. *Mediators Inflamm* **2006:** 1–4.

Simon SA, Liu L, Erickson RP (2003). Neuropeptides modulate rat chorda tympani responses. *Am J Physiol Regul Integr Comp Physiol* **284:** R1494–14505.

Smith KG, Yates JM, Robinson PP (2004). The effect of nerve growth factor on functional recovery after injury to the chorda tympani and lingual nerves. *Brain Res* **1020:** 62–72.

St John MA, Li Y, Zhou X *et al* (2004). Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* **130:** 929–935.

Svensson P, Bjerring P, Arendt-Nielsen L, Kaaber S (1993). Sensory and pain thresholds to orofacial argon laser stimulation in patients with chronic burning mouth syndrome. *C J Pain* **9:** 207–215.

Tamatani M, Senba E, Tohyama M (1989). Calcitonin gene-related peptide- and substance P-containing primary afferent fibers in the dorsal column of the rat. *Brain Res* **495:** 122–130.

Thacker MA, Clark AK, Marchand F, McMahon SB (2007). Pathophysiology of peripheral neuropathic pain: immune cells and molecules. *Anesth Analg* **105:** 838–847.

Thoenen H (1991). The changing scene of neurotrophic factors. *Trends Neurosci* **14:** 165–170.

Thomas EL, Jefferson MM, Joyner RE, Cook GS, King CC (1994). Leukocyte myeloperoxidase and salivary lactoperoxidase: identification and quantitation in human mixed saliva. *J Dent Res* **73:** 544–555.

Vergnolle N, Bunnett NW, Sharkey KA *et al* (2001). Proteinase activated receptor-2 and hyperalgesia: a novel pain pathway. *Nat Med* **7:** 821–826.

Wakisaka S (1990). Neuropeptides in the dental pulp: their distributions, origins and correlation. *J Endodont* **16:** 67–72.

Walsh LJ. (2003). Mast cells and oral inflammation. *Crit Rev Oral Biol Med* **14:** 188–198.

Wesselmann U, Reich S (1996). The dynias. *Semin Neurol* **16:** 63–74.

White DM, Basbaum AI, Goetzl EJ, Levine JD (1990). The 15-lipoxygenase product, 8R,15S-diHETE, stereospecifically sensitizes C-fibermechanoheat nociceptors in hairy skin of rat. *J Neurophysiol* **63:** 966–970.

Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L (2000). Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* **80:** 617–653.

Woda A, Pinchon P. (2000). A unified concept of idiopathic orofacial pain: pathophysiologic features. *J Orofac Pain* **14:** 196–212.

Yilmaz Z, Renton T, Yiangou Y *et al* (2007). Burning mouth syndrome as a trigeminal small fiber neuropathy: Increased heat and capsaicin receptor TRPV1 in nerve fibers correlates with pain score. *J Clin Neurosci* **14:** 864–871.

Zabucchi G, Soranzo MR, Menegazzi R *et al* (1988). Uptake of human eosinophil peroxidase and myeloperoxidase by cells involved in the inflammatory process. *J Histochem Cytochem* **37:** 499–508.

Zakrzewska JM (1995). The burning mouth syndrome remains an enigma. *Pain* **62:** 253–257.

Zakrzewska JM (2009). Facial pain: an update. *Curr Opin Support Palliat Care* **3:** 125–130.

Zidverc-Trajkovic J, Stanimirovic D, Obrenovic R *et al* (2009). Calcitonin gene-related peptide levels in saliva of patients with burning mouth syndrome. *J Oral Pathol Med* **38:** 29–33.

Zuo Y, Perkins NM, Tracey DJ, Geczy CL (2003). Inflammation and hyperalgesia induced by nerve injury in the rat: a key role of mast cells. *Pain* **105:** 467–479.