Salivary concentration of the antimicrobial peptide LL-37 in patients with oral lichen planus

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**Background**: The antimicrobial peptide LL-37 is a significant molecule of innate immunity and recent studies indicate that it plays an important role in maintaining oral health. Yet limited knowledge exists on its role in oral diseases and oral lichen planus (OLP) in particular.

**Objective**: The study aimed to examine: 1) the salivary concentration of LL-37 in patients with OLP and healthy subjects, 2) the relation between the type (reticular or erosive) and size of OLP lesions and LL-37 concentration, and 3) the effect of the therapeutic modalities on LL-37 levels.

**Design**: The salivary peptide concentration in samples from 20 patients and 30 healthy subjects at the same age range was determined by ELISA.

**Results**: Despite the wide variation in peptide concentration found in both groups, the healthy subjects exhibited significantly lower levels than patients. Patients with the erosive form had significantly higher peptide concentrations than patients with the reticular form. Systemic treatment with corticosteroids resulted in a significant decrease of the salivary peptide concentration, while other treatment modalities, such as administration of vitamins A and E or local application of corticosteroids had no effect. Improved clinical appearance of the lesions was followed by a decrease in the salivary LL-37 level.

**Conclusions**: Salivary concentration of LL-37 correlates to the manifestation of mucosa lesions in OLP patients, the highest levels being observed in the most severe cases. This increase in peptide levels may protect against lesion infection and promote a quick wound healing.

**Keywords**: antimicrobial peptide LL-37; oral lichen planus; saliva

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LL-37 is released at the inflamed sites mainly by migrated neutrophils (22, 24).

Besides being antimicrobial (20, 26, 27), a wide range of other biological activities has been attributed to LL-37. They include inhibition of biofilm formation, stimulation of angiogenesis, mast cell degranulation, tissue degeneration, and enhancement of keratinocyte migration and proliferation (28, 29). Moreover, LL-37 has contrasting effects on apoptosis in different cells such as neutrophils, epithelial cells, and keratinocytes (30, 31), and may contribute to adaptive immunity by mobilizing T cells (32). LL-37 can also up-regulate genes encoding chemokines and chemokine receptors without stimulating the proinflammatory cytokine TNF-α, thus contributing to the immune response by recruiting immune cells to the site of inflammation and by limiting the damage caused by bacterial products (33). Recent studies revealed that LL-37 is present in saliva of all healthy subjects, even from early childhood, although its concentration varies widely with women to exhibit higher peptide levels than men (34, 35). Furthermore, edentulism leads to a considerable decrease in salivary LL-37 concentration, which implies the gingival sulcus as an important source of the peptide in the oral cavity (35). Inheritable neutrophil defects leading to a lack of the LL-37 peptide have been associated to aggressive periodontitis (36, 37), while low levels of LL-37 have been associated with high caries activity (34).

In patients with oral diseases, such as OLP, the salivary concentration of HNP-1 (α-defensin 1) was significantly higher than in healthy subjects (38, 39). The expression of HBD2 (β-defensin 2) is also up-regulated in OLP (40) and a stronger signal for HBD3 (β-defensin 3) is detected by in situ hybridization in OLP than in normal oral epithelium. A recent study (41) has shown a positive correlation between salivary levels of LL-37 and the number of monthly oral ulcers in patients with Behçet’s disease, which may indicate that topical inflammation may result in an increase in salivary LL-37. If the latter suggestion is true, an increase in peptide concentration is to be expected in patients with OLP. Consequently, this study was conducted to examine this hypothesis. Furthermore, the study aimed to determine any correlation between: 1) the concentration of LL-37 and the OLP form (reticular or erosive), and 2) changes in the LL-37 level and the therapeutic approach and clinical outcome.

Materials and methods
The study was approved by the Ethics Committee of the Dental School of the Aristotle University of Thessaloniki, Greece. All the participants signed consent forms, in accordance with the Helsinki declaration, after they were informed about the purpose and the procedures of the study.

The study group consisted of 20 patients with OLP (mean age 61.8 ± 12). For comparative purposes, 30 healthy subjects in the same age range (mean age 57.5 ± 12.1) with no history of systemic diseases or inflammatory oral lesions were included. A detailed medical history of each participant was obtained and none of the subjects had received any medication either topical or systemic that could cause lichenoid reaction 3 months prior to the study. Moreover, none of the patients of OLP had received any topical, or systemic medication for treatment of OLP at any point, prior to saliva sampling. All patient subjects were selected from the Outpatient Clinic, Department of Oral Medicine in the Dental School of the Aristotle University of Thessaloniki. The OLP diagnosis was established by clinical and histological examinations, according to the World Health Organization’s clinicopathological criteria for OLP (42). Based on the clinical appearance of their lesions, OLP patients were categorized into the following groups: Group A included patients with the reticular form, group B patients with one small erosion (<5 mm), and group C patients with one erosion >5 mm or with more than one erosion.

Treatment of the OLP patients was according to the therapeutic protocols of the collaborating outpatient clinic. Depending on the kind of treatment (topical administration of antifungal agents and corticosteroids, per os administration of corticosteroids or vitamins A and E), the patients were divided into three groups (Table 1). Saliva samples were obtained at least three times from each patient, that is, before and during the treatment every seventh day, upon clinical examination. Oral photographs were taken on these occasions to enable evaluation of the lesions. Any improvement in the disease condition recorded was based on the clinical picture as judged by the examiner clinician (42) and on the patients’ subjective symptoms. The examining clinician had received no information about the nature of the study, thus was objective regarding the clinical examination and selection of treatment. Corticosteroids per os were administered in the following manner: for the first 7 days 10 mg

Table 1. Distribution of patients in groups according to the type of OLP lesions they exhibited at their first visit at the clinic and the treatment modality they received

| OLP group | Topical corticosteroids and antifungals | Vitamins A + E per os | Corticosteroids per os | Total |
|-----------|----------------------------------------|-----------------------|------------------------|-------|
| A         | 3                                      | 3                     | 0                      | 6     |
| B         | 3                                      | 2                     | 0                      | 5     |
| C         | 4                                      | 0                     | 5                      | 9     |
| Total     | 10                                     | 5                     | 5                      | 20    |
prednisolone every 12 h and for the following 7 days 5 mg every 12 h.

Unstimulated saliva was collected by spitting, all samples being taken at the same time, around 11 a.m., to avoid any possible diurnal variation (43). The samples were stored at \(-70^\circ\text{C}\) until examined. On the day of analysis, the samples were thawed and cleared by centrifugation at 10,000 \(x g\) for 5 min. The concentration of LL-37 in the supernatant was determined by an ELISA method, using a commercially available kit (HyCult Biotechnology, Uden, the Netherlands) and following the manufacturer’s instructions. Each sample was analyzed in duplicate and the mean value was calculated. If the individual absorbance values differed by more than 15% from the corresponding mean value, the sample was retested. If required, the saliva samples were diluted 5–10 times with distilled water. For calculation of the LL-37 concentration, the equation of regression analysis was used, obtained with standard peptide solutions (concentration range 0.1–100 ng/ml).

Data were summarized by means of descriptive statistical indices. The statistical comparison between different groups was performed with the Mann-Whitney test and the Wilcoxon test. Non-parametric statistical methods were preferred because the normality of the LL-37 concentration in saliva could not be assumed or confirmed. In all hypothesis-testing procedures, the observed significance level (\(p\)-value) was computed by the Monte-Carlo simulation method (44). This approach leads to valid inferences even in cases where the methodological presuppositions of the non-parametric tests are not satisfied and the number of patients is small. All analyses were performed with SPSS v15.0 statistical package enhanced with the module Exact Tests. The significance level of all statistical tests was predetermined at \(p < 0.05\).

Results
LL-37 was detected in all saliva samples and its concentration varied widely both in patients with OLP and in healthy subjects (Fig. 1). Compared to healthy subjects, patients with erosive OLP (groups B and C) had significantly \((p < 0.05)\) higher LL-37 concentrations, while patients with reticular OLP exhibited similar peptide concentrations (Fig. 1). The median LL-37 concentration for healthy subjects was 30.5 ng/ml. Corresponding levels for OLP groups A, B, and C were 34, 64, and 68.5 ng/ml, respectively.

The salivary concentration of LL-37 was significantly \((p < 0.001)\) decreased upon systemic treatment with corticosteroids (Fig. 2). The median value of concentration change was \(-34.5\) ng/ml after a 7-day treatment. None of the other treatment modalities showed any correlation with the changes in the salivary LL-37 level (Fig. 2). Reducing the dose of corticosteroids \(\text{per os}\) by half resulted in a significant increase in the concentration of LL-37

\[ \text{Fig. 1. Box plot of data on LL-37 concentration (in ng/ml saliva) in samples from healthy subjects and OLP patients with: (a) reticular form, (b) erosive form with small lesion, and (c) erosive form with large or multiple lesions. For each group the median values (bold line), the minimum and maximum values (bars) and the quartiles Q1 and Q3 (box) are shown.} \]

\[ \text{Fig. 2. Box plot of data on change in salivary LL-37 concentration in relation to treatment modality. The change is defined as the difference: LL-37 concentration after 7 days of treatment minus LL-37 concentration found immediately before treatment. For each group the median values (bold line), the minimum and maximum values (bars) and the quartiles Q1 and Q3 (box) are shown.} \]
at the first examination, 21 ng/ml (min–max 1.1–52.5 ng/ml) after 7 days of treatment, and 44 ng/ml (min–max 7.5–69 ng/ml) after 7 days of receiving half treatment dose.

Irrespective of the treatment modality, improvement of the lesions’ appearance, as judged by the clinical examiner, correlated with a decrease in salivary LL-37 level (Fig. 3). The median change was −20 ng/ml. The median changes for patients that were systemically treated with steroids or not were −34.5 and −2 ng/ml, respectively. The differences between these groups were statistically significant as indicated in Fig. 3.

Discussion
It is evident from the present findings that emergence of erosive OLP lesions leads to an increase in the salivary level of LL-37. The increase relates to the presence of erosive lesions, while improvement of the clinical status of the lesions coincides with changes toward a decreased peptide level. Thus, the results support the hypothesis stated above, that inflammatory conditions in the oral mucosa might raise the concentration of LL-37 in saliva.

The levels of the peptide recorded for healthy subjects and the wide intra-subject variation observed in all groups examined are similar with previous findings (35). There was no difference in peptide concentration between the patients with reticular OLP and healthy subjects, while the extent of the erosive lesions seems to influence the salivary LL-37 levels. LL-37 in saliva is derived from epithelial cells of the oral mucosa and gingival as well as from neutrophils that enter the oral cavity through the junctional epithelium and inflamed sites (13). Our results are in line with a previously reported relation between the LL-37 concentration in saliva and the number of monthly ulcers in patients with Behc¸et’s disease (41), which indicates a contribution of the ulcerated oral mucosa to the increase of the peptide levels. Taken together, these observations indicate that changes in the salivary LL-37 level will take place if the inflammatory disease creates ulcers in the oral mucosa.

Systemic administration of steroids dramatically reduced the LL-37 concentration in saliva. On the contrary, topical application of steroids had no effect. The immunosuppressant and anti-inflammatory properties of corticosteroids are well established (45, 46). A recent study (47) revealed that the systemic rather than the topical administration influences the mucosal tolerance. The authors concluded that the route of administration rather than the doses of corticosteroids administered affects the immune response, this finding being in agreement with our results. There is no data on the possible effect of steroid therapy on LL-37 or other antimicrobial peptides. Although it seems logical to assume that immunosuppressant drugs will reduce the production of antimicrobial peptides, the impact of such a therapy on innate immune response remains to be elucidated.

Based on the multifunctional nature of LL-37 that exhibits a variety of biological actions both as an antimicrobial component of broad spectrum and as a regulatory molecule that affects immunological response and tissue regeneration (29), it is tempting to suggest that the changes in the LL-37 concentration, found presently in OLP patients and in previous studies on ulcerating oral diseases, arise from the targeted action of the innate defense mechanisms in the local soft tissues to prevent the establishment of infection in the ulcers and to enable healing of the lesions. Further studies are needed to clarify the possible role of the peptide as an objective indicator of the severity of the inflammatory condition and of the treatment outcome and also in therapeutic applications.

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There is no conflict of interest in the present study for any of the authors.

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