Cathelicidin LL-37 levels in the gingival crevicular fluid and the saliva of smokers and non-smokers with stage III, IV periodontitis: An Observational study

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ABSTRACT: **Background** This cross-sectional study aimed to assess the levels of LL-37 in the gingival crevicular fluid (GCF) and saliva in non-smokers and smokers with stage III and IV periodontitis. **Materials and Methods:** 60 participants in a sample from Egyptian patients seeking dental treatment at the Diagnostic Center, Faculty of Dentistry, Cairo University. Periodontal examination involving Pocket depth, clinical attachment level, gingival recession depth, bleeding on probing and plaque index were measured using a manual UNC periodontal probe. GCF samples were collected from the deepest site by inserting absorbent paper strips into the gingival sulcus for 30 seconds. Five ml of unstimulated whole expectorated saliva samples were collected from all subjects participating in this study. GCF & salivary LL-37 levels were quantified by using a commercial Enzyme-Linked Immunosorbent Assay kit. **Results:** There was a statistically significant difference in LL-37 levels in GCF between smokers and non-smokers patients with periodontitis while there was no statistically significant difference in LL-37 levels in saliva between smokers and non-smokers patients with periodontitis. **Conclusion:** LL-37 levels in the GCF and saliva might be considered as a biomarker of advanced stages of periodontitis in smokers and non-smokers.

Keywords: Tobacco smoking; periodontitis; antimicrobial peptides; Cathelicidins; ll-37

DOI: 10.48047/ecb/2023.12.Si8.619

Introduction

Periodontitis is an inflammatory disease that disrupts oral homeostasis. It is established by the dental plaque biofilm and is influenced by genetic factors, environmental factors and systemic diseases **(Nishihara and Koseki, 2004, Socransky and Haffajee, 2005, Bartold and Van Dyke, 2019)**. It is characterized by progressive destruction of the tooth-supporting apparatus, which is demonstrated by clinical attachment loss (CAL), radiographic alveolar bone loss, periodontal pockets, and gingival bleeding **(Papapanou et al., 2018)**. It is well known that smoking is a risk factor for developing periodontitis **(Leite et al., 2018, Tonetti et al., 2018)**. Smokers have significantly higher levels of plaque index, pocket depth, and clinical attachment loss than non-smokers **(Javed et al., 2017)**. Smoking tobacco has a pro-inflammatory effect by inducing the release of certain cytokines and reactive oxygen species (ROS), which are involved in the destruction of periodontal tissues **(Katz et al., 2005)**.The longer the duration of smoking, the more the periodontal destruction **(Genco and Borgnakke, 2013)**.

Antimicrobial peptides (AMPs) are soluble molecules involved in the innate immunity of mucosal surfaces against pathogens that provide a balance between disease and health **(Nguyen et al., 2011)**. Moreover, they play an important role in initiating an immune response against the microbes of the gingival epithelium **(Gorr, 2012)**. AMPs are produced in the oral cavity by epithelial cells, neutrophils, and salivary gland secretions **(Tomasinsig and Zanetti, 2005, Mallapragada et al., 2017)**. They are found mostly in saliva and subset of it in GCF. Saliva, GCF and the innate immune system of the oral epithelia serve as the first line of defense against the invasion of harmful bacteria via AMPs **(Gorr, 2009, Gorr and Abdolhosseini, 2011)**. Dysregulation in the expression of AMPs might link in the microbial dysbiosis in the periodontal disease with some risk factors as tobacco smoking, diabetes mellitus, and obesity **(Li and Schmalz, 2018)**.

Cathelicidins, are a group of peptides released from neutrophil granules present only in humans in the form of LL-37. They play an important role in regulating the immunological response of oral tissues **(Karsiyaka Hendek et al., 2019).** They are antimicrobial for many pathogens, including both Gram-positive and Gram-negative bacteria, fungi, parasites **(Wessely-Szponder et al., 2010, JA Veldhuizen et al., 2017)**. Thus, by periodically estimating their relative GCF and salivary levels, this diverse spectrum of AMPs expressed in the oral cavity can be effectively utilized as potential biomarkers for understanding periodontal disease activity **(Mallapragada et al., 2017)**.

A previous study by **Türkoğlu et al., (2016)** reported that smoking might have a suppressive effect on the gingival crevicular fluid (GCF) LL-37 levels in chronic periodontitis patients. Furthermore, they suggested that LL-37 levels in GCF of smokers with chronic periodontitis are lower than those in non-smokers with chronic periodontitis **(Takeuchi et al., 2012)**. Another study by **Ertugrul et al., (2014)** found a higher LL-37 level in GCF in smoker periodontitis patients than smokers and non-smokers with periodontitis. Moreover, **Soldati**

et al., (2020) suggested that smoking was associated with reduced levels of LL-37 in GCF of patients with periodontitis. Recently, **Lopes et al., (2021)** concluded that smoking did not influence the levels of LL-37 in the gingival crevicular fluid in periodontitis. Few studies in the current literature investigated the levels of LL-37 in smokers with periodontal disease. Accordingly, the aim of the current study was to assess the levels of LL-37 in the GCF and saliva of non-smokers and smokers with stage III and IV periodontitis.

MATERIALS AND METHODS

Study Design and Participants

The present observational cross-sectional study with the Identifier: ID: NCT04861493 at ClinicalTrials.gov, was accepted by the Ethics Committee for Scientific Research at Cairo University (April 2021) (Reference code: 9221). The purpose of the study was described to all subjects who signed a written consent and committed to participate in this work prior to filling out the questionnaire. The current study included 60 participants of nonsmokers and smokers with stage III and IV periodontitis of Egyptian patients seeking dental treatment at Cairo University's Diagnostic Center, Faculty of Dentistry. This study was conducted from February 2020 till April 2021 and screening of patients was continued until the target sample was achieved. Identifying and recruiting potential subjects was achieved through patients' database. The study included 3 groups, smokers with stage III, IV periodontitis, non-smokers with stage III, IV periodontitis and healthy subjects. Patients were diagnosed by proper history and periodontal clinical examination.

Inclusion criteria included subjects with stage III and IV periodontitis with presence of a minimum of 15 natural teeth, at least two non-adjacent teeth sites in each jaw having clinical attachment level (CAL) ≥ 5 mm and pocket depth (PD) > 5 mm in one or more sites, bleeding on probing (BOP) ≥ 30%, tooth loss due to periodontitis ≤ 4 teeth. For smoker's patients; study included patients who regularly smoked at least 10 cigarettes/day for a minimum of 5 years. For non-smokers patients, study included patients who had never smoked. Exclusion criteria were those individuals with a history of systemic diseases or immunological disorders, individuals using hormone replacement therapy, pregnant and lactating women, individuals that received periodontal treatment within the last 6 months, individuals with a history of systemic antibiotics and anti-inflammatory drugs within the last 3 months.

Clinical periodontal examination

Clinical examinations were performed by a trained examiner (AM) to reach a case identification and diagnose the periodontal condition. For case identification, to diagnose and define the stage of periodontitis, PD, clinical attachment level, gingival recession depth (RD), BoP and plaque index (PI) were measured. These parameters were measured using a manual UNC periodontal probe at the site of interproximal defect and were rounded up to the nearest millimeters. All permanent fully erupted teeth, excluding third molars, were evaluated at six different sites for each tooth; mesio-buccal, mid-buccal, disto-buccal, disto-lingual, mid-lingual, and mesio-lingual. Diagnosis and case identification of

the periodontal disease was performed based on the new classification of periodontal disease **(Papapanou et al., 2018).** BoP measured by gently probing the gingival crevice orifice. Starting in one interproximal area and moving to the other, the periodontal probe was inserted 1 to 2 mm into the gingival sulcus. A positive finding is recorded if bleeding begins within 10 seconds **(Ainamo and Bay, 1975)**. PI was examined by the presence of visible dental plaque and supragingival calculus was documented after drying the teeth in each quadrant with a blast of air **(Löe, 1967)**. Each of the four tooth surfaces (buccal, lingual, mesial, and distal) was assigned a score ranging from 0 to 3. The PI for the tooth was calculated by adding the scores from the four sections of the tooth and dividing them by four. PD and CAL measurements were recorded from the free gingival margin till the base of the pocket and the cemento-enamel junction (CEJ) till the base of the pocket, correspondingly **(Ramfjord, 1967).**

Saliva sampling

All subjects who took part in this investigation provided unstimulated entire expectorated saliva samples. To eliminate circadian rhythms, five ml of saliva were retrieved from each patient between 9:00 and 11:00 a.m. **(Navazesh, 1993)**. Saliva samples were obtained in the morning following an overnight fast, patient was instructed not to drink (except water) or chew gum. Subjects were rinse their mouth with tape water. The saliva sample was obtained by expectoration of patient into Eppendorf tube while seated in an upright position. All the Eppendorf tubes with saliva samples were immediately frozen & stored at (-70 ºc) until the time of LL-37

Sample collection:

GCF sampling: (Soldati et al., 2020)

GCF samples were obtained from the deepest site by a single examiner. Before sampling, the teeth were isolated with cotton rolls, supragingival plaque was eliminated using a sterile curette, and the surfaces were gently air-dried. GCF was collected by introducing absorbent paper strips into the gingival sulcus or periodontal pocket for 30 seconds. Paper strips contaminated with blood and saliva were thrown. Samples from all individuals were immediately pooled into a dry sterile polypropylene micro centrifuge tube (five paper strips per tube) and stored at -80°C until analysis. The electronic device measurements were converted to real volume (µL) using a standard curve. GCF LL-37 levels were determined using a commercial Enzyme-Linked Immunosorbent Assay kit provided from Bioassay Technology Laboratory (Shanghai, China) Cat. No E4039Hu.

assay. Salivary LL-37 levels were quantified by using a commercial Enzyme-Linked Immunosorbent Assay kit. provided from Bioassay Technology Laboratory (Shanghai, China) Cat. No E4039Hu.

Statistical and power analysis:

The primary outcome in this power analysis was cathelicidin LL-37 levels in the GCF of smokers and nonsmokers with stage III, IV periodontitis. Based on past research, the anticipated difference in cathelicidin LL-37 in the GCF is 0.91 **(Soldati et al., 2020)**. With a power of 80% and a significance level of 5%, 20 patients in each group were sufficient. The sample size was determined using the G-power tool. Data was analyzed using

MedCalc software, version 19 for windows (MedCalc Software Ltd, Ostend, Belgium). Data was explored for normality using Kolmogorov Smirnov test and Shapiro Wilk test. The mean and standard deviation were used to describe continuous data with a normal distribution. Intergroup comparison between continuous data was performed using one-way ANOVA followed by tukey post-hoc test. Non-parametric data were described as median and range and were compared using the Kruskal-Wallis test followed by Dunn post-hoc test. A value of 0.05 or less was considered statistically significant and all tests were two tailed.

Results

1. Results of Clinical Parameters:

Table 1 shows periodontal parameters for the groups. There was statistically significant difference in all periodontal parameters between healthy and smokers with periodontitis, healthy and non-smokers with periodontitis ($P < 0.000001$), while there was no statistically significant difference between smokers with periodontitis and non-smokers with periodontitis.

Table (1): Clinical periodontal parameters throughout the study period (Mean ± SD).

Group / Outcome	Healthy	Smokers + Periodontitis	Non-smokers + Periodontitis	P value
	Mean±SD	Mean±SD	Mean±SD	
Plaque index	$0.00^{a} \pm 0.00$	$2.85^{b} \pm 0.36$	$2.60^{b} \pm 0.50$	P< 0.000001*
Bleeding on probing	$0.00^{a} \pm 0.00$	$100.00^{b} \pm 0.00$	$96.47^{b} \pm 10.86$	P < 0.001*
Pocket depth	$1.19^{a} \pm 0.18$	$5.83^{b} \pm 0.57$	$6.09^{b} \pm 0.60$	P < 0.001*
Clinical attachment level	$0.00^{a} \pm 0.00$	$6.33^{b} \pm 0.78$	$6.11^{b} \pm 0.77$	P < 0.001*

Means that do not share a letter are significantly different, * corresponds to statistically significant difference.

2. Levels of Cathelicidin LL-37 in the GCF and saliva (ng/ml):

The levels of Cathelicidin LL-37 in the GCF and saliva throughout this study are showed in table (2). There was statistically significant statistically significant difference in Cathelicidin LL-37 levels in the GCF between healthy and smokers with periodontitis, healthy and non-smokers with periodontitis, and smokers with periodontitis and non-smokers with periodontitis ($P < 0.001$). Regarding the levels of Cathelicidin LL-37 in the saliva, there was statistically significant difference between healthy and smokers with periodontitis, healthy and non-smokers with periodontitis, ($P < 0.001$) while there was no statistically significant

difference between smokers with periodontitis and non-smokers with periodontitis

Table (2): Mean and standard deviation of LL-37 GCF and saliva (ng/ml) for all groups.

Group / Outcome	Healthy	Smokers + Periodontitis	Non-smokers + Periodontitis	P value
	Mean±SD	Mean±SD	Mean±SD	
LL-37 GCF (ng/ml)	177.25[a] ± 42.16	458.37[b] ± 155.57	280.37[c] ± 49.24	P < 0.001*
LL-37 saliva (ng/ml)	145.00[a] ± 52.54	257.25[b] ± 81.56	222.25[b]± 93.30	P < 0.001*

Means that do not share a letter are significantly different, * corresponds to statistically significant difference.

DISCUSSION

There are controversies in the current periodontal literature regarding the levels of LL-37 in the GCF and saliva of smokers with periodontitis. Accordingly, the current observational study aimed to evaluate levels of LL-37 in the GCF and saliva of non-smokers and smokers with periodontitis patients with varying degrees of the severity to have better understanding concerning of the effect of smoking on periodontal disease pathogenesis. To the best of the authors' knowledge, this is the first observational study investigating the levels of LL-37 in both GCF and saliva of non-smokers and smokers with stage III and IV periodontitis.

The results presented in the current study showed a statistically significant difference in PI scores and BOP between healthy individuals and smokers with periodontitis as well as healthy individuals compared to non-smokers with periodontitis. Yet, there was no statistically significant difference between smokers with periodontitis and non-smokers with periodontitis. These results were consistent with **Lopes et al., (2021)** who stated that there was no statistically significant difference between smokers and non-smokers in BOP and PI scores. However, **Türkoğlu et al., (2016)** found that smokers with periodontitis group have lower BOP and PI scores than non-smoker periodontitis group. Although smoking inhibits blood flow **(Henemyre et al., 2003)**, the current study found that the bleeding tendency was slightly higher among the smokers as compared to the non-smokers. This was supported by previous studies showing more BOP among smokers compared to the non-smokers. A possible explanation is that active smoking was linked to an increase in intra-oral temperature, which was thought to cause an increase in gingival blood flow followed by a gradual collapse after around 5 minutes **(Haber et al., 1993, Meekin et al., 2000, Mavropoulos et al., 2003)**. In addition,

deeper pockets display a higher percentage of BOP **(Radvar et al., 2011)**.

Regarding PPD and CAL there was a statistically significant difference between healthy individuals and smokers with periodontitis as well as healthy and non-smokers with periodontitis. However, there was no statistically significant difference between smokers with periodontitis and non-smokers with periodontitis. These findings are consistent with numerous studies which concluded that smokers have deeper pockets, more extensive and severe attachment loss, higher levels of bone destruction, and a higher rate of tooth loss **(Grossi et al., 1995, Baljoon et al., 2005, Johnson and Guthmiller, 2007, Radvar et al., 2011)**. Although a recent study by **Lopes et al., (2021)** stated that there was no statistically significant difference between smokers and non-smokers in PPD, yet a higher CAL was observed in smokers.

The main findings of this study were the significantly higher concentrations of LL-37 in the GCF of smokers with periodontitis compared to non-smokers with periodontitis and healthy patients. These findings were supported by the results of **Ertugrul et al., (2014)** who reported higher levels of LL-37 in the GCF of smoker patients with generalized aggressive periodontitis in comparison with non-smokers. This could be explained by the severity of periodontal disease as well as the frequency and duration of smoking habit. Smoking increases bacterial colonization by decreasing the bactericidal capacity of the periodontal tissues. As a result of increased microbial colonization, increased AMPs may be produced from periodontal tissue into the GCF as a form of protection against increased microbial invasion.

It is well-known that smoking causes neutrophil dysfunction which could result in a decrease in the AMPs produced by neutrophils **(Bagewadi and Keluskar, 2015)**. Nevertheless, tobacco use might increase LL-37 expression from the epithelial cells. AMPs are also increased during epithelial cell development, as well as in response to inflammatory stimuli and microbial infection. Furthermore, non-surgical periodontal therapy was reported to be beneficial in lowering LL-37 levels in non-smokers with chronic periodontitis but not in smokers with chronic periodontitis **(Nishida et al., 2008, Sanders et al., 2011)**.

A previous study demonstrated that GCF levels of cathelicidin LL-37 were significantly elevated in patients with chronic periodontitis compared to healthy controls **(Puklo et al., 2008)**. The authors hypothesized that LL-37 was produced from lysed neutrophils in healthy tissues, but most neutrophils moving into the gingival crevice from healthy periodontium were not activated. The reason for decreased GCF levels of cathelicidin LL-37 in periodontally healthy areas was attributed to a lack of active neutrophils in the gingival crevice while the increase in GCF levels of LL-37 in chronic periodontitis was suggested to be due to severe inflammation and periodontal destruction **(Türkoğlu et al., 2009)**.

Moreover, the concentrations of LL-37 in the gingival tissue, whether derived from neutrophils or from gingival epithelium, was associate positively with the depth of the gingival crevice REF. In addition, the LL-37 peptide was detected in saliva and GCF. The

LL-37 levels in GCF were significantly elevated in patients with chronic periodontitis compared with those in patients with gingivitis or those in healthy volunteers (**Krisanaprakonkit and Supanchart, 2010**). LL-37 production has been shown to be proportional to the extent and severity of periodontal damage during infection and inflammation **(Puklo et al., 2008, Türkoğlu et al., 2009, Turkoglu et al., 2017)**. As a result, by estimating their GCF and salivary levels, AMPs can be used as potential biomarkers to assess the course of periodontal disease (**Mallapragada et al., 2017**).

On the contrary to these present findings, a previous study reported lower concentrations of LL-37 in the GCF of smokers with chronic periodontitis than in non-smokers with chronic periodontitis. The authors also stated that smoking has no effect on GCF LL-37 levels in periodontal health **(Türkoğlu et al., 2016)**. This contradictory observation might be due to the differences between aggressive and chronic periodontitis. The current investigation studied the levels of LL-37 in periodontitis stages III and IV, which is characterized by more periodontal destruction. Likewise, **Soldati et al., (2020)** demonstrated that smoking was associated with lower GCF levels of LL-37 in periodontitis patients. The fact that LL-37 levels were lower in smokers suggests a compromised immune response, which might be linked to reduced resistance to the microorganisms. Another recent study by **Lopes et al., (2021)** concluded that smoking had no effect on the levels of LL-37 in the GCF in periodontitis. Also **Grant et al., (2019)** have discovered that there is no correlation between smoking and LL-37 levels in saliva in patients with periodontal disease.

One of the tenable explanations of the increased LL-37 levels in GCF and saliva in smokers with periodontitis in the current investigation is that nicotine causes the release of more AMPs from periodontal tissues once specific amounts are consumed. The consequent colonization of microorganisms by such elevated AMPs can thus be avoided, however this response may be advantageous in bacterial defense mechanisms (**Ertugrul et al., 2014**). In another explanation, previous researchers stated that AMPs increase with increasing periodontal pathological pocket formation **(Dale et al., 2001, Türkoğlu et al., 2009)**.

In agreement with the present findings, **Takeuchi et al., (2012)** reported that continued aggressiveness of the periodontopathic bacterial may result in inflammation, more periodontal tissue degradation, and consequently increased LL-37 production. Furthermore, LL-37's protective role is dependent on both a direct bactericidal impact and an immunomodulatory effect, which account for most of its activity in suppressing periodontitis. In addition, **Türkoğlu et al., (2009)** and **(2017)** suggested that periodontal diseases increase the production of LL-37 in a proportionate manner to the extent and severity of periodontal destruction.

One of the limitations of the present study was the cross-sectional design. Longitudinal studies involving larger populations are warranted to provide stronger evidence. Another limitation was that the patient's smoking state was determined by a

questioning which should be confirmed by cotinine level analysis for exact discrimination. Also, patients in non-smoker group might be passive smokers and thus the biochemical data might be affected by this inaccurate discrimination in the current study.

CONCLUSION

Within the limitations of this investigation, it might be concluded that the highest amounts of LL-37 were detected in the GCF of smokers with stage III and IV periodontitis patients while the lowest levels of LL-37 in the GCF and saliva were found in healthy group. AMPs, namely LL-37 might play a pathological role in the progression of periodontal disease in smokers. Since there are relatively few studies investigating the effect of smoking on LL-37 levels in body fluids in periodontitis patients, further research will aid in defining the interactions and mechanistic relationship between smoking and AMPs in the periodontal disease progression. Understanding how AMPs are controlled and function is crucial for creating innovative periodontal disease prevention, diagnostic, and therapeutic methods.

Acknowledgements

The authors declare that there is no conflict of interest and the study is self-funded.

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