Salivary Levels of Antimicrobial Peptides in Chronic Periodontitis Patients with Type 2 Diabetes

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Abstract:

Objective: The aim of this study was to evaluate the interrelationship between salivary levels of human neutrophil antimicrobial peptides (AMP) 1-3 (HNP 1-3), LL-37 and periodontitis in individuals with and without type 2 diabetes (T2DM).

Methods: Eighty individuals were enrolled and grouped as follows: Group I: 20 healthy individuals, Group II: 20 systemically healthy individuals with chronic periodontitis (CP), Group III: 20 individuals with T2DM only and Group IV: 20 individuals T2DM and CP. Plaque index, probing pocket depth, bleeding on probing and clinical attachment levels were evaluated. The diabetic status was established by assessing the levels of fasting plasma glucose and HbA1c. Salivary levels of HNP 1-3 and LL-37 were assessed by ELISA.

Results: The present study demonstrated that individuals with T2DM and CP had significantly higher salivary levels of LL-37 (443.00 ±221.52 ng/ml) and HNP 1-3 (149.52 ± 35.07 ng/ml) (p<0.001) compared to other groups. Additionally, salivary LL-37 and HNP 1-3 were significantly correlated with clinical and laboratory parameters (p<0.001). A significant positive correlation was seen between salivary levels of LL-37 and HNP 1-3 (r=0.69; p<0.001).

Conclusion: LL-37 concentrations were highest in patients with T2DM+CP when compared with controls. LL-37 was positively correlated with age. HNP 1-3 levels were increased in groups with DM when compared to the groups without periodontitis. The role of AMPs is vital in the immunoinflammatory response in the pathogenesis of periodontitis and DM.

Key words: diabetes, periodontitis, antimicrobial peptides, LL-37, HNP 1-3

Introduction

Periodontitis is a broadly perceived inflammatory disease. An important component of the inflammatory process in periodontitis is the presence of neutrophils (Mariano *et al.*, 2012). All antimicrobial peptides (AMPs) are extracted from larger precursors and comprise a signal sequence with post-translational modification that includes glycosylation, proteolysis, amino-acids isomerization, carboxy-terminal (C-terminal) amidation and halogenations (Khurshid *et al.*, 2015). The microbial membranes are negatively charged and are disrupted by the AMPs due to their cationic and amphiphatic structure (Girnita *et al.*, 2012).

AMPs are found in saliva, in the epithelium and in neutrophils (Gursoy *et al.*, 2013). To date, around 106 human host defense peptides have been identified. Cathelicidins and defensins form two important types of AMPs (Khurshid *et al.*, 2015). Only one cathelicidin, LL-37, is expressed in humans and this belongs to the family of α helical peptides. The precursor peptide human cationic antimicrobial peptide of 18 kDa (hCAP18) is digested by enzymes such as elastase or proteinase 3 which frees the C-terminal thereby forming a peptide. The name LL-37 is used because it contains 2 leucine residues and 37 amino-acid residues (Greer *et al.*, 2013).

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LL37/hCAP18 can stimulate monocytes, neutrophils, mast cells and T-cells. It neutralizes bacteria very quickly by forming ionic channels in the cell membranes of the microorganisms and by its ability to bind lipopolysaccharides (LPS) of bacterial membranes (Girnita *et al.*, 2012; Khurshid *et al.*, 2015; Mariano *et al.*, 2012). Defensins are rich in arginine, containing 29-35 amino acids (Moreno-Navarrette and Fernandes-Real, 2011). Human defensins are classified as alpha or beta. Alpha defensins comprise 6 different types of peptides, out of which human neutrophil peptides (HNP) -1, -2, -3, and -4 are mainly expressed in the azurophilic granules of neutrophils. The most abundant type of HNPs are HNP 1-3 which have highly similar attributes of their amino acid sequences making it arduous to individually analyze these peptides (Mariano *et al.*, 2012). Alpha defensins induce different chemokines for neutrophils, macrophages, monocytes, dendritic cells, T-lymphocytes and mast cells. Neutrophils containing LL-37 and HNPs migrate to sites of inflammation thereby constituting the innate host response (Greer *et al.*, 2013).

The incidence of diabetes mellitus is increasing worldwide and is reported to affect up to 346 million individuals worldwide. It affects general health, life span and healthcare maintenance. The World Health Organization (WHO) predicts an increase to 439 million by 2030 (World Health Organization, 2011). Inflammation is a critical component of diabetes which leads to beta-cell dysfunction in the pancreas and later apoptosis. This leads to insulin resistance and ultimately to diabetes (Chapple and Genco, 2013). Chronic periodontitis and diabetes often co-exist together and individuals with diabetes show a 3 - 4 times increased likelihood of developing periodontitis (Takeda *et al.*, 2006). Studies in Caucasian men have shown that plasma α-defensin (DEFA1–3) concentrations have a positive correlation with insulin sensitivity, vascular function and non-atherogenic lipid profile (Moreno-Navarrette and Fernandes-Real, 2011; Saraheimo *et al.*, 2008). An increased expression of LL-37 has also been recently reported in type 2 diabetes (Yilmaz *et al.*, 2015).

Saliva is an important component of the host defense against repeated microbial challenges and can provide a unique, non-invasive diagnostic fluid to determine the current disease status, to monitor patient response to therapy and to assess future disease progression. Traditionally, assessment of the probing pocket depth, clinical attachment level, oral hygiene status and bleeding on probing have been used for the diagnosis of periodontal disease but have proved inadequate. The identification of specific biomarkers from saliva through technological advancement has shown great promise to overcome this problem. In addition to providing a mechanical rinsing action, saliva carries significant amounts of AMPs which contribute to its defense mechanism. Salivary concentrations of antimicrobial defensins have also shown to alter with the exacerbation and remission of periodontal disease (Gursoy *et al.*, 2013; Sorsa *et al.*, 2016; Guentsch *et al.*, 2012).

Defensins and LL-37 are key components of the mucosal antimicrobial defense system. In addition to their antibacterial action, they may also contribute to wound healing in the periodontal tissues as they promote epithelial cell migration and differentiation (Greer *et al.*, 2013). AMPs also provide a chemical barrier which is mainly responsible for maintaining the oral health by fighting pathogenic bacteria (Dommisch *et al.*, 2015).

Both periodontitis and diabetes mellitus (DM) share common mechanisms of pathogenesis that are related to altered immune-inflammatory responses at local and/or systemic levels (Duarte *et al.*, 2012). However, despite the remarkable clinical evidence of the negative influence of DM on periodontal breakdown, to date there are scarce data concerning the cascade of immune-inflammatory mediators at areas of chronic periodontitis (CP) in subjects with Type 2 DM (T2DM). There is also limited information on alpha-defensin and cathelicidin expression in diabetics. We hypothesized that chronic periodontitis may aggravate metabolic control in diabetes and alter salivary peptide levels in patients with diabetes. Therefore, the present study aimed to evaluate the relationship between salivary levels of antimicrobial peptides LL-37, HNP 1-3 and chronic periodontitis in individuals with and without T2DM.

Material and Methods

Eighty Indian patients (40 males and 40 females) aged between 35-75 years were recruited after obtaining their written informed consent. The patients were advised about the nature of the study according to a protocol which was approved by the Ethics Committee of our institution (AECS/179). The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. Medical history was recorded before the periodontal examination. A questionnaire was completed by each patient that included information regarding age, gender, oral hygiene habits, diabetic history, smoking history, family history and medications used. Patients were categorized into four groups as follows:

- Group I – 20 healthy individuals without chronic periodontitis or diabetes mellitus or any systemic disease.
- Group II – 20 patients who had only chronic periodontitis without diabetes mellitus or any other systemic disease.
- Group III– 20 patients who had only type 2 diabetes mellitus without chronic periodontitis.
- Group IV – 20 patients who had both type 2 diabetes and chronic periodontitis

Each group contained an equal number of males and females. The diagnosis of patients with chronic periodontitis was based on the criteria arising from the 1999 American Academy of Periodontology Workshop (Flemmig, 1999). The inclusion criteria for generalized chronic periodontitis was ≥5 mm of attachment loss present at more than 30% of the sites in patients who were aged ≥35 years and who also showed bone loss radiographically. Patients were included only if they had ≥20 functional teeth. Periodontally healthy individuals showed no evidence of periodontitis, i.e. probing depth ≤3 mm with no attachment loss, absence of bleeding on probing, and no radiographic alveolar bone loss.

The diagnosis of patients with type 2 diabetes was made according to the WHO guidelines (World Health Organization, 2011). The glucose levels of previously diagnosed diabetic patients were confirmed by their fasting blood sugar (FBS) and glycosylated hemoglobin (HbA1c) levels (FBS levels ≥126 mg/dl, HbA1c levels ≥6.5%).

Patients were excluded if they experienced any systemic conditions (aside from diabetes mellitus) that could influence the advancement of periodontitis, if they were smokers or were former smokers, obese, alcoholics or were former alcoholics, pregnant or lactating or those who took oral contraceptive drugs. They were also excluded if they had taken antibiotics/corticosteroids and/or non-steroidal anti-inflammatory drugs during the previous 4 weeks or if they had received professional periodontal treatment 6 months prior to the study.

A full mouth evaluation of the periodontal conditions was done by a single, calibrated examiner (excluding third molars) with the help of a Williams graduated probe (Hu Friedy, Chicago, IL).

The intraexaminer variability for probing depth (PD) and clinical attachment level (CAL) was 0.20 mm and 0.21 mm respectively. Bleeding on probing (BOP) was analyzed dichotomously and evaluated by κ-light test, where the intra-examiner agreement was >0.85. Periodontal parameters including PI (Silness and Loe, 1964), BOP (absent or present), PD in mm, CAL in mm were analyzed at 6 sites per tooth (mesio-buccal, disto-buccal, mesio-lingual, disto-lingual, mid-buccal, mid-lingual).

Fasting Blood Sugar (FBS) and Glycosylated Hemoglobin (HbA1c) levels

Ten ml of venous blood was collected between 8 am to 10 am before the evaluation of periodontal parameters and was analyzed by a single laboratory technician who was blinded to the study groups. The levels of glucose were analyzed using an automated enzymatic method and the values were expressed in milligrams per deciliter (mg/dl). The HbA1c values were expressed as a percentage and were measured by using a high-performance liquid chromatography.

Saliva Sampling

The saliva samples were collected in the morning around 10 am in order to avoid a fluctuation in peptide levels because of circadian rhythm. Whole human saliva was obtained from the seated patients, as saliva flowed into the anterior floor of the oral cavity, into a 10 ml sterile polypropylene container over a period of 10 minutes. No oral stimulus was permitted for 120 minutes prior to saliva collection in order to avoid any impact of mastication of food stuff. The samples were then quickly frozen at -80°C until the time of investigation and soon thereafter the specimens were defrosted and were cleared by centrifugation at 14000×g for 5 minutes.

Analysis of Salivary LL-37 and HNP 1-3

The concentrations of free LL-37 and HNP 1-3 were determined using ELISA kits (Biotechnology, Uden, The Netherlands) as per the guidelines of the manufacturer. The ELISA was a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 3 and a half hours. The efficient format of a plate with 12 disposable 8-well strips allowed free choice of batch size for the assay. Samples and standards were incubated in micro titer wells coated with antibodies recognizing the peptide. Biotinylated tracer antibody was bound to captured peptide. Streptavidin-peroxidase conjugate was bound to the biotinylated tracer antibody and reacted with the substrate, tetramethylbenzidine (TMB). The enzyme reaction was stopped by the addition of oxalic acid. The absorbance at 450 nm was measured with a spectrophotometer. A standard curve was obtained by plotting the absorbance (linear) versus the corresponding concentrations of the peptide standards (log). Samples (neat or diluted five times) and standards (in the range 0.1-100 ng/ml) were tested in duplicate and the mean values were calculated. The mean values of standards and samples were tested in duplicate and if there was a difference of more than 15% in the individual absorbance values the samples were re-assessed. The minimum detection limit (MDL) of the assay was 0.14 ng/ml. The intra- and inter-assay coefficients of variation were less than 4.2 and 3.2 respectively.

Statistics

A confidence interval of 95% with a 0.05 probability and aggregate of square of means measured up to 6.852 when the standard deviation for the sample was 4.21 and when a sample size of 20 was considered from a population of 80. Consequently, the power of the study was computed by utilizing the above values and evaluated to be 0.90. The Statistical Package for Social Sciences software (SPSS) and Palisade Stat Tools v15.0 statistical package was used for data analysis. In the present study we completed a descriptive in-

vestigation and the outcomes which had constant estimations were displayed as mean ± SD (min-max) and categorical estimations were exhibited as number (n) and percent (%). We likewise utilized analysis of variance (ANOVA) to discover the significance between the study groups. Pearsons correlation coefficient investigation was utilized to analyze the correlation between laboratory and periodontal parameters. A value of $p<0.05$ was considered statistically significant. Multiple regression analysis was conducted to examine the relationship between, LL-37 and HNP 1-3 and various potential predictors using a stepwise method.

Results

LL- 37 and HNP 1-3 were detected in all the salivary samples with varied concentrations. The mean concentrations of LL-37 and HNP 1-3 were 213.33 ng/ml and 91.89 ng/ml respectively in healthy individuals. The concentration of antimicrobial peptides increased significantly in other groups compared to the controls ($p<0.001$). The peptide concentration significantly correlated with age in all the four groups. No statistical difference between gender was observed in the study population. *Table 1* shows comparison of age, periodontal parameters and laboratory parameters between all the four groups including all the participants (n=80) using ANOVA. The periodontal parameters (PI, PPD, CAL and BOP), the concentrations of LL-37 and HNP 1-3 in saliva and the laboratory parameters FPG and HbA1c were greatest in group IV followed by groups III and II. The concentrations were least in group I when compared to other groups. Correlation between both laboratory and periodontal parameters in all 4 groups was done using Pearson's correlation test. Our results showed significant correlation between age, periodontal parameters (PI, PPD, CAL and BOP) and laboratory parameters (FPG, HbA1c, LL-37, HNP 1-3) in all the groups ($p<0.001$**;** *Table 2*). A multiple regression analysis was carried out to predict LL-37 and HNP 1-3 from age, FPG, HbA1c, PI, PPD, CAL and study groups. Age variables statistically significantly predicted LL-37 ($\beta=8.775$, $F= 21.127$, $p<0.001$, $R^2= 0.213$). HbA1c variables statistically significantly predicted HNP 1-3 ($\beta=18.862$, $F=64.984$, $p<0.001$, $R^2= 0.454$. All other variables added no statistical significance to the prediction ($p> 0.05$; *Table 3*). A graph was plotted for comparison of age, gender, FPG, HbA1c, salivary levels of LL-37 and HNP 1-3, and periodontal parameters between the four groups *(Figure 1)*.

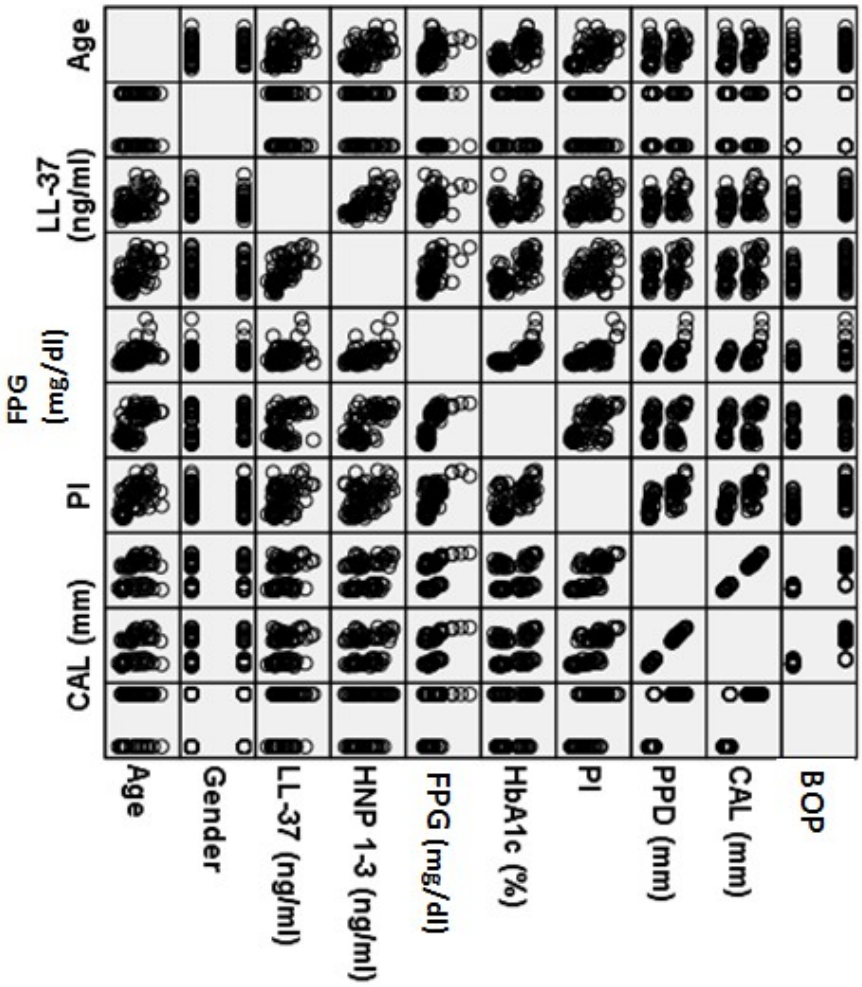

Figure 1: Graphical representation of age, gender, FPG, HbA1c, salivary levels of LL-37 and HNP 1-3, and periodontal parameters between the four groups.

Table 1. Comparison of age, gender, FPG, HbA1c, salivary levels of LL-37 and HNP 1-3, and periodontal parameters between the four groups including all the participants (n=80).

Parameter	Mean ± SD				*p* - value
	Group I (Healthy)	**Group II (Chronic periodontitis)**	**Group III (Diabetes)**	**Group IV (Diabetes and chronic periodontitis)**	
DEMOGRAPHIC DATA					
Age (yrs)	40.05 ± 5.56	43.5 ± 4.81	50.95 ± 9.92	56.85 ± 6.95	<0.001*
Gender	Male – 10 Female – 10	Male – 10 Female – 10	Male – 10 Female – 10	Male – 10 Female – 10	0.838
CLINICAL DATA					
PI	0.39 ± 0.11 I vs II,III,IV (*p*<0.001*)	1.44 ± 0.57 II vs IV (*p*<0.001*)	1.34 ± 0.38 III vs IV (*p*<0.004*)	2.10 ± 0.41	<0.001*
PPD (mm)	1.40 ± 0.16 I vs II,IV (*p*<0.001*)	6.05 ± 0.50 II vs III,IV (*p*<0.001*)	1.80 ± 0.43 III vs IV (*p*<0.004*)	7.06 ± 0.83	<0.001*
CAL (mm)	1.41 ± 0.17 I vs II,IV (*p*<0.001*)	6.28 ± 0.56 I vs III (*p*<0.001*)	1.92 ± 0.40 II vs III,IV (*p*<0.001*)	7.23 ± 0.83 III vs IV (*p*<0.001*)	<0.001*
BOP	Absent-20 Present-0	Absent-0 Present-20	Absent-15 Present-5	Absent-0 Present-20	<0.001*
LABORATORY DATA					
FPG (mg/dl)	106.35 ± 8.22 I vs III,IV (*p*<0.001*)	107.00 ± 9.28 II vs III,IV (*p*<0.001*)	150.80 ± 20.63 III vs IV (*p*<0.001*)	191.10 ± 55.31	<0.001*
HbA1c (%)	4.85 ± 0.29 I vs III,IV *(p*<0.001*)	4.85 ± 0.39 II vs III,IV (p<0.001*)	7.03 ± 0.50 III vs IV (p<0.001*)	7.49 ± 0.40	<0.001*
LL-37 (ng/ml)	213.33 ± 150.05 I vs IV (*p*<0.001*)	299.65 ± 167.98	302.06 ± 174.18	443.00 ± 221.52	<0.002*
HNP 1-3 (ng/ml)	91.890 ± 20.49 I vs III (*p*<0.001*)	92.97 ± 20.21 I vs IV (*p*<0.001*)	127.21 ± 29.18 II vs III (*p*<0.001*)	149.52 ± 35.07 II vs IV (*p*<0.001*)	<0.001*

PI - Plaque Index;
PPD - Probing pocket depth in mm;
CAL - Clinical attachment level in mm;
BOP - Bleeding on probing;
FPG – Fasting plasma glucose in mg/dl;
HbA1c – Glycated hemoglobin in percentage;
LL-37 – Cathelicidin in ng/ml;
*Significance at (*p*<0.001)

Discussion

The present study is a descriptive, cross sectional study that included 80 individuals with an equal number of males and females who were divided into four groups. The levels of salivary LL-37 and HNP 1-3 were determined in adults who had periodontitis and/or diabetes. Healthy individuals were used as controls. The concentrations of LL-37 and HNP 1-3 in saliva and the laboratory parameters fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c) were greatest in group IV followed by groups III and II. The concentrations were least in group I when compared to other groups. After adjusting for age, a step-wise multiple linear regression analysis showed significant correlations of salivary LL-37 levels with age and of salivary HNP 1-3 with HbA1c levels. No statistical difference in gender was observed in the study population.

To the best of our knowledge this is the first study to evaluate the levels of LL-37 and HNP 1-3 in the saliva of individuals with periodontal health and chronic periodontitis, with or without type 2 diabetes mellitus.

Table 2. Comparison of age, gender, FPG, HbA1c, salivary levels of LL-37 and HNP 1-3, and periodontal parameters in all groups

Correlations		**Age**	**Gender**	**LL-37 (ng/ml)**	**HNP 1-3 (ng/ml)**	**FPG (mg/dl)**	**HbA1c (%)**	**PI**	**PPD (mm)**	**CAL (mm)**	**BOP**
Age	R	1.00	-0.02	0.46	0.56	0.53	0.687	0.58	0.398	0.40	0.33
	p-Value		0.84	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	0.002*
Gender	R	-0.02	1.00	-0.14	-0.09	-0.02	0.01	0.02	-0.03	-0.03	0.03
	p-Value	0.84		0.21	0.44	0.87	0.95	0.880	0.81	0.78	0.82
LL-37 (ng/ml)	R	0.46	-0.14	1.00	0.69	0.38	0.36	0.42	0.36	0.35	0.33
	p-Value	<0.001*	0.21		<0.001*	0.001*	0.001*	<0.001*	0.001*	0.001*	0.003*
HNP 1-3 (ng/ml)	R	0.56	-0.09	0.69	1.00	0.58	0.67	0.44	0.28	0.28	0.23
	p-Value	<0.001*	0.44	<0.001*		<0.001*	<0.001*	<0.001*	0.013*	0.013*	0.037*
FPG (mg/dl)	R	0.53	-0.02	0.38	0.58	1.00	0.78	0.64	0.42	0.41	0.31
	p-Value	<0.001*	0.87	0.001*	<0.001*		<0.001*	<0.001*	<0.001*	<0.001*	0.005*
HbA1c (%)	R	0.69	0.01	0.36	0.67	0.78	1.00	0.59	0.24	0.24	0.23
	p-Value	<0.001*	0.95	0.001*	<0.001*	<0.001*		<0.001*	0.031*	0.033*	0.043*
PI	R	0.58	0.02	0.42	0.44	0.64	0.59	1.00	0.71	0.71	0.68
	p-Value	<0.001*	0.88	<0.001*	<0.001*	<0.001*	<0.001*		<0.001*	<0.001*	<0.001*
PPD (mm)	R	0.39	-0.03	0.36	0.28	0.42	0.24	0.71	1.00	0.99	0.89
	p-Value	<0.001*	0.81	0.001*	0.013*	<0.001*	0.031*	<0.001*		<0.001*	<0.001*
CAL (mm)	R	0.40	-0.03	0.35	0.28	0.41	0.24	0.71	0.99	1.00	0.89
	p-Value	<0.001*	0.78	0.001*	0.013*	<0.001*	0.033*	<0.001*	<0.001*		<0.001*
BOP	R	0.33	0.03	0.33	0.23	0.31	0.23	0.68	0.89	0.89	1.00
	p-Value	0.002*	0.82	0.003*	0.037*	0.005*	0.043*	<0.001*	<0.001*	<0.001*	

p<0.001*
r = correlation coefficient
PI - Plaque Index;
PPD - Probing pocket depth in mm;
CAL - Clinical attachment level in mm;
BOP - Bleeding on probing;
FPG – Fasting plasma glucose in mg/dl;
HbA1c – Glycated hemoglobin in percentage;
LL-37 – Cathelicidin in ng/ml;
HNP 1-3 – Human neutrophil peptide 1-3 in ng/ml;

A limitation of this study was the inability to collect information about the status of diabetic control by the patients included in the study. Additionally, data collected including the duration of diabetes and current diabetes treatment could have been taken into consideration.

Primarily, antimicrobial peptides arise in the oral cavity from the gingival epithelium and neutrophils, although defensins and LL-37 are also secreted by salivary glands. In humans, activation of neutrophil-derived LL-37 is carried out by proteinase-3. Both elastase and proteinase-3 are stored in the azurophil granules, which prevents unwanted intracellular processing of cathelicidin molecules into active antimicrobial peptides within resting neutrophils. HNPs are synthesized as precursors that are proteolytically activated before storage in granules. These neutrophilic peptides regulate gingival homeostasis by their actions on the attachment, spread and proliferation of epithelial cells (Gursoy, 2013; Greer *et al.*, 2013).

Gutner *et al.* (2009), in an *in vitro* study, revealed that saliva enabled the antimicrobial activity of LL-37 when there was active periodontal destruction. Continued periodontopathic bacterial presence may lead to inflammation and result in even more extensive periodontal tissue destruction and elevated LL-37 production. The protective role of LL-37 is based on both a direct bactericidal effect and an immunomodulatory effect that accounts for the biggest share of its activity in suppressing periodontitis. Wound fluids containing high levels of glycosaminoglycans have shown to markedly inhibit the antibacterial action of LL-37 (Baranska-Rybak *et al.*, 2006). Binding of LL-37 to mucin is also inhibited by bacterial endotoxins, indicating a possible competition between mucins and lipopolysaccharide (LPS) for binding LL-37 (Bucki *et al.*, 2008). Karilysin, a metalloprotease from *Tannerella forsythia,* can inactivate the bactericidal activity of LL-37 in a time- and concentration- dependent manner through limited proteolysis (Koziel *et al.*, 2010). Expression of defensins in the oral cavity can be regulated by both infection and inflammation (Gursoy *et al.*, 2016). Salt concentration in saliva is also a crucial factor for the antimicrobial activity of defensins. The activity of HNP1-4 is completely lost in the presence of 150 mM NaCl (Abiko and Saitoh, 2007).

Table 3. Adjusted associations between the antimicrobial peptides (LL-37 and HNP 1-3) in saliva, glycemic and clinical parameters of the various groups

Independent Variable	Dependent Variable (Antimicrobial peptides)					
	LL-37 (ng/ml)			HNP 1-3 (ng/ml)		
	β	SE	*p* value*	β	SE	*p* value*
Constant	-96.503	91.522	0.295	1.255	14.472	0.931
Age	8.775	1.909	***<0.001***	0.186		0.106
FPG (mg/dl)	0.183		0.123	0.129		0.339
HbA1c (%)	0.086		0.535	18.862	2.340	***<0.001***
PI	0.222		0.071	0.064		0.541
PPD (mm)	0.207		0.058	0.122		0.158
CAL (mm)	0.200		0.069	0.121		0.162
Group 1	-0.031		0.809	-0.017		0.864
Group 2	0.044		0.666	0.007		0.941
Group 3	-0.153		0.141	-0.133		0.154
Group 4	0.178		0.146	0.196		0.073
R Square	0.213			0.454		
F Value	21.127			64.984		
p value	<0.001			<0.001		

P<0.001*
R square - Coefficient of determination,
F - Variance of group means
B - constant
SE - Standard error
PI - Plaque Index;
PPD - Probing pocket depth in mm;
CAL - Clinical attachment level in mm;
BOP - Bleeding on probing;
FPG - Fasting plasma glucose in mg/dl;
HbA1c - Glycated hemoglobin in percentage;
LL-37 - Cathelicidin in ng/ml;
HNP 1-3 - Human neutrophil peptide 1-3 in ng/ml;

Several studies have assessed cathelicidin LL-37 levels in the saliva, gingival tissues or gingival crevicular fluid in subjects with periodontal disease (Gorr and Abdolhosseini, 2011; Puklo *et al.*, 2008; Dommisch *et al.*, 2009; Takeuchi *et al.*, 2012; Davidopoulou *et al.*, 2013; Turkoglu *et al.*, 2009). These have shown a prominent LL-37 expression by neutrophils was in the periodontal lesions. Turkoglu *et al.* (2016), in his study, observed that LL-37 levels in gingival crevicular fluid were higher in non-smokers with chronic periodontitis than smokers with chronic periodontitis and that there was no significant reduction in LL-37 levels after non-surgical periodontal therapy in smokers with chronic periodontitis while significant decrease was observed in non-smokers with chronic periodontitis.

In the present study a positive, significant relationship was seen with LL-37 and the periodontal parameters in patients with chronic periodontitis. This is in agreement with earlier studies (Turkoglu *et al.*, 2009; Gorr *et al.*, 2011). Another study reported that patients with systemic diseases and reduced LL-37 production are more likely to develop periodontitis as a result of impaired ability to eliminate periodontopathic bacteria (Takeuchi *et al.,* 2012). However, it was noted that higher levels of salivary LL-37 was observed in patients who had severe chronic periodontitis which is in agreement with our study.

The positive association could be attributed to the fact that microbes invoke immune and inflammatory responses during the advancement of periodontal disease. After stimulation, neutrophils migrate to the site of infection, which leads to the release of mature LL-37 from stimulated neutrophils. Although the salivary LL-37 levels were high in the chronic periodontitis group, they were not sufficient to prevent the periodontal destruction seen in chronic periodontitis.

The results of the present study showed significant positive correlations between LL-37, FPG and HbA1c which differed from the results obtained of Meguro *et al.* (2014) who demonstrated that plasma LL-37 was not significantly correlated with FPG and HbA1c. A recent study also showed an increased expression of LL-37 along with other defensins in the gingiva of diabetics with periodontitis and that LL-37. In periodontitis patients, the peptides were visible at all the epithelial layers (Yilmaz *et al.*, 2015). In the present study, levels of salivary LL-37 tended to be the greatest in patients with chronic periodontitis and type 2 diabetes as compared to the other study groups. It was noted that levels of salivary LL-37 increased with the inflammatory response to microorganisms and this increase was more prominent in Group IV because of the severe inflammation and destruction in this group. Therefore, the present study indicates that cathelicidin LL-37 could play a role as a mediator between innate immunity and adaptive immunity as suggested by Takeuchi *et al.* (2012).

A number of studies have evaluated HNP 1-3 levels in saliva, gingival tissues or GCF of patients with periodontal disease (Guentsch *et al.*, 2012; Turkoglu *et al.*, 2010; Range *et al.*, 2012). The results of our study showed that levels of salivary HNP 1-3 were significantly increased in patients with chronic periodontitis and type 2 diabetes followed by diabetic patients and chronic periodontitis patients respectively. The levels were least in the healthy group. The total amount of HNP 1-3 was similar in periodontitis and healthy groups and furthermore no significant correlation was found between HNP 1-3 levels and periodontal parameters in the chronic periodontitis group. This is in agreement with the study performed by Turkoglu *et al.* (2010). This could be attributed to the defensive role of HNP 1-3 and its effect on innate immunity.

We also found a significant positive association between HNP 1-3 levels and HbA1c. This is in agreement with the study done by Nemeth *et al.* (2014) who evaluated the plasma HNP 1-3 levels in type 2 diabetics and observed that the diabetic patients displayed higher plasma HNP 1-3 levels when compared to the healthy controls. Saraheimo *et al.* (2008) have stated that diabetic nephropathy has an impact on the circulating HNP 1-3 levels and this could be due to the possible reduced renal degradation of α-defensin peptides with progressive nephropathy. Diabetic patients have altered polymorphonuclear neutrophil (PMN) functions, and it has often been suggested that PMN activity could be restored by controlling hyperglycemia with insulin. Antimicrobial protein production in PMNs is also altered in association with insulin resistance and in the elderly. Hyperglycemia, or the presence of advanced glycation end products (AGEs), leads to persistent activation of neutrophils, as evidenced by the increased activity of neutrophil alkaline phosphatase. Insulin is able to stimulate PMN chemotaxis to N-formylmethionyl-leucyl-phenylalanine (fMLP). In adition, antimicrobial protein production in PMNs is also altered in association with insulin resistance (Walrand *et al.* 2006). Metabolic dysfunction is also associated with decreased production and/or secretion of lactoferrin, BPI (Bactericidal/Permeability-Inducing Protein), and a-defensins from neutrophils (Moreno-Navarrette and Fernandes-Real., 2011).

Results from the present study also revealed that HNP 1-3 and LL-37 were positively and significantly correlated in all groups. Our hypothesis was that LL-37 and HNP 1-3 could contribute to innate immunity in periodontal disease by affecting each other. Defensins and LL-37, key components of the mucosal antimicrobial defense, are induced by bacterial stimulation of gingival epithelial cells while only LL-37 expression is induced in peripheral blood neutrophils. In some cases where AMPs were measured in saliva or gingival crevicular fluid, down-regulation was caused by proteolytic degradation of the AMP by bacterial proteases. It is not clear if the regulation of AMP expression is a functional response to the bacterial insult or if it is part of a more general stimulation of target cells. In fact, even the stimulated levels of several AMPs have been found to be below the minimal inhibitory concentration for oral pathogens (Gorr and Abdolhosseini, 2011). Therefore, despite increased levels of antimicrobial peptides, the protective functions of defensins and cathelicidins are still inadequate to resist inflammation and promote wound healing (Yilmaz *et al.*, 2015).

Conclusion

To the best of our knowledge this is the first report describing LL-37 and HNP 1-3 levels in four distinct groups in an Indian cohort. Within the confines of this study, the outcomes indicate that HNP 1-3 and LL-37 may participate in the pathogenesis of both diabetes and periodontitis. Future research with a larger cohort and interactions of antimicrobial peptides with other proteins in saliva are essential to verify the role of antimicrobial peptides as biomarkers in the diagnosis and therapy of numerous pathological processes in the oral cavity.

Conflict of Interest and Sources of Funding Statement

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