DETERMINATION OF SALIVARY LACTOFERRIN LEVELS BEFORE AND AFTER NON-SURGICAL PERIODONTAL TREATMENT IN PATIENTS WITH AGGRESSIVE PERIODONTITIS

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Abstract

Objective: This study determined levels of Salivary lactoferrin (sLf) before and after non-surgical periodontal treatment (NSPT) in individuals with localized and generalized aggressive periodontitis compared to healthy controls.

Methodology: The study was an interventional and prospective design. Nineteen participants were clinically and radiographically diagnosed as having AP and 18 participants who did not show evidence of the disease or other diseases, served as controls. All participants received NSPT which included scaling and polishing for the controls and scaling and root planing (SRP) for the cases. Unstimulated whole saliva was collected from participants before and after NSPT. Salivary Lactoferrin levels were quantified using the Human Lactoferrin ELISA kit.

Results: The mean ages were: GAP=33.80±8.93, LAP=32.11±8.07 and Controls = 31.39±8.98 years. Concentration of sLf before NSPT among the cases was 70.92±26.84 (-µg/ml-) and the controls had 48.39±28.56 (-µg/ml-). After treatment, concentration of sLf was 68.57±25.34 (-µg/ml-) for cases and 43.42±21.54(-µg/ml-) for controls. A significant difference was observed at baseline between cases and controls (p= 0.018) and after treatment (p=0.003) which indicated sLf levels were higher in the diseased than non-diseased individuals.

Conclusion: Elevated sLf levels serve as an indication for increased degree of inflammation and this biomarker can be used to determine the severity of AP.

Key words: *Saliva, Proteomic biomarker, Salivary lactoferrin, Localized Aggressive Periodontitis, Generalized Aggressive Periodontitis.*

Introduction

Aggressive periodontitis (AP) just as its name suggests, is an inflammatory disease of the periodontium, characterized by rapid attachment loss (AL) of the periodontal tissues as well as alveolar bone destruction in otherwise systemically healthy individuals.[1] There are two clinical varieties of AP: the Localized Aggressive Periodontitis (LAP) and the Generalized Aggressive Periodontitis (GAP) forms.

LAP frequently has an age onset at about puberty and is clinically characterized by interproximal tissue AL on at least two permanent teeth, one of which is a first molar, and involving no more than two teeth other than the first molars and incisors".[2,3] GAP usually affects individuals under age 30, but older patients may be affected.[2] Clinically, GAP is characterized by "generalized interproximal tissue AL affecting at least three permanent teeth other than first molars and incisors."[2] The bacteria *Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis* have been associated with LAP and GAP in mostly teenagers and young African adults.[3,4]

Arowojolu and Nwokorie[5] and Harley and Floyd [6] recorded the prevalence of AP in Ibadan and Lagos among Nigerian teenagers and young adults to be 0.8-1.6% and 0.8% respectively. In the United States, a national survey of adolescents aged 14 to 17 years reported that 0.53% had LAP [7] and 0.13% had GAP.[7] In addition, blacks were at much higher risk than whites for all forms of AP, and male teenagers were more likely to have GAP than female adolescents. Salivary Lactoferrin levels are raised in patients with periodontitis and AP not being an exception.[8]

Saliva is secreted mainly by three pairs of major salivary glands (parotid, submandibular and sublingual) and numerous minor salivary glands (450-750). [9] Human saliva is a plasma ultra-filtrate and contains proteins either synthesized in situ in the salivary glands or derived from blood and contains biomarkers derived from serum, gingival crevicular fluid, and mucosal transudate. [11]Lactoferrin is a biomarker secreted in saliva and can be used to monitor the levels of hormones, drugs and medications, bone turnover, biologic markers, forensic evidence and oral diseases which include caries and periodontal disease.[10]

The use of proteomic biomarkers such as sLF have been found to have favorable diagnostic value in addition to genomic and microbiological markers.[10] We

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hypothesized that sLf levels will be higher at baseline in patients with AP and reduced after non-surgical periodontal treatment (NSPT). The aim of this study was to determine the levels of sLf before and after NSPT in individuals with AP and control participants.

Materials and Methods

The study was an interventional and prospective design. Ethical approval for the study was obtained from the Ethical and Protocol Review Committee of the College of Health Sciences, University of Ghana (CHS-Et/M.8-P 4.6/ 2015-2016). Respondents aged 20-50 years who met the inclusion criteria according to Armitage[1] were selected for this study. Participants who had comorbid conditions, those on antibiotics and medications for systemic diseases, known alcoholics, smokers, pregnant or lactating women, and individuals who had had scaling and root planing (SRP) in the past six months were excluded.

The sample size was estimated based on effect size in terms of standard deviation of the sample differences of 0.76 as reported in a similar study by Pagano and Ganvreau.[12] Using the equation for comparisons of two means, a total of 37 individuals were estimated made up of 19 cases and 18 controls. Thirty-seven individuals who consented and met the inclusion criteria were recruited. The participants were selected consecutively from May 2016- May 2017 at the Oral Diagnosis unit of the Dental Clinic of the then UGSMD. Nineteen participants were clinically and radiographically diagnosed as having AP and 18 participants who did not show evidence of the disease or other diseases, served as controls. The controls were patients attending the dental clinic for routine dental care who did not show evidence of periodontal disease or other systemic diseases per the inclusion and exclusion criteria and after obtaining informed consent. The sociodemographic data of all the participants was captured using a structured questionnaire

Clinical Visits and Treatment of Cases and Controls

The cases had four clinical visits whiles the controls had three over a period of four weeks. At the first visit, a comprehensive periodontal examination was done and an OPG of each participant was taken. The clinical examination of all the controls and AP individuals was carried out using a periodontal chart and a form for saliva collection which had the participants initials and identification number, the tube weight before and after saliva collection, the volume of saliva collected and the duration of the collection. Protocols and guidelines for saliva collection by Wang et al.[9] was explained to the participants. At the second visit, unstimulated whole saliva was expectorated into falcon tubes provided every minute under ice in a mug over a period of 15 min. At least 3-5 ml of saliva was collected for each participant. In the dental clinic, the samples were stored in an ice chest with ice packs for 15-30 min. They were then transported to the Chest clinic laboratory for centrifugation and subsequently stored at -80ºC at the Pathology Department at Korle-Bu. All the samples were kept at the Korle-Bu Teaching Hospital and later sent to Noguchi Memorial Institute for Medical Research, Legon for laboratory analysis.

A full mouth scaling and polishing for controls which is done routinely for patients who come for regular dental visits and SRP for the cases (individuals with disease) was carried out. For the cases, 10ml of 0.2% Chlorhexidine gluconate mouthwash was used to irrigate all probing pocket depths (PPD'S) ≥ 4 mm and the solution gargled for one minute and expectorated. Oral hygiene instructions were given, and the controls seen at four weeks (third visit) after which the saliva samples were collected again using the same protocols and the comprehensive periodontal examination repeated. The cases were seen within 24 h for the second session of SRP (third visit) and then at four weeks for re-evaluation (fourth visit). Saliva samples were collected again at this time using the same protocols and a comprehensive periodontal examination done.

The samples were transferred into a centrifuge (Eppendorf 5810 R) and run at 1,071xg for 25 min at 4°C to remove insoluble materials, cell debris and food remnants. The supernatants were carefully decanted into four cryo tubes and the pellets placed into one tube. Pierce protease inhibitor cocktail EDTA-Free (5µl) was added to the supernatants and mixed. The treated samples were then transferred and stored in a −80°C freezer awaiting laboratory analysis.

Salivary Lactoferrin (sLf) measurement by ELISA

The laboratory work for sLf determination was done at the Noguchi Memorial Institute for Medical Research (NMIMR) of the College of Health Sciences, University of Ghana, Legon. Salivary Lactoferrin was quantified using the Human Lactoferrin ELISA kit (ab108882- Lot: GR232543-29 Lactoferrin (HLF2) based on the manufacturer's instructions. Saliva samples and lactoferrin standards were added to the plate in duplicate. For colour development, 50 µl/well of chromogen ($3,3^1,5,5^1$-tetramethylbenzidine (TMB) was added with the samples turning blue after the plate was incubated in the dark for 15 min. The colour reaction was subsequently stopped by the addition of 50 µl/well of stop solution (0.2 N H_2SO_4) with the colour of samples turning yellow. The samples were transferred to an ELISA plate reader (BioTek, VT, USA) and optical density (OD) read at 450 nm. Optical density data was converted to lactoferrin concentration using a 4-parameter logistic curve fit.

Data Analysis

Data was analysed using Statistical Package of Social Sciences (SPSS version 22). Chi-square test was used to compare proportions of socio-demographic data. Mean lactoferrin levels were compared using T- test for two means and ANOVA for more than two means between cases and controls. The data was presented as mean±SD. Significance level was set at $p < 0.05$.

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Results

Background Characteristics and Sociodemographic Data

The age range of respondents who voluntarily participated in the research work were from 20-50 years. The mean age and distribution of respondents with GAP, LAP and the controls can be found in Table 1. Data pertaining to the tribe, religion and educational background details of the participants were collected (Table 1).

Table 1: Sociodemographic characteristics of all respondents

	Participant category				
Demographics	**GAP n (%)**	**LAP n (%)**	**Controls n (%)**	**Total N (%)**	**P-Value**
Mean age ± Mean SD (yrs)	10(33.80 ± 8.93)	9(32.11 ± 8.07)	18(31.39± 8.96)	37(32.22 ± 8.56)	0.78
Gender					
Male	4(40.0)	6(60.0)	10(55.6)	20(54.0)	0.06
Female	6 (60.0)	3(40.0)	8(44.4)	17(46.0)	
Total	10 (100.00)	9(100.00)	18(100.00)	37(100.00)	
Marital Status					
Never Married	6(60.0)	7(77.8)	12(66.7)	25(67.6)	0.84
Married	3(30.0)	2(22.2)	3(16.7)	8(21.6)	
Divorced	1(10.0)	0(0.0)	2(11.0)	33(8.1)	
Separated	0(0.0)	0(0.0)	1(5.6)	1(2.7)	
Widowed	0(0.0)	0(0.0)	0(0.0)	0(0.0)	
Total	10(100.00)	9(100.00)	18(100.00)	37(100.00)	
Tribe					
Ga/Dangme	3(30.0)	1(11.1)	3(16.7)	7(18.9)	0.57
Akan	5(50.0)	4(44.4)	12(66.7)	21(56.8)	
Ewe	1(10.0)	3(33.3)	2(11.0)	6(16.2)	
Northern	1(10.0)	1(11.1)	0(0.0)	2(5.4)	
Other	0(0.0)	0(0.0)	1(5.6)	1(2.7)	
Total	10(100.00)	9(100.00)	18(100.00)	37(100.00)	
Religion					
Christian	10(100)	8(88.9)	18(100.00)	36(97.3)	
Islam	0(0.0)	1(11.1)	0(0.0)	1(2.7)	0.20
Traditional	0(0.0)	0(0.0)	0(0.0)	0(0.0)	
Total	10(100.00)	9(100.00)	18(100.00)	37(100.00)	
Educational background					
No Formal Education	0(0.0)	0(0.0)	1(5.6)	1(2.7)	0.25
Completed only Primary education	2(20.0)	0(0.0)	1(5.6)	3(8.1)	
Completed only Secondary education	7(70.0)	5(55.6)	7(38.8)	19(51.4)	
Completed Tertiary education	1(10.0)	4(44.4)	9(50.0)	14(37.8)	
Total	10(100.00)	9(100.00)	18(100.00)	37(100.00)	

F-test did not show any significant difference in the mean ages of the three participant groups.
Chi-square test showed no significant associations between the sociodemographic characteristics and the participant categories.

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Concentration of Salivary Lactoferrin (sLf) Measured for the Participants

The mean concentration of sLf measured before and after treatment for the cases and controls can be found in Table 2 and the details of the subgroup analysis for the cases (LAP and GAP) before and after treatment are captured in Table 3.

Table 2: Concentration of sLf levels before and after treatment for the cases and controls

	Mean Concentrations of sLf		P-value
Participant category	**sLf conc(µg/ml) Before treatment**	**sLf conc (µg/ml) After treatment**	
Cases (n=19)	70.92 ± 26.84	68.57 ± 25.34	0.44
Controls (n=18)	48.39 ± 28.56	43.42 ± 21.54	0.37
Total (n=37)	59.96 ±29.59	56.33 ±26.51	
P-value	0.018	0.003	

T- test showed significant differences in sLf between cases and controls before treatment (p= 0.018) and after treatment (p= 0.003).

Table 3: Concentration of sLf levels before and after treatment for the LAP and GAP cases

	Mean Concentrations of sLf		P-value
Participant category	**sLf conc (µg/ml) Before treatment**	**sLf conc (µg/ml) After treatment**	
LAP (n=9)	70.08 ± 26.12	74.40 ± 15.42	**0.53**
GAP (n=10)	76.94 ± 20.12	63.84 ± 31.81	
Total (n=19)	73.69 ± 22.76	68.84 ± 25.32	
P-value	**0.84**		

Repeated measure F- test showed no significant difference in the sLf concentration of the LAP and GAP groups before and after treatment (P value= 0.53 and 0.84).

Discussion

This study was conducted primarily to determine the levels of sLf and to find out if there was any association between sLf levels in participants with AP at baseline and after NSPT. Our literature search did not reveal work on this subject in sub-Saharan African populations. The present study was therefore regarded as part of an early effort to determine whether a link exists between AP and the concentration of sLf among sub-Saharan African populations.

There were 20 males and 17 females who participated in the study. Among the cases, 10 (54.0%) and 9 (46.0%) of the males and females respectively had AP (Table 1). Among the cases, 4 (40%) males and 6 (60.0%) females had GAP whilst 6 (66.7%) males and 3 (33.3%) females had LAP (Table 1). Comparing our results to a study by Fine et al.,[13] 10 patients with LAP and equal number of controls were matched for age, gender and race. The African Americans were 7 in each group whiles the Caucasians were three 3 in each group. In yet another study by Suomalainen et al.,[14] 7 participants with LAP and the same number of controls were recruited in Finland. This reveals the low numbers of Caucasians with the disease.

The mean age in this study for GAP (33.8 ± 8.93) years, LAP (32.11± 8.07) years and control (31.39 ± 8.96) years respondents was slightly higher compared to the mean age of the respondents in a study by Fine et al.,[13] which recorded the mean age of participants with LAP to be 18.9 ± 7.0 years and that for the controls to be 19.7±7.4 years. In another study by Rocha et al.,[15] on 'Differential expression of salivary glycoproteins in aggressive and chronic periodontitis,' the average age for AP participants was 19-28 years which was close to the age range captured in our study.

The normal lactoferrin concentration in saliva is 8µg/ml. [8] Among salivary proteins, sLf is the most important factor of natural immunity. Its concentration corresponds to 8.96 and 7.11 µg/ml in unstimulated and stimulated saliva, respectively.[8] Our literature search did not reveal baseline levels of lactoferrin in saliva of individuals with AP.

The sLf concentration in the oral cavity is related to different fluid samples to be assayed (whether stimulated or unstimulated saliva or gingival crevicular fluid)[8]. The results of the concentration of sLf in this present study compares with those of other published work even though differences existed in the samples collected, methods and participants used.[15,16,17] In this present study, the concentration of lactoferrin was quantified using ELISA. The concentration of lactoferrin obtained at baseline and also comparing before and after NSPT using whole unstimulated saliva were consistent with the views expressed by other investigators even though some studies focused on the iron content of sLf, use of stimulated saliva, gingival crevicular fluid and serum.[8,15,16,17]

Unique to this work was the introduction of GAP cases which most studies in the literature did not report on (Table 3). Re-evaluation after initial periodontal therapy in one month was considered in this study which was not indicated in some of the reports read.

In this study, the results obtained at baseline and after treatment between the cases and controls were significant (P = 0.018) and (P = 0.003) respectively (Table 2). The observed drop in lactoferrin concentration among the cases after treatment was not significant. It did not approach the levels of the healthy controls. The differences in sLf before and after the

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NSPT for both the GAP and LAP cases were not significant. A study by Berlutti et al. [19] confirmed an increase in lactoferrin levels from 20 to 60 μg/ml during infection and inflammatory processes and this was attributed to the recruitment of neutrophils which increases sLf concentration. It was also reported by Malathi et al. [20] that, during gingival inflammation, lactoferrin was strongly upregulated and detected at a higher concentration in saliva of patients with periodontal diseases as compared to healthy patients. The reduction in the levels of lactoferrin as recorded in the literature can reach normal levels in a non-inflammatory state.[18]

Furthermore, lactoferrin was found to be strongly up-regulated in mucosal secretions during gingival inflammation and detected at high concentrations in saliva of patients with periodontal disease compared with healthy patients in a study by Giannobile et al.[21] An elevated level of lactoferrin was also observed in the saliva of participants with periodontitis in a study by Kumar et al. [22] and their levels decreased following NSPT. In this study, the respondents were re-evaluated at four weeks and a decrease in sLf levels were detected between the cases and controls. These findings are in agreement with another study by Buchmann et al.[23] who concluded that clinical healing in chronic periodontal disease is associated with a down regulation of local polymorphonuclear responses following NSPT.

In a review by Narang et al. [24] on 'Salivary Biomarkers for Periodontal Diseases', it was reported that the biomarkers during gingival inflammation were detected at high concentrations in saliva of patients with periodontal disease compared with healthy patients. These results were consistent with the current findings with regards to the cases and controls. The overall increase and decrease in sLf concentration obtained in this study among AP participants before and after NSPT respectively are consistent with some studies with designated increased levels of this biomarker when compared to control participants without periodontal disease.

The results of the study did not support our hypothesis probably due to the low sample size and re-evaluation period, but a significant difference was observed between cases and controls at baseline (p= 0.018) and after treatment (p=0.003) which indicated sLf levels are higher in the diseased state than the non-diseased state. The elevated sLf levels serve as an indication for increased degree of inflammation and this is a biomarker to determine the severity of the periodontal disease. After treatment, this can be measured to indicate possible effective treatment outcomes.

Conclusion

At baseline, sLf levels were significantly different from the cases and controls. The significant difference persisted even after treatment. There were no significant changes in sLf levels for both cases and controls after treatment. This study demonstrated that, sLf levels change with the LAP and GAP conditions.

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Declaration

Part of the original data has also been published in an earlier article under a different title with a different outcome as, "*Total Salivary Immunoglobulin A Determination Before and After Non-Surgical Periodontal Treatment in Patients with Aggressive Periodontitis*". (J Surg Res 2022; 5 (2): 322-331)".

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