Salivary Matrix Metalloproteinase-8 Levels and Periodontal Health Status among Adults Attending the University of Nairobi Dental Hospital.

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Abstract

Objective: To determine the relationship between Salivary MMP-8 levels and periodontal health status.

Methods: In this descriptive cross-sectional study, 114 participants had about 5mL of unstimulated whole saliva collected over 3 minutes, between 7:30 am and 10:00 am at the University of Nairobi Dental Hospital between July 2014 and April 2015.

Clinical examination assessed oral hygiene (Silness and Loe, 1964), gingival status (Loe and Silness, 1963) and periodontal health status was classified using the CDC/AAP consensus definitions for epidemiological studies. Salivary MMP-8 was quantified using specific Quantikine double antibody ELISA technique.

Results: Statistically significant higher levels of salivary MMP-8 were noted with increasing periodontal disease severity. The participants with no periodontitis were found to have 22.68ng/mL, mild periodontitis 44.55ng/mL, moderate periodontitis 46.34ng/mL and severe periodontitis 156.62ng/mL. A mean level of salivary MMP-8 of 40.52ng/mL (±66.38 SD ng/mL) with a range of 0.0ng/mL to 295.9ng/mL was found. A predictive value of 0.8 (AUC=0.8, p>0.001) and an optimal diagnostic concentration of 114.55ng/mL was observed.

Conclusion: A strong positive association was drawn between salivary MMP-8 levels and periodontal health status among adults visiting the UoN dental hospital. The adjunctive diagnostic concentration of salivary MMP-8 was comparable to those of other studies. This study provides new knowledge on salivary MMP-8 in Kenya.

Clinical relevance: Effective diagnosis, quantification and monitoring of periodontal disease are critical in management. The establishment of consensus novel biologic diagnostic approaches will provide clinicians a key guide to treatment.

Scientific rationale: MMP-8 plays a key role in periodontal tissue destruction thus great potential as a screening, diagnostic and monitoring tool. Inherent differences in MMP-8 expression have been shown to exist between individuals, races and regions.

Principal findings: Statistically significant higher levels of salivary MMP-8 were noted with increasing periodontal disease severity. A predictive value of 0.8 (AUC=0.8, p>0.001) and an optimal diagnostic concentration of 114.55ng/mL was observed.

Practical implications: Further randomized studies should be considered to evaluate this putative biomarker. Salivary MMP-8 should be considered a potential adjunctive diagnostic and monitoring tool as well as a periodontal disease susceptibility test.

Keywords: Salivary; MMP-8; Biomarker; ELISA; Kenya; CDC/AAP; Periodontitis

I. Introduction

Periodontitis is a multifactorial, progressively destructive process initiated by microbial plaque biofilms. It is characterized by initial inflammation of the gingiva, progressive destruction of tooth supporting structures causing clinical attachment loss, alveolar bone loss and pocket formation if the disease goes on unabated. In Africa, gingivitis affects 50% of the population while 35% are affected by varying degrees of periodontitis(1). In Kenya, gingivitis affects up to 90% of the population while 1-10% suffer from chronic periodontitis(2).

Conventional diagnostic techniques lack the ability to distinguish between current and previous effects disease and are also unable to identify those patients at greatest risk of further periodontal disease progression(3). They have been also found to be labour intensive, cumbersome and poorly utilized in screening programs(4).

Matrix metalloproteinase-8(MMP-8) plays a role in periodontal tissue destruction and may be found in saliva hence promising as an indicator for periodontal and peri-implant tissue destruction(5). Quantification of salivary MMP-8 levels may provide a non-invasive technique for detecting the presence and activity state of...
periodontal disease. This may enable large populations to be screened accurately, in particular, resource-limited communities. This will by and large allow timely intervention, facilitate well targeted periodontal therapy, hence mitigate the effects of progressive periodontal destruction(6).

A meta analysis in 2008 found statistically significant associations between cytokine gene polymorphisms and periodontal disease with statistically significant differences noted between Caucasians and Asians(7). These inflammatory cytokines have been shown to play a key role in regulating MMP-8 expression, secretion and activity in the periodontium(8, 9). Genetic, racial and socioeconomic variability in periodontal disease pathogenesis including MMP-8 enzyme expression has also been established (10,11). Notably, some of the genetic variations may also be protective and beneficial to the host(12).

Disease management would also be more targeted, based on individual’s periodontal disease activity. The development of techniques with universally standardized methodology and establishing regional reference values in view of factors influencing disease is key in making salivary diagnostics an adjunctive gold standard(13).

Saliva collection is relatively simple and does not require a skilled work force, hence sample collection can be carried out by patients for self-monitoring at home or care givers in clinical settings including in remote areas. Saliva can be used in clinically challenging situations, for example obtaining samples from children, physically and mentally challenged individuals or anxious patients. It is thus ideal for population based screening activities.

II. Materials And Methods

Study population and design

Ethical clearance to conduct this study was obtained from the Kenyatta National Hospital / University of Nairobi Ethics and Research committee (P 179/04/2014). A descriptive cross sectional study was carried out among 120 consenting adults with and without periodontal diseases of various severities and visiting University of Nairobi Dental Hospital between July 2014 and April 2015.

The sample size was determined by a one-way analysis of variance power calculation using a formula from R software along the lines of Cohen methods. A sample size of 16 for each of the four groups was derived giving a total of 64 participants with an effect size of 0.4, 95% confidence level and power of 80%. Anticipating possible losses during laboratory stages, a total sample size of 120 participants was used. Convenience sampling method was used to select the adult patients with the following excluded: Persons who had had periodontal treatment procedures taken any antibiotic within the past 6 months, persons with concurrent systemic illness for example rheumatoid arthritis, Female patients who were pregnant or lactating, smokers or smoking history in the last 3 years, edentulous patients and patients who had less than 20 teeth. Socio-demographic data was collected from participants using serialized interviewer administered questionnaires.

Saliva collection

Prior to saliva sample collection, the participants were allowed to rest seated comfortably for a few minutes and then asked to thoroughly rinse their mouth with plain water into the spittoon. They were then asked to slightly lean forward and not to swallow or speak. After about 5 minutes, the saliva had pooled in the anterior floor of the mouth. About 5mL whole saliva was collected by passively drooling into 50mL pre-weighed, airtight, serialized, centrifuge compatible polystyrene tubes(14). Saliva collection was done between 8:00 a.m and 11:00a.m. Sealed sample tubes were placed in ice cubes and gel ice packs inside a cooler box for transportation to the laboratory (within maximum 2 hours) for processing.

Clinical examination

This was done on a dental chair by the calibrated investigator. Plaque scores were taken using Silness-Loe index (1964) and gingival index using the Loe and Silness index (1963). Full mouth periodontal examination was done whereby probing depths, recession and clinical attachment loss was measured in millimeters at six sites per tooth (mesiobuccal, mid-buccal, distobuccal, mesiolingual, midlingual and distolingual) using a sterile Marquis periodontal probe at all teeth excluding third molars. Periodontitis category was then determined using the CDC/AAP consensus definitions.

Laboratory evaluation of MMP-8

Serialized sealed saliva collection tubes were stored in gel ice in a cooler box and transported to the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) laboratory where were received. Samples were then weighed and centrifuged at 3,000 rpm for 3 minutes in a refrigerated centrifuge. The aliquots of 500 μL were pipetted into similarly serialized serum vials and stored at -70°C until ready for processing. Precautions were made to avoid repeated freezing and thawing of samples.
Reagents and samples were brought to room temperature and constituted according to the manufacturer’s instructions. A commercially available ELISA kit, Quantikine® Colorimetric double antibody sandwich ELISA kit, catalog number DY908 by R&D Systems (United Kingdom), was used to detect and quantify concentrations of MMP-8 in every saliva sample. A pilot run was carried out using samples from test subjects, the MMP-8 standards as well as negative controls (blanks); to pretest, optimize and streamline the analysis protocols. Following salivary MMP-8 sandwich antibody assay, a calibrated microplate reader Emax spectrophotometer - Molecular devices x®; set at 450nm filter was used to measure the optical density of each duplicate sample and standards. A linked computer software (SOFTmax® PRO 4.3.1 Life Sciences edition by Carey Farquhar, University of Washington) was used to automatically tabulate the resultant optical densities. A 4-parameter logistic (4PL), non-linear regression line of best fit curve was plotted for the optical densities. Trend line formulae converted the optical densities to the corresponding salivary MMP-8 concentrations in ng/mL.

Data analysis
Data was cleaned, coded and processed with Statistical Packages for Social Sciences (SPSS) 20.0 for Windows, Microsoft-Excel as well as R software (ver. 3.1.2). Descriptive statistics applied were measures of central tendency and dispersion for continuous variables for example age, gender, plaque scores, gingival status and periodontal health status.

Comparison of means and proportions was done using t-test and Chi-squared test. Where appropriate, ANOVA, Pearson’s and Spearman’s rank correlation tests were used to determine the associations between key variables including periodontitis severity, oral hygiene practices, gingival status, socio-demographic and other variables when compared with the salivary MMP-8 levels. Receiver operating characteristic (ROC) curve applying area under the curve (AUC) value was used evaluate the diagnostic value of MMP-8. Confidence level was set at 95% (α level <0.05) to assess strength of association. The data met the assumptions of the various statistical tests applied.

III. Results
A total of 120 patients were recruited into the study. However, six participants were later excluded as extreme outliers on the basis of extremely high levels of MMP-8 using Grubb’s test (R-software) to avoid an overtly skewed distribution. Removing these extreme outliers was deemed safe in retaining the general characteristics of the parameters under study as the remaining number of participants (114) was still far above the computed sample size of 80.

Of the 114 participants evaluated, 64(56.1%) were female and 50 (43.9%) were male. The age ranged from 18 - 77 years with a mean of 36 years (±13.9SD). All the participants were observed to have some plaque deposits despite the fact that all reported to brush their teeth at least once in a day. The mean plaque score of participants was 0.94(±0.49SD). The mean gingival index was 1.1 (±0.33SD) with a range of 0.3-2.4. Regarding severity, out of the 114 participants, 48(42.1%) had mild gingivitis, 63(55.3%) had moderate gingivitis while 3(2.6%) had severe gingivitis. Out of the 114 participants, 69(60.5%) had no periodontitis, whereas 45(39.5%) had periodontitis. Out of those with periodontitis, 9(7.9 %) had severe periodontitis, 23(20.2%) had moderate periodontitis, 13(11.4 %) had mild periodontitis.

Salivary MMP-8
Salivary MMP-8 was present in majority of participants 91(79.8%) and not detectable in 23 (20.2%). The MMP-8 values ranged from undetectable values to 295.9 ng/mL with a mean of 40.5ng/mL (±66.4 SD).

Salivary MMP-8 and severity of Gingivitis.

The mean salivary MMP-8 levels tended to be higher with increasing gingivitis severity (Graph 1). Participants with mild gingivitis had 14.9ng/mL (±42.68 SD), moderate gingivitis had 56.4ng/mL (±69.43 SD) while those with severe gingivitis had 117.8ng/mL (±154.88SD). One way ANOVA analysis revealed a statistically significant positive association between the gingivitis groups based on salivary MMP-8 levels (F=8.398,df=2, p<0.001).
Salivary Matrix Metalloproteinase-8 Levels and Periodontal Health Status among Adults Atten....

The mean salivary MMP-8 levels tended to rise with increasing severity of periodontitis (Graph 2). The group with no periodontitis (69) had 22.7 ng/mL (±50.32 SD). The MMP-8 then increased 44.6 ng/mL (±59.11 SD) in those with mild periodontitis to 156.6 ng/mL (±96.36 SD) in those with severe periodontitis. The salivary MMP-8 concentration was generally higher in the 45 participants with periodontitis (67.9 ng/mL ±78.38 SD) compared to those with no periodontitis (22.7 ng/mL ±50.32SD). ANOVA analysis revealed a statistically significant difference between the four groups of periodontitis severity (F=14.96, df=3, p<0.001).

Multiple linear regression analysis.

This was used to evaluate the association between: gender, increase in age, gingival index, mean probing depths, mean clinical attachment levels and salivary MMP-8 levels after controlling for plaque scores and toothbrushing frequency for participants. The regression analysis yielded a coefficient of determination R squared ($R^2$) of 0.24.
Therefore, 24% of the variation in salivary MMP-8 levels was accounted for by the independent/predictor variables studied. There was a statistically significant association between increase in age, plaque score, gingival inflammation and salivary MMP-8 levels.

**Table 1: MULTIPLE LINEAR REGRESSION ANALYSIS TO PREDICT CHANGES IN MMP-8 LEVELS FROM CHANGE IN PREDICTOR VARIABLES**

<table>
<thead>
<tr>
<th>Variable</th>
<th>B</th>
<th>t value</th>
<th>95% Confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Years)</td>
<td>0.940</td>
<td>2.18</td>
<td>Upper: 1.793 Lower: 0.087</td>
<td>0.031*</td>
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<tr>
<td>Gingival index</td>
<td>70.47</td>
<td>3.88</td>
<td>Upper: 106.49 Lower: 34.44</td>
<td>0.000*</td>
</tr>
<tr>
<td>Mean periodontal probing depth</td>
<td>-14.34</td>
<td>-0.95</td>
<td>Upper: 15.48 Lower: -44.17</td>
<td>0.342</td>
</tr>
<tr>
<td>Mean clinical attachment loss</td>
<td>-5.37</td>
<td>-0.45</td>
<td>Upper: 18.27 Lower: -29.00</td>
<td>0.654</td>
</tr>
<tr>
<td>Gender</td>
<td>9.84</td>
<td>0.88</td>
<td>Upper: 32.14 Lower: -12.46</td>
<td>0.384</td>
</tr>
</tbody>
</table>

*There was a statistically significant association between increase in age, gingival inflammation and salivary MMP-8 levels.

**Receiver Operator Characteristics (ROC) curve analysis.**

This analysis was performed to determine the diagnostic accuracy of salivary MMP-8(Graph 3). A statistically significant Area under the curve (AUC) value of 0.774 was noted. This indicated that the measure of salivary MMP-8 can distinguish between individuals with and without periodontitis with an accuracy of about 80%. Coordinates of the curve noted a trade-off sensitivity (0.133) and specificity (0.05). This estimated that the approximate cut-off value was114.55 ng/mL of MMP-8 above which there was a likelihood of periodontitis in participants.
IV. Discussion

In this study, the finding that salivary MMP-8 ranged from undetectable levels to 295.9ng/mL and that the group with periodontitis had a higher mean MMP-8 levels (67.9ng/mL) than the group with no periodontitis (22.7ng/mL) suggested that there was a wide range in the levels of severity of periodontitis among the participants studied.

A statistically significant, positive correlation was found between increasing age and the salivary MMP-8 levels (Pearson's r = 0.316, p>0.001). These findings were in agreement with a study on age-related changes in salivary biomarkers. A plausible explanation for increased periodontal disease severity with increasing age is prolonged exposure to risk factors over the years and possible influence of undiagnosed concurrent systemic diseases predisposing periodontal changes and breakdown.

Statistically significant positive associations were also observed between salivary MMP-8 and increasing periodontitis severity. It is also notable that differences between the mean salivary MMP-8 concentration in participants diagnosed with periodontitis compared to those with no periodontitis was statistically significant. These finding concur with those of Miller CS et al (2006) studies which reported significantly lower salivary MMP-8 concentrations of the healthy patients at 64.6 ng/ml (± 16.4SD) than that of periodontally diseased patients with 623.8ng/ml (± 204.0SD, p <0.05). Genetic and racial differences in the Kenyan population may be responsible for the varied mean concentrations of salivary MMP-8 between these findings and those of our study.

Individuals in the mild and moderate periodontitis groups had closely approximated means of salivary MMP-8 of 44.5ng/mL and 46.3ng/mL respectively. A plausible explanation for the varied levels and close figures is the existence of different disease activity states within individuals within the study. Studies have demonstrated that cyclic periods of “quiescent or linear” and “burst” episodic periods and subsequent repair occur in periodontitis. A study done by Herr in 2007 in the United States similarly demonstrated that, salivary MMP-8 concentrations exhibit variability, even among periodontally diseased groups. This further confirmed the dynamism in periodontitis disease activity. Another plausible explanation may be that some individuals innately produce higher amounts of MMP-8 that predispose them to more severe periodontitis, while those who produce less may be less susceptible. A study by Chou in 2011 among Taiwanese adults associated susceptibility to chronic and aggressive forms of periodontitis to genetic polymorphisms in genomic DNA for MMP-8-799 C and T alleles. The T allele is related to increased expression of MMP-8 (11). The role of cytokine and receptor gene polymorphisms in altered immuno inflammatory pathways leading to susceptibility to periodontal tissue destruction has also been described (20).

Statistically significant positive correlations were also found between plaque scores (r=0.389, p<0.001), gingival index (F=8.398,df=2, p=0.005) and salivary MMP-8 levels. This was in agreement with a study by Gupta et al in 2014 that found similar relationships between gingival indices (r =0.49, p<0.00) and plaque indices (r =0.63, p<0.001) in the periodontitis group studied. It is well established that the major determinant of periodontitis susceptibility is the host immune-inflammatory response to the subgingival biofilm (22).

A multiple linear regression analysis indicated that 24.0% of the variation in salivary MMP-8 levels was accounted for by gender, increase in age, gingival index, mean probing depths and mean clinical attachment levels (R²= 0.24) after controlling for plaque scores. This highlighted the influential role of other factors, possibly genetic polymorphisms in inducing variations in MMP-8 concentrations. Individually, the change in gingival scores was observed to be a statistically significant predictor of salivary MMP-8 levels (r=3.88,df=2,p<0.01). This reinforces the immuno-inflammatory concepts that salivary MMP-8 levels do indicate individual disease activity.

The generation of receiver operator characteristics (ROC) curve demonstrated that salivary MMP-8 could distinguish between individuals with and without periodontitis. A statistically significant area under the curve (AUC) of 0.8 was noted. The findings were in agreement with a study by Rameiser, Kenney and colleagues where an AUC of 0.9 was derived. The more robust AUC value in the Rameiser study was possibly due to the analysis of a combination of biomarkers (MMP-8, MMP-9 and osteoprotegerin) as well as genomic identification of red complex periodontal pathogens.

Further analysis was carried out to establish cut off concentrations of MMP-8. In this study, the ROC coordinates of sensitivity and specificity indicated an approximate threshold cut-off concentration of 114.55ng/mL of salivary MMP-8 above which indicated a likelihood of periodontitis. Within the limits of this study, salivary MMP-8 is thus valuable as a putative diagnostic biomarker for periodontal disease.

Limitations of the study included it being a cross sectional study and as such could not monitor the prospective causal relationship between salivary MMP-8 levels and changes in disease severity. Secondly, the study was carried out in a hospital setting and was not randomized potentially introducing selection bias. This also posed a challenge in making inferences to the general population.
In conclusion, salivary MMP-8 levels have a strong positive correlation with increasing periodontitis and gingivitis assessed with the clinical parameters and should be considered as a putative adjunctive diagnostic tool. However there is need for larger longitudinal cohort studies and randomized controlled studies to make longitudinal associations of periodontal disease activity as well as evaluating outcomes of non surgical and surgical periodontal interventions in the Kenyan set up. Due to the drawbacks of current disease classifications, the use of putative salivary biomarkers should be developed as an adjunct in identifying individuals’ disease activity and susceptibility to periodontal destruction.

Declarations

This study was carried out with accordance to Declaration of Helsinki 2008 and approval from the Kenyatta National Hospital and University of Nairobi research ethics and standards committee Ref: KNH-ERC/A/228. Permission to conduct the study was also granted by the University of Nairobi Institute of Tropical and Infectious Diseases laboratories. The cost of the study was met solely by the principal investigator for scientific and academic purposes and there was no conflict of interest related to this study. The dataset and materials supporting the conclusions of this article are available. Kindly contact the principal author for data requests.

Consent for publication: Not applicable

Acknowledgements

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References

[22] Preshaw PM, Taylor JJ. How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis? Journal of clinical periodontology. 2011;38:60-84.
V. Legend Of Tables And Figures

Table 1. Association between salivary MMP – 8 levels and severity of gingivitis

<table>
<thead>
<tr>
<th>Severity of gingivitis</th>
<th>Number of participants (%)</th>
<th>Mean Salivary MMP-8 levels in ng/mL (sd)</th>
</tr>
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<tbody>
<tr>
<td>Mild</td>
<td>48 (42.1)</td>
<td>14.8 (±42.7)</td>
</tr>
<tr>
<td>Moderate</td>
<td>63 (55.3)</td>
<td>56.8 (±69.4)</td>
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<td>Severe</td>
<td>3 (2.6)</td>
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<tr>
<td>No Periodontitis</td>
<td>69 (60.5)</td>
<td>22.7 (±50.3)</td>
</tr>
<tr>
<td>Mild</td>
<td>13 (11.4)</td>
<td>44.6 (±59.1)</td>
</tr>
<tr>
<td>Moderate</td>
<td>23 (20.2)</td>
<td>46.3 (±54.4)</td>
</tr>
<tr>
<td>Severe</td>
<td>9 (7.9)</td>
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Table 3: Multiple Linear Regression Analysis To Predict Changes In Mmp-8 Levels From Change In Predictor Variables

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Fig.1. Receiver operating characteristic (roc) curve analysis of salivary mmp-8 on periodontitis.